Analysis of the efficacy of aminophenylamidines and cyclooctadepsipeptides and their mode of action

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Declaration on plagiarism:

I hereby confirm that I have independently composed this thesis and that no other than the indicated aid and sources have been used. This work has not been presented to any other examination board.

Berlin, April 2014

Daniel Kulke

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List of abbreviations

5-HT .... Serotonin
® .... Registered trademark
AAD .... Amino acetonitrile derivative
AChE .... Acetylcholine esterase
AChR .... Acetylcholine receptor
AdAMD .... Acetylated deacylated amidantel
AUC .... Area under the curve
B.C. .... Before Christ
$[Ca^{2+}]_i$ .... intracellular calcium concentration
CI .... Confidence intervals
C_{max} .... Maximal plasma concentration
DALY .... Disability-adjusted life year
dAMD .... Deacylated amidantel
DMSO .... Dimethyl sulphoxide
DPP .... Days post purification
e.g.. .... *Example gratia* (for example)
EC_{50} .... Effective concentration (50%)
$ED_{50/90/95/99}$ .... Effective dose (50%, 90%, 95%, 99%)
epg .... Eggs per gram
ES system .... Excretory-secretory system
FKPM .... Fragments per kilobase of exons per million fragments mapped
GABA .... γ-aminobutyric acid
GluCl .... Glutamate gated chloride channel
i.e. .... *id est* (that means)
IVC .... Current-voltage curve
L1 .... First-stage larvae
L2 .... Second-stage larvae
L3 .... Third-stage larvae
L4 .... Fourth-stage larvae
LANUV .... Landesamt für Natur-, Umwelt- und Verbraucherschutz
LAVES .... Landesamt für Verbraucherschutz und Lebensmittelsicherheit
$LD_{50/99}$ .... Lethal dose (50%, 99%)

LF .... Lymphatic filariosis
MDA... Mass drug administration
nAChR... Nicotinic acetylcholine receptor
NFR ....Normal frog ringer
NNI.. Nearest neighbour interchange
NO .... Nitric oxide
OTU.. Operational taxonomic unit
PKC .... Protein kinase C
ppm.. Parts per million
p.i... *Post infectionem*
R^2 .... Coefficients of determination
RCK.. Regulator of conduction of potassium
(RT-)PCR .... (Reverse transcriptase) polymerase chain reaction
SD... Standard deviation
SEM.. Standard error of the mean
SNP... Single nucleotide polymorphism
SPR... Subtree pruning and regraftment
(SSU) rDNA ....(Small subunit) ribosomal DNA
STH .... Soil-transmitted helminthosis
TPAC... Terephalic acid
TPAL... Terephthalaldehyde
WHO ....World Health Organisation

Chapter 1

General Introduction

1 General introduction

1.1 Historical contemplation on the diversity of parasitic helminths

"At the approach of dentition, pruritus of the gums, fevers, convulsions, diarrhea, especially when cutting the canine teeth… To persons somewhat older, affections of the tonsils, incurvation of the spine at the vertebra next the occiput, asthma, calculus, round worms [helminths], ascarides, acrochordon, satyriasmus, struma, and other tubercles [phymata], but especially the aforesaid"

(Aphorisms 3, 25-26) [1]

The first reference of specific types of intestinal worms and descriptions of anthelmintic prescriptions is dated back around 1550 B.C. in the Ebers Papyrus [2]. As long ago as the times of the great physician of antiquity, Hippocrates (460-377 B.C.), it has been known that there is more than one type of parasitic helminths, primarily striking children right after teething [1]. In the Hippocratic Corpus three types of helminths are described: "*helmins strongyle*" (roundworms), "*helmins plateia*" (flatworms), and "*ascaris*" (which corresponds to *Enterobius vermicularis*) [2]. Based on the Hippocratic doctrine, Aristotle (384-322 B.C.) continued with the categorisation of the three types of intestinal worms [3]. Furthermore, instead of having reproduction, Aristotle postulated the spontaneous creation of several small animals (*Generatio automatica*), including helminths and intestinal worms being generated "from excrement yet within the living animal" [3]. Considered as an exception, Aristotle described the reproduction of flatworms in the following way:

"The flatworm, however, in an exceptional way, clings fast to the gut, and lays a thing like a melon-seed, by observing which indication the physician concludes that his patient is troubled with the worm"

(Historia animalium Book V, Part 19) [3]

Aristotle further described several parasites of domestic and wildlife animals, including the occurrence of *Taenia solium* metacestodes in pig muscles [3]. Pliny the Elder (23-79), Celsus, Lucius Junius Moderatus Columella and Aelius Galenus (130-206) also reported on human and animal infections with intestinal worms and the morphology of these parasites [4]. Moreover, Publius Flavius Vegetius Renatus (450-510) and Alexander Trallianus (525-605)

extensively mentioned infections of pets and humans with intestinal worms and the appropriate therapy in their writings, respectively [4].

The Hippocratic classification – "*ascaris*" affect the large intestine and "*helmins strongyle"* the small intestine – was challenged with the introduction of the classification by Linné (1758) only [4]. Since Linné termed the roundworms of the small intestine as ascarids and the roundworms of the large intestine strongylids, a lot of confusion concerning the classification of intestinal worms existed [4].

With the publication of the writing "Versuch einer Naturgeschichte der Eingeweidewürmer thierischer Körper" (1782), Johann August Ephraim Goeze established a basis for fundamental helminthology [5]. In 1806, Johannes Gottfried Bremser built a centre for the classification of helminths including a massive collection of worms [5]. Two years later, in 1808, Karl Asmund Rudolphi published his writing "Entozoorum Historia naturalis", serving as the fundament of modern systematic of helminths [5].

In the following two centuries an incredible number of discoveries of major importance for the field of helminthology have been made, including the original description of several species, the disclosure of transmission paths as well as the identification of whole life cycles. Furthermore, tremendous progress in experimental biology, human and veterinary medicine, biochemistry and genetics, such as the sequencing of the first whole genome of a metazoan organism, the free-living nematode *Caenorhabditis elegans* [6], had an exceptional influence on the systematic classification of the diverse group of helminths.

Nowadays, the collective term helminths still represents a group of metazoa with a more or less worm-like shape. Parasitic helminths can be found in a large number of taxa, including the phyla Acanthocephala, Arthropoda (subclass Pentastomida), Nematoda and Platyhelminthes. However, most parasitic helminths of major importance belong either to the phylum Nematoda or to the phylum Platyhelminthes. Whereas the phylum Nematoda contains for example the gastrointestinal roundworms and filariae, the phylum Platyhelminthes includes the classes Cestoda or tapeworms and Trematoda or flukes. Since the variety of species is that diverse and most species addressed in this thesis, belong to the Nematoda, only this taxon will be introduced in detail.

1.2 Nematodes

Nematodes are metazoan organisms with a cylindrical to filiform body shape [7]. With an estimated 0.1 – 100 million species, the taxon Nematoda is considered to be one of the most diverse taxa in the animal kingdom [8]. Furthermore, free-living nematodes are one of the most widely distributed organisms in marine, freshwater and terrestrial habitats [9]. Next to the variety of free-living species, parasitism is a common life form for a large number of nematodes. According to a phylogenetic analysis comparing small subunit ribosomal DNA (SSU rDNA) sequences from a wide range of nematodes, Blaxter *et al.* [10] suggest that animal parasitism arose at least four times and plant parasitism three times independently. Despite a deceptively simple and consistent anatomical pattern, fascinating adaptions to their environments gave rise to a complex and biologically specialized group of metazoans [11]. Even after 350 years of studies on life cycles and relationships of nematodes, the lack of objective criteria for assessing homology of morphological characters hampers the reconstruction of the phylogeny of nematodes [8].

1.2.1 Classification of nematodes

Chitwood & Chitwood [12] and Chitwood [13,14] published some of the earliest and most influential classifications of nematodes by dividing the phylum Nematoda into the Aphasmidia (later: Adenophorea) and the Phasmdia (later: Secernentea) [8,9]. The classification was based on the presence or absence of sensory organs, the phasmids. Besides phasmids, Secernentea share several morphological characteristics, whereas Adenophorea were found to be a less homogenous group. Therefore, Andrássy [15] subdivided the Adenophorea into the Torquentia and the Penetrantia and ranked them equally to Secernentea [8]. Using molecular biological techniques, Blaxter *et al.* [10] published a molecular evolutionary framework for the phylum Nematoda by comparing SSU rDNA sequences from 53 nematodes. They confirmed the paraphyly of the Adenophorea and defined a clade system of nematodes, with parasitic species in each of the 5 major clades [9]. Holterman *et al.* [9] further itemised the phylogenetic tree of nematodes by analysing 339 SSU rDNA sequences and subdividing the 5 major clades into 12 clades. However, the more basic system by Blaxter *et al.* [10] is still widely used.

However, comparisons of rDNAs had not only a lasting influence on the view of nematode phylogeny, but also gave valuable insight into the phylogenetic relationship to other phyla.

Sequencing of several complete 18S rDNAs resulted in the hypothesis that nematodes are related to arthropods and not to other worm-shaped phyla. The new class of Ecdysozoa, containing moulting animals, drastically diminishes the phylogenetic distance between insects and nematodes and therefore, influences conclusions drawn from knowledge on the model organisms *C. elegans* and *Drosophila melanogaster* [16]. Assuming that common attributes of *C. elegans* and *D. melanogaster* originated very early in the evolution of Bilateria, it was concluded that these characteristics might be found in *Homo sapiens*. However, due to the close relationship of nematodes and arthropods, this hypotheses for *H. sapiens* might not be supportable any longer [16].

1.2.2 General Organisation of nematodes

The basically uniform anatomical pattern of nematodes is described in several publications [7,11,17,18]. Nematodes are bilateral symmetrical and unsegmented organisms with a cylindrical, worm-like shape. However, nematodes vary in length from microscopic sizes to several meters in length. Species are generally bisexual with slight to striking sexual dimorphisms (females are mostly larger than males). The body wall is composed of a characteristic acellular cuticule with an epidermis (hypodermis) and a layer of muscle cells laying beneath, forming the exoskeleton. Together with the fluid-filled body cavity, the pseudocoel, the hydrostatic skeleton is formed. The Pseudocoel incloses the nervous system, the digestive tract as well as the reproductive system. The digestive system starts cranial with the buccal cavity followed by the pharynx, the intestine and the caudal rectum. In females and larvae the digestive tract terminates with an anus, whereas males have a cloaca formed by the vas deferens joining the rectum. Nematodes have a complex nervous system. The nerve ring encircles the pharynx and is connected to the anterior and posterior via longitudinal nerves. The dorsal and ventral nerve cords arise from the nerve ring and run caudal. Several commissures connect the longitudinal nerves. Submedian nerves are without a ganglion, whereas the lateral nerve contains a few and the ventral nerve a chain of ganglia. Cranial, six nerves connect the nerve ring to the cephalic sensory organs (sensilla). A sensillum is either a mechanoreceptor or has chemosensoric functions. Next to the cranial sensilla, a number of tactile papillae or sensory organs can be found on the body surface including amphids, cervical papillae, male caudal papillae, phasmids and female perivulval probes (for review of sense organs, see [19]). Due to the importance in the mode of action of several anthelmintic drug classes, more details on the neuromuscular organisation and the involvement in the

stereotypic dorsoventral sinusoidal body movement will be given in the following chapter.
The female reproductive tract consists mostly of paired ovaries connected to the uterus via the oviduct, the terminal vulva and the receptaculum seminis. The reproductive system of male nematodes consists of the testes and the seminal gland connected via the vas deferens. Male worms may also have a species-specific bursa copulatrix and paired spicules. Nematodes have an excretory-secretory (ES) system but no circulatory system. During the development from egg to adult worms, nematodes undergo four moults (from first-stage (L1) to second-stage (L2) to third-stage (L3) to fourth-stage (L4) larvae to preadult worms) by completely shedding the cuticule each time, followed by the development of sexual maturity (mature adult). Life cycles are very diverse and characteristic for systematical groups within the phylum Nematoda, including stage-specific parasitism combined with free-living stages as well as parasitism throughout all life cycle stages (*e.g. Trichinella spiralis*).

1.2.3 Neuromuscular organisation of nematodes

In order to understand the mode of action of several anthelmintics, profound knowledge of the organisation of nematode neuromuscular transmission is essential. Therefore, the organisation of nematode neuroanatomy, somatic muscle cells as well as neuromuscular signal transmission are described (according to [20]). The nervous systems of *Ascaris suum* and *C. elegans* were described to consist of approximately 250 and 320 neurons, respectively. Each nematode neuron can have between one and 30 different synaptic contacts, resulting in a total of approximately 5000 chemical synapses, 600 gap junctions and 2000 neuromuscular junctions [20]. Locomotion is controlled by the motor nervous system (excludes the nerve ring and the cranial neuronal network), consisting of five repeated segments with eleven motoneurons, each.
Based on their morphology (distribution of their axons and dendrites) these neurons are classified into seven types (V1, VI, V2, D1, DE1, DE2 and DE3). D1, DE2 and DE3 are present once, whereas the remaining types occur twice in each of the segments (see Fig. 1-1A). D1 and VI are inhibitory motoneurons, whereas DEI, D2, D3, V1, V2 and V3 are excitatory motoneurons [20]. Next to junctions to interneurons as well as synaptic contacts to other motoneurons, they have neuromuscular connections with somatic muscle cells. The longitudinal oriented, spindle-shaped somatic muscle cells are found grouped in quartiles between the hypodermal cords (ventral and dorsal nerve cord, and lateral cords containing nerves and the H cell constituting the excretory system). The cross-section of nematodes can

therefore be divided into four parts (see Fig. 1-1B). Each somatic muscle cell (see Fig. 1-1C) is of the obliquely striated muscle cell type. The contractile part contains thin and thick filaments arranged in a well-defined angle, allowing the muscle to highly extend while maintaining the velocity of contraction. The non-contractile portion contains the cell nucleus.

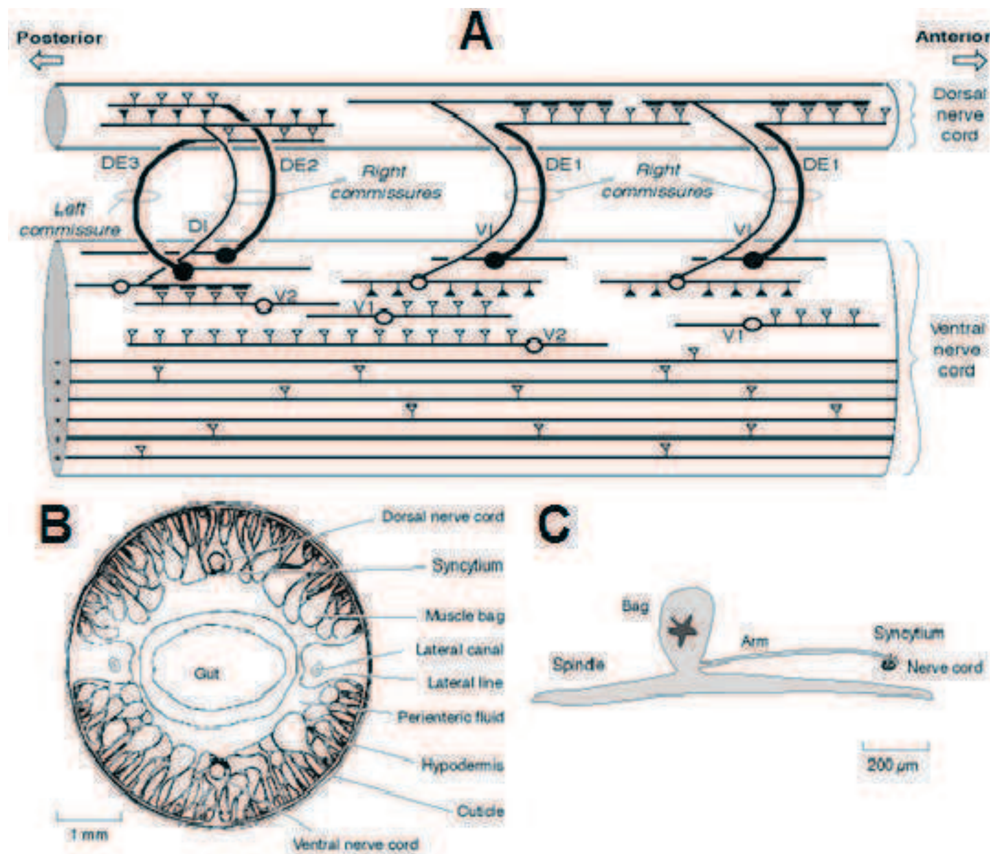

Fig. 1-1. Neuromuscular organisation of nematodes (modified by Richard Martin from [20])

A) Schematic illustration of one of the five repeated segments of the motor nervous system controlling locomotion. The ventral and dorsal nerve cords are connected via four commissures (three right-hand and one left-hand). Each commissure contains one or two axons of motoneurons, which has its cell body in the ventral nerve cord. Distribution of the eleven motoneurons V1 (2x excitatory), VI (2x inhibitory), V2 (2x excitatory), DEI (2x excitatory), D1 (1x inhibitory), D2 (1x excitatory) and D3 (1x excitatory) is also shown. B) Illustration of the cross-section of a nematode, showing the division of somatic muscle cells into quartiles by the ventral and dorsal nerve cord as well as the lateral cords (lateral lines). C) Cartoon of a somatic muscle cell showing the spindle (contractile part), which contains the thin and thick filaments responsible for contraction, and the bag (non-contractile portion), which contains the cell nucleus. In addition, an arm (projection to the nerve) is illustrated, passing from the base of the non-contractile portion to the nerve cord.

Somatic muscle cells are divided into dorsal or ventral localised cells, showing reciprocal behaviour in terms of contraction and relaxation. This results in the typical dorsoventral sinusoidal locomotion of nematodes.

In contrast to other animals, somatic muscle cells of nematodes send projections to the nerves and not vice versa. On average each somatic muscle cell has 2.7 of so-called arms passing from the base of the non-contractile portion to the nerve cord. Each arm branches into several fingers, which form junctions with fingers of adjacent arms, building a so-called syncytium [20]. The formed network accounts for the signal transmission between individual somatic muscle cells. Neuromuscular junctions are formed between the syncytium and extensions of the longitudinal axons of the motoneurons. Since, dorsal muscle cells are connected only to the dorsal nerve cord, whereas ventral muscle cells are connected to the ventral nerve cord, neuromuscular signalling on each side is homogenous. Signalling in dorsal and ventral side is reciprocal, resulting in locomotion along the dorso-ventral plane, only.

Electrophysiological studies on somatic muscles cells of *A. suum* showed, that the membrane potential is approximately -30 mV and largely depends on chloride, followed by sodium and potassium (7:4:1). A large number of electrophysiological experiments with known neurotransmitters (summarised in [20]), resulted in contraction of the muscle or in de- or hyperpolarisation of cells demonstrating that signalling across the neuronal and the neuromuscular network is ensured by neurotransmitters, many of which are also conserved in vertebrate nervous systems. In nematodes, the major excitatory transmitter in the neuromuscular junction is acetylcholine, whereas the main inhibitory neurotransmitter is γ-aminobutyric acid (GABA). In nematodes, nicotinic acetylcholine receptors (nAChRs) (sensitive to nicotine and acetylcholine) were found to be expressed in neurons and muscle cells and their activation results in intracellular calcium increase and finally in depolarisation of the cell. In addition, $GABA_A$ receptors are also expressed in neurons and somatic muscle cells. However, opening of the channel results in hyperpolarisation, due to an increase of intracellular chloride. Interestingly, besides its function as main inhibitory neurotransmitter, GABA is also an excitatory neurotransmitter in nematodes at some interneuron synapses.

The neurotransmitter glutamate acts on glutamate-gated chloride channels (GluCls). Their activation also results in hyperpolarisation due to intracellular increase of chloride. Furthermore, serotonin (5-HT), a large number of different FMRFamide-like peptides and nitric oxide (NO) were shown to be involved in neuromuscular control in nematodes.

1.2.4 Relevance of nematodes

As outlined before, nematodes constitute one of the most diverse and most widely distributed phyla in the animal kingdom with an enormous variety of plant and animal pathogenic organisms. Therefore, traditional research in nematology mainly addressed questions related to disease management. However, given the fact, that free-living nematodes inhabit almost every soil, freshwater and saltwater habitat [8,9], nowadays free-living nematodes have been identified as pivotal players in ecosystems. Due to the increased interest in the evaluation of the impacts of global climate change and human activities on biodiversity as well as the increased demand for animal and plant fibres in a growing world population, nematodes are frequently used as indicator species in biodiversity assessment and biomonitoring of agricultural and natural ecosystems [21-23].

In addition to their importance as indicator species for biodiversity and biomonitoring, free-living nematodes contain one of the best characterised model organisms. *C. elegans* was the first metazoan organism whose whole genome was sequenced [6] and the first metazoan for which development has been described completely at cellular level [24]. Attributes such as a short and prolific life cycle and a small but manageable size resulted in a convenient maintenance [25]. Besides a handful of other organisms, *C. elegans* is essential for research in basic life sciences including neurobiology, developmental biology and genetics [26]. Up to now, research using *C. elegans* as a model won three Nobel prizes. In 2002, the Nobel Prize in physiology or medicine was awarded jointly to Sydney Brenner, H. Robert Horvitz and John E. Sulston "for their discoveries concerning genetic regulation of organ development and programmed cell death'" [27]. In 2006, the Nobel Prize in physiology or medicine was awarded jointly to Andrew Z. Fire and Craig C. Mello "for their discovery of RNA interference - gene silencing by double-stranded RNA" [28] and in 2008, the Nobel Prize in chemistry was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien "for the discovery and development of the green fluorescent protein, GFP" [29].

However, next to free-living species, parasitism is a common life form for a large number of nematodes, including plant and animal parasitic species, having a strong impact on human and animal health and cause tremendous financial losses in agriculture and livestock rearing [30]. In the following two sections the impact of nematodes on animal and human health are described.

1.2.4.1 Impact on animal health

A tremendous variety of nematodes infect companion animals, including cats and dogs, as well as grazing animals as cattle, sheep, goats and horses. In livestock industry, infections with parasitic nematodes result in enormous economic losses due to reductions in growth rates and fitness and thus reduced milk, meat and wool production [31]. Ruminants are predominantly infected with gastrointestinal nematodes belonging to the trichostrongylids. *Haemonchus contortus*, *Teladorsagia circumcincta*, *Ostertagia* spp. and *Trichostrongylus axei* inhabit the abomasum, whereas *Cooperia oncophora*, *Cooperia pectinata*, *Nematodirus* spp., *Trichostrongylus colubriformis, Trichostrongylus vitrinus* are found in the small intestine. In most cases more than one species are present in the individual host. These multiple infections can result in very heterogeneous disease pattern, depending on the predominant species and the intensity of the infection. Infections with *Cooperia* spp. are of minor pathological significance, whereas infections with *Ostertagia* spp. are considered to be the most important parasite in grazing sheep and cattle in temperate climatic zones throughout the world [18]. Clinical disease is characterised by symptoms including partial anorexia, mild anaemia, hypalbuminaemia and diarrhoea resulting in significant weight loss and dehydration and in some cases in death [18]. Due to sustainable pasture management symptoms of *Ostertagia* spp. infections are mostly subclinical or limited to reduced fitness. In contrast, infections with blood-sucking *H. contortus* frequently result in severe symptoms including chronic anaemia in small ruminants. Especially in young animals persisting infections affect the respiration and in combination with the significant blood loss, haemonchosis can be lethal. Next to trichostrongylids, *Strongyloides papillosus, Capillaria* spp. and *Oesophagostomum* spp. are of importance in ruminants [18].

Companion animals are commonly infected with a variety of gastrointestinal nematodes including the clade III nematodes *Toxocara canis* (dogs), *Toxocara cati* (cats) and *Toxascaris leonina* (carnivores), the clade V species *Ancylostoma tubaeformae* (cats), *Ancylostoma caninum* (dogs) and *Uncinaria stenocephala* (carnivores) as well as the clade I whipworm *Trichuris vulpis* (dogs). These infections can result in mucus and/or blood in faeces, anorexia, anaemia, dehydration, diarrhea and cachexia [32]. Due to global warming and increased travel activity infections with parasites previously restricted to Mediterranean areas in Europe such as the filariae *Dirofilaria repens* and *Dirofilaria immitis* are of increasing importance in Germany [33-35].

1.2.4.2 Impact on human health

Helminth infections are among the most prevalent and persistent infections in poverty-stricken and resource-constrained regions, typically associated with suboptimal hygienic conditions in sub-Saharan Africa, the Americas, and parts of Asia [36]. Therefore, helminth infections belong to both the “neglected tropical diseases” and the “neglected infections of poverty” [37]. It has been estimated recently that more than a billion people are infected with one or more worm species [36]. An important part of global human helminth infections is caused by soil-transmitted nematodes, including the roundworm *Ascaris lumbricoides* with 800 million infections, the whipworm *Trichuris trichiura* with 600 million infections, and the hookworms *Ancylostoma duodenale* and *Necator americanus* with 600 million infections [38]. The threadworm *Strongyloides stercoralis* and the pinworm *E. vermicularis* are also highly prevalent gastrointestinal helminth infection with approximately 30-100 million and 500 million infections, respectively [39,40]. Worldwide, annually approximately 135,000 deaths are estimated to be a direct result of soil-transmitted helminthoses [41]. High prevalence often comes along with high abundance of protein energy malnutrition and anaemia as well as limited access to medical care and educational opportunities [42]. Mild infections are often asymptomatic but severe and chronic infections can result in iron-deficiency anaemia, chronic inflammation of the intestine, rectal prolapse, poor growth, clubbing of the fingers as well as intellectual retardation [42-45]. The impact of helminth infections on human health is frequently expressed by referring to disability-adjusted life years (DALYs). One DALY is defined as one lost year of “healthy” life. Addition of all DALYs across the world caused by helminth infections can be used as a measurement of the gap between current health status and the optimal situation, where humans live to an advanced age, free of any diseases and disabilities caused by parasitic worms [46]. For 2011, the impact of gastrointestinal nematode infections was estimated to approximately 50 million DALYs [47]. In addition to a tremendous burden of diseases due to infections with soil-transmitted helminths, more than 150 million people of these unprivileged populations are infected with vector-borne filarial parasites. Eight species are considered as filariae of humans: *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*, *Onchocerca volvulus*, *Loa loa*, *Mansonella perstans*, *Mansonella streptocerca* and *Mansonella ozzardi*. The most prevalent species, which are also responsible for the vast majority of morbidity, are *W. bancrofti*, *B. malayi* and *B. timori* (lymphatic filariosis, LF) as well as *O. volvulus* (riverblindness, onchocerciasis). Lymphatic filariae are endemic in 73 countries across the globe, where more

than 1.4 billion people are at risk of infection [48]. Together, LF and onchocerciasis are responsible for 6.3 million DALYs [49]. Onchocerciasis is the second most frequent cause of infectious blindness after trachoma, with 99% of the cases in sub-Saharan Africa [50] and was also found to significantly increase human mortality due to high microfilarial loads [51]. Symptoms due to LF range from sub-clinical lymphangiectasia to severe oedema and dramatic sequelae of elephantiasis.

1.3 Helminth control

As pointed out before, helminth infections have a tremendous impact on both, veterinary medicine as well as human tropical medicine. Furthermore, the increased demand for animal and plant fibres and food in a growing world population can only be ensured with a successful and lasting parasite control in crops and livestock [30]. In addition to the biological control of crop pests, the potential immunological control of animal and human parasitic nematodes using vaccines as well as a sustainable pasture management in livestock farming, the principle part of the control of helminth infections currently and in the foreseeable relies on chemotherapy using anthelmintics.

As pointed out earlier, parasitic helminths of vertebrates belong to several taxa (*e.g.* trematodes, cestodes and nematodes) and are very diverse in terms of their physiology, location in the host and genetic background and therefore respond to different anthelmintic drugs. Based on their mode of action and shared chemical structures, anthelmintics are divided into different classes [52]. The principle classes of modern broad-spectrum drugs to control helminth infections of both humans and animals are described below. However, there are a notable number of older, narrow-spectrum anthelmintics or even highly toxic drugs, which are no longer in use and are not mentioned here, but can be found in some excellent reviews [18,53,54].

1.3.1 Control of platyhelminths

Since platyhelminths have not been introduced in detail in section 1.2, some important parasites of humans and animals are introduced here before summarising the respective chemotherapeutics. In humans, food-borne trematodes and cestodes are often considered to be problems of only regional interest. However, flukes and tapeworms occur all over the world,

with the highest prevalence in developing countries [55]. In humans the most prevalent trematodes are *Clonorchis sinensis* with 35 million, *Paragonimus* spp. with 20.7 million, *Fasciola* spp. with 2.4-17 million, *Opisthorchis viverrini* with 10 million, *Fasciolopsis buski* with 1.3 million and *Opisthorchis felineus* with 1.2 million infections worldwide [56]. Importantly, not only humans but also a wide range of animals act as definitive hosts for the above-mentioned and several other food-borne trematode species. Fluke infections in humans are characterised by the long-term chronic and debilitating nature of infection and in addition complications such as cholangiocarcinoma. The veterinary impact of the infections is mainly associated with significant economic losses such as losses in meat and milk production mainly due to infections with *Fasciola hepatica* [56]. The most common cestodes *Taenia saginata*, *T. solium, Hymenolepis nana*, and *Diphyllobothrium latum* are estimated to cause 170-200 million human infections [57]. Infections with adult tapeworms mostly result in negligible or only mild symptoms such as diarrhoea, abdominal discomfort and weight loss [58,59]. However, infections with larval stages of *T. solium* can result in life-threatening neurocysticercosis [57]. Furthermore, zoonoses caused by metacestode stages of *Echinococcus multilocularis* (alveolar echinococcosis) and *Echinococcus granulosus* (cystic echinococcosis) occur in the northern hemisphere and are even widespread globally. In particular, alveolar echinococcosis is lethal within 10-15 years if left untreated [60].
Treatment with praziquantel, albendazole or niclosamide against intestinal cestode infections is usually highly effective in human and animals [57]. Praziquantel is also the drug of choice against all fluke infections, except of infections with *Fasciola* spp. Human and animal infections with *Fasciola* spp. are treated with triclabendazole [39]. To highlight the enormous contribution to the safe and reliable treatment against food-borne trematodes, *Schistosoma* spp. and cestodes, the next section introduces praziquantel in more detail.

1.3.1.1 Praziquantel

Praziquantel, synthesised in the mid 1970s with the intention to develop a human tranquilizer [39], remains the gold standard drug for the treatment of all human infections with blood flukes [61], food-borne flukes, except for *Fasciola* spp. [39] and cestodes [55]. In 1979 the safety and broad-spectrum efficacy of praziquantel against *Schistosoma* spp. were shown in clinical human trials [61]. It is still frequently used against infections with immature and mature stages of *Schistosoma mansoni* and *Schistosoma japonicum* (locally *Schistosoma intercalatum* and *Schistosoma mekongi*) as well as *Schistosoma haematobium* and

Schistosoma matheii [54] as well as against human and animal cestode infections. However, the mode of action of praziquantel is not completely understood. It is known that treatments with praziquantel result in disintegration of the parasite´s membrane, affecting several membrane functions [55]. Furthermore, it has been shown, that praziquantel disrupts the calcium homeostasis in adult schistosomes by interaction with a β-subunit of a voltage-gated calcium channel [62]. Since praziquantel is the gold standard drug against platyhelminth infections it is alarming that resistance to praziquantel has already been observed in schistosomes [63] and in *T. saginata* [64].

1.3.2 Control of nematodes

Promising results in vaccination trials against *H. contortus* in sheep [65] and against *N. americanus* in humans [66,67] suggest that less-chemotherapy dependent control strategies might be feasible in future. But since no vaccine has successfully passed clinical trials yet, use of anthelmintics will remain the gold standard to control animal and human helminth infection in the foreseeable future. The World Health Organization (WHO) list for essential medicines for adults and children contains only four drugs (the benzimidazoles albendazole, mebendazole and the cholinergic drugs levamisole and pyrantel) for the treatment against infections with gastrointestinal nematodes and only three drugs (albendazole, diethylcarbamazine and ivermectin) for the treatment of filariasis [68,69]. Similar to almost all anthelmintics used in humans, these drugs were initially developed for veterinary use since there is a significant commercial veterinary market for anthelmintics [70]. Since access to health services and education, epidemiological monitoring and effective sanitation are hardly available in many of the endemic areas for human helminthosis, the mass drug administration (MDA) campaigns became a successful strategy for the treatment against soil-transmitted helminthosis and filariasis [70,71]. The chemotherapeutic control of nematode infections of animals and humans strongly relies on three drug classes: The benzimidazoles, the cholinergic drugs (consisting of the imidazothiazoles and tetrahydropyrimidines) and the macrocyclic lactones. However, before the introduction of the first modern broad-spectrum anthelmintics with reasonable safety profiles in the 1960s, a variety of narrow-spectrum drugs with severe toxicology profiles were widely used. Due to the extensive use of piperazine in the past and the ongoing importance of diethylcarbamazine as filaricidal drug, they will be introduced in addition to the three major classes.

1.3.2.1 Diethylcarbamazine

In 1948, diethylcarbamazine was introduced for the treatment of human filariasis [53]. After more than fifty years of use in filarial control, it still remains a common drug against infections with *W. bancrofti*, *B. malayi, B. timori* [72] and *L. loa* [73]. Treatment with diethylcarbamazine also appears to substantially decrease *M. perstans* microfilarial loads in human blood [74]. Additionally to its therapeutical effects against acute and chronic cases of microfilaraemia [72], it also has partial macrofilarial efficacy against the above mentioned species in annual treatment regimens [75]. Due to the rapid death of microfilariae, significant inflammation and pain in the surrounding tissue are the consequence [75]. Therefore, diethylcarbamazine is not used in areas where onchocerciasis is co-endemic, because it induces strong local inflammation in patients with ocular microfilariae [72]. The mode of action of diethylcarbamazine is not completely understood. For microfilariae, an interaction of diethylcarbamazine with the endothelial cells of the host and the eicosanoid production of the worms were suggested, making the microfilariae susceptible to an immunological attack by the immune system of the host [76]. In addition to its use in human medicine, diethylcarbamazine was largely used in the prevention of canine heartworm diseases. Due to the macrocyclic lactones the application of diethylcarbamazine against *D. immitis* is significantly reduced nowadays [77].

1.3.2.2 Piperazine

Piperazine was launched in the mid 1950s as a drug to treat human *A. lumbricoides* and *E. vermicularis* infections [52,76]. It was synthetically designed as a result of the antifilarial potency of diethylcarbamazine [53]. However, the mode of action was found to be different. Piperazine acts as a weak GABA-mimetic and directly activates $GABA_A$ channels (*e.g.* homomeric and heteromeric UNC-49 channels of *H. contortus*) [78]. The activation of the channels causes hyperpolarisation of the body wall muscle and finally results in flaccid paralyses of the worm [76]. Since piperazine binds only reversible to $GABA_A$ channels, shown in single channel recordings of *A. suum* body muscles [79], expelled worms are still alive after treatment [53]. Due to the limited spectrum, the use of piperazine has declined since the introduction of the newer broad-spectrum anthelmintics [76].

1.3.2.3 Benzimidazoles

Thiabendazole, which was introduced into the marked in 1961, represents the first modern broad-spectrum anthelmintic [80]. Due to the exceptional profile of thiabendazole, several thousand benzimidazoles were synthesised for evaluation of anthelmintic efficacy. However, only less than twenty have reached commercial use [81]. Using fairly low dosages (1-5 dosages of 5-10 mg/kg) of either benzimidazoles, benzimidazole carbamates or pro-benzimidazoles, safe control of gastrointestinal nematode infections including immature stages in a variety of hosts was ensured [76,82]. Furthermore, the benzimidazoles also have potent ovicidal effects [83]. Next to their importance in animal health, the benzimidazoles thiabendazole, albendazole and mebendazole were and still are essential for the anthelmintic chemotherapy in human medicine [82]. Albendazole and mebendazole are highly effective against adult *A. lumbricoides* in a single dose. However, only albendazole is used for the treatment against tissue migrating larvae since mebendazole is poorly absorbed from the gastrointestinal tract. Therefore, its *in vivo* efficacy is limited to intestinal stages [84]. Furthermore, the efficacy of both drugs is unsatisfactory against hookworms and *T. trichiura* in single dose regimen [70]. Higher efficacies were observed when albendazole or mebendazole were administered using multiple drug administration [39].

Besides their nematicidal effects, several benzimidazoles are active against protozoa, cestodes and trematodes. In general, benzimidazoles have low toxicity in mammals, with the exception of teratogenicity of some derivatives [76].

A significant number of biological effects for benzimidazoles have been published. However, all observed effects are linked to an interaction of benzimidazoles with tubulin [85]. Benzimidazoles selectively bind to helminth β-tubulin, which in an α-/β-tubulin complex is considered to result in an unfolded region of the carboxyl-terminus of the respective protein. The change in quaternary structure prevents further addition of tubulin heterodimers to microtubule and therefore inhibits polymerisation of microtubules and finally essential processes including cell proliferation and intracellular transport are impaired [86].

1.3.2.4 Cholinergic drugs

Based on their mode of action (agonists of acetylcholine receptors, AChRs), imidazothiazoles (levamisole and tetramisole) [87] and tetrahydropyrimidines (pyrantel, morantel and oxantel) [88] are categorised as a collective class of broad-spectrum anthelmintics. Furthermore, the

newer drug classes, aminophenylamidines (section 1.5.1), spiroindoles (section 1.5.3) and amino-acetonitrile derivatives (section 1.5.4) were found to act on AChRs. However, details on the newer drug classes are given in the respective sections.

As pointed out earlier, acetylcholine is considered to be the major excitatory neurotransmitter in the neuromuscular junction of nematodes [89]. Since perfusion of *A. suum* muscle flaps with either imidazothiazoles or tetrahydropyrimidines resulted in depolarisation, increased spiking frequency and muscle contraction – effects which were blocked in the presence tubocurarine – it has been suggested that both classes are nicotinic. Similar observations were made on *Oesophagostomum dentatum* and *B. malayi* muscles [90].

However, it has been demonstrated that diverse subtypes of AChR are present in nematodes and that both, levamisole and pyrantel selectively activate a restricted subgroup of AChRs. Each AChR is composed of five subunits including α- and non-α- or β-subunits [90].

In *C. elegans* at least 27 genes encode for AChR subunits. The channels sensitive to levamisole and pyrantel consist of the α-subunits UNC-63, UNC-38 and LEV-8 as well as of the β-subunits UNC-29 and LEV-1 [91]. In all nematodes, homologs for *unc-29*, *unc-38* and *unc-63* have been identified. Interestingly, in *H. contortus* and *T. circumcincta* four distinct *unc-29* paralogs have been found [92]. However, homologs to *lev-1* have only been identified in clade V nematodes but not in clade III and clade I. Furthermore, trichostrongylid *lev-1* lacks a signal peptide, suggesting that this subunit might not be involved in AChRs sensitive to levamisole. Finally, there is also no evidence for homologs to *lev-8* in trichostrongylids. However, homologs of *acr-8*, which are closely related to *lev-8*, have been described for parasitic nematodes [90].

Heterologous expression of different subsets of the receptor subunits in *Xenopus laevis* oocytes gave further insight into the mode of action of the cholinergic drugs. Microinjection of *C. elegans* unc-38, unc-63, lev-8, unc-29 and lev-1 cRNAs resulted in a channel with highest response to acetylcholine, followed by the response to levamisole and no response to nicotine. Interestingly, two different levamisole sensitive AChRs were identified in *A. suum*. Depending on the stoichiometric combination of UNC-29 and UNC-38, channels were either more sensitive to levamisole and pyrantel than to nicotine or more sensitive to oxantel and nicotine than to levamisole, respectively [93]. Microinjection of *H. contortus* subunits showed, that four distinct subunits (UNC-63, UNC-29, UNC-38 and ACR-8*)* were required to form a channel highly sensitive to levamisole followed by the sensitivity to acetylcholine and poorly responsive to pyrantel and nicotine. However, injection of only unc-63, unc-29, unc-38 cRNAs resulted in channels more responsive to pyrantel and acetylcholine than to levamisole,

indicating that ACR-8 plays a crucial role in levamisole but potentially not in pyrantel susceptibility [90]. Similar findings concerning the necessity of ACR-8 for effects of levamisole but not pyrantel have been observed in *O. dentatum* [94]. These observations clearly show, that anthelmintic effects of cholinergic AChR agonists have to be analysed in each nematode species of interest individually and that *C. elegans* might not be a suitable model for screening of new anthelmintics acting on AChRs.
It should be mentioned that levamisole and pyrantel are highly effective against infections with *A. lumbricoides*, but only moderately effective against hookworm species. Efficacies against *T. trichiura* were reported to be only 10-31% using either 10 mg/kg pyrantel or 2.5 mg/kg levamisole [39].

1.3.2.5 Macrocyclic lactones

Avermectins and milbemycins are closely related 16-membered macrocyclic lactones, showing the most important structural difference in a bisoleandrosyloxy substituent in avermectins, whereas milbemycins have no substituent at the respective position. Both classes are fermentation products of soil dwelling actinomycetes from the genus *Streptomyces* [95]. In 1973, Sankyo discovered milbemycins as acaricidal and insecticidal compounds for crop protection. However, the full parasiticidal profile, including acaricidal, insecticidal and nematocidal efficacy, was only recognised with the discovery of avermectins by Merck in 1975. This combination of efficacies opened a new chapter in the treatment of endoparasitosis and ectoparasitosis in human and animal health. Macrocyclic lactones are therefore often called endectocides [95].
A semisynthetic derivative of the fermentation product avermectin, named ivermectin, showed an exceptional broad-spectrum and long-lasting efficacy against parasites using very low dosages [96]. Due to the exceptional success of the introduction of ivermectin into the market in over 60 countries in 1981, a large number of avermectin derivatives (including abamectin, doramectin, eprinomectin and selamectin) as well as milbemycins (including milbemycin D, milbemycin oxime and moxidectin) have followed. Macrocyclic lactones became the gold standard broad-spectrum parasiticides in small and large ruminants [95] and the first drug to prevent heartworm infections in dogs (macrocyclic lactones still remain the only class to do so) [77]. Due to the donation by Merck & Co. in 1987, ivermectin is also used to treat human onchocercosis [97] within the framework of two major projects: The Onchocerciasis Elimination Program of the Americas [98], which is close to achieving its

goal to eliminate onchocercosis form the continent, and the African Program for Onchocerciasis Control are moving towards the elimination of the infection [99]. Furthermore, the Global Program to Eliminate Lymphatic Filariasis treats people in regions where *O. volvulus* and LF are co-endemic with a combination of 150 µg/kg ivermectin and 400 mg albendazole [100].

The mode of action of macrocyclic lactones against nematodes is well investigated. They bind with an extremely high affinity to nematode GluCls [101]. Site-directed mutagenesis studies suggesting the binding site of ivermectin to GluCl [102,103] have recently been confirmed by the publication of the 3D structure of ivermectin bound to the *C. elegans* GluCl [104]. The activation of GluCls is very slow but essentially irreversible. Macrocyclic lactones also potentiate the activation of the channel by glutamate, which might be of major importance for *in vivo* efficacy of macrocyclic lactones. It is not known if macrocyclic lactones are able to bind to the closed state of channel. However, once GluCls are opened by glutamate, binding of macrocyclic lactones holds the channel in an open state resulting in a lasting hyperpolarisation [105].

1.4 Resistance

Resistance is the heritable ability of a parasite population to withstand the effects of a drug at a concentration, which was previously known to be effective. This ability is either due to the presence and selection of specific alleles in the original, susceptible population, which can confer at least partial resistance or via mutation or recombination, the production of new alleles or combinations of alleles, which are then selected [106].

The extensive utilisation of anthelmintics as major means of the managed control of economically important nematode species, particular in livestock, resulted in strong selection of highly resistant nematode populations [107,108]. Thus, nowadays the spread of resistance against all drug classes including the benzimidazoles, cholinergic drugs and macrocyclic lactones is severe enough to threaten effective worm control in cattle, goats, sheep and horses [106,109-111]. Two examples are given for the rapid onset of anthelminthic resistance in the veterinary field. Thiabendazole was introduced to the market in 1961 and first resistant *H. contortus* populations in sheep where identified only three years later [112]. Similar observations have been made for monepantel. Only five years after the introduction of monepantel (Zolvix®) first reports of resistant isolates of *T. circumcincta* and *T. colubriformis* in goats have been found [113].

The development of resistance is based on a handful of changes: (i) changes in the molecular target of the drug, resulting in a loss of drug target interaction, (ii) changes in metabolism that a) inactivate or remove the drug or b) prevents its activation, (iii) changes in distribution of the drug preventing the drug from reaching its target or increasing its efflux (iv) amplification of target genes to overcome drug action [114] and (v) compensation of the molecular target by expression of closely related proteins not sensitive to the drug [90,94].

Accordingly, mechanisms of resistance against benzimidazoles, cholinergic drugs and macrocyclic lactones are diverse. Resistance against benzimidazoles was shown to be associated with single nucleotide polymorphisms (SNPs) in the target gene *ben-1* of *C. elegans* encoding for β-tubulin. Alteration of codon 200 (TTC to TAC) of orthologs in *C. elegans* and *H. contortus*, of codon 198 (GAA to GCA) in the *H. contortus* ortholog as well as of codon 167 (TTC to TAC) in orthologs of several parasitic nematodes result in benzimidazole resistance [115]. The changes associated with cholinergic drug resistance were shown to be species and drug dependent. In *C. elegans* null mutations in 21 genes, encoding for proteins categorised in (i) subunits of the target (AChRs), (ii) proteins responsible for distribution and proper function of AChRs, (iii) proteins associated with calcium release and calcium sensitive contraction and (iv) accessory proteins associated with processing, assembly and delivery of AChR to the membrane, resulted in levamisole resistance. In *O. dentatum* a reduced number of receptors in the muscle membrane and a loss of a specific AChR subtype (G35 pS, related to the single-channel conductance) were associated with levamisole resistance, whereas pyrantel resistance was associated with a reduced number of all conductance subtypes. In *A. caninum* pyrantel resistance was associated with a reduced expression of the AChR subunits UNC-38, UNC-63 and UNC-29 but not of ACR-8. In *H. contortus* levamisole resistance is associated with a truncated form of UNC-63. Truncation or loss of ACR-8 also results in levamisole, but not pyrantel resistance. This suggests that loss of ACR-8 results in levamisole resistance only, whereas loss of UNC-63 leads to resistance against both, levamisole and pyrantel [90]. Resistance against macrocyclic lactones involves several of the above-mentioned resistance mechanisms including changes in the target (*H. contortus avr-14, ggr-3, glc-5* and *lgc-37*), changes in the macrocyclic lactone metabolism (mixed-function oxidases, carboxylesterases and glutathione S-transferases) and changes in the macrocyclic lactone distribution (substrate of the ABC-Transporter sub-family P-glycoproteins) [106]. Especially, since macrocyclic lactones represent the only class of anthelmintics successfully used as preventative in heartworm disease in dogs, detected genetic polymorphisms in β-tubulin, heat shock protein 60, P-glycoproteins and the sarco-

endoplasmic reticulum calcium ATPase of potentially resistant *D. immitis*, are currently of major interest [116].

Currently, reports on resistance of human helminths against anthelmintics listed on the essential list of medicines are rare. However, the onset and rapid spread of anthelmintic resistance in the veterinary field [108] must be considered as a realistic scenario for the treatment of human helminthosis as well, even though it might develop slower because of less frequent treatments. Persistent underdosing of hookworms and whipworms within the recently increased MDA campaigns against filariosis may favor selection of highly resistant genotypes [70] as already described for *T. trichiura* [117]. Furthermore, observations in *O. volvulus* populations showed sub-optimal responses and genetic changes under high ivermectin pressure [118]. In addition, resistance to praziquantel has been observed in schistosomes [63], benzimidazole resistance has been frequently reported in liver flukes [119] and both praziquantel and niclosamide resistance have been found in *T. saginata* [64,120], indicating that lower treatment frequencies do not prevent the onset of resistance. Thus, efficient and strategic monitoring for emergence of resistance is essential (*e.g.* by the usage of genetic markers for identification of single nucleotide polymorphisms in reporter/target genes) for a sustainable helminth control in humans [70].

1.5 Future control of nematode infections – new drug classes

It has been highlighted that the largest part of the control of helminth infections in veterinary and human medicine relies on chemotherapy using anthelmintics. Especially with the introduction of the macrocyclic lactones in the 1980s, but also due to the benzimidazoles and cholinergic drugs (imidazothiazoles and tetrahydropyrimidines), the control of nematode infections was safe and highly effective and therefore, clinical and economic problems were considered to be solved. Consequently, research and development of new drug classes was largely discontinued by the pharmaceutical industry [121].

However, due to the increasing prevalence of highly resistant worm populations, as pointed out before, future control is severely threatened [39,121]. In the very recent past only a single new anthelmintic drug class, the amino acetonitrile derivatives (AADs) including monepantel, has been discovered [122]. But also several classes such as the aminophenylamidines including tribendimidine [123], the cyclooctadepsipeptides including emodepside [124], the thiazolides including nitazoxanide [125] and the spiroindole class including derquantel [126], which all were originally discovered between the late 1970s and early 1990s, became of

interest again [39,121]. Following a detailed description of the aminophenylamidines and cyclooctadepsipeptides, the main features of derquantel, monepantel and nitazoxanide will also be highlighted.

1.5.1 Aminophenylamidines

In the late 1970s the first aminophenylamidine, amidantel, was attributed to have anthelmintic efficacy. Multiple drug administrations with relatively high dosages (100-500 mg/kg body weight) given to rodents resulted in complete elimination of a wide range of parasitic nematodes, *e.g.* of *Dipetalonema witei*, *Heligmosomoides bakeri*, *Nippostrongylus brasiliensis* and *Strongyloides ratti*, whereas only low efficacy against *Trichuris muris* was observed [123]. In addition to the nematicidal effects, amidantel was also described to have baseline efficacy against some platyhelminths (*e.g.* moderate efficacy against *Hymenolepis diminuta* but not against *Hymenolepis nana*) [123].

These results indicated that amidantel might be an interesting lead structure for the development of a new broad-spectrum anthelmintic. While studying aminophenylamidine structure-activity relationship, a symmetrical diamidine derivative of amidantel, tribendimidine, was synthesised and tested by the Chinese National Institute of Parasitic Diseases [127]. Fairly low dosages of tribendimidine showed high efficacy against several parasitic nematodes in rodents (50-200 mg/kg body weight) and dogs (6-25 mg/kg body weight) [127]. Again, in addition to the nematicidal effects, tribendimidine was also found to be efficacious against several trematodes (i.e. *Echinostoma caproni*, *C. sinensis*, *Opisthorchis viverrini*), whereas no effects against *F. hepatica* and *S. mansoni* were observed [127-129]**.** Furthermore, tribendimidine was described to be effective against unstated cestodes in chicken, but detailed data were not shown [127].

Interestingly, amidantel was found to be rapidly metabolised to its primary metabolite, p-(1-dimethylamino ethylimino) aniline (dAMD) (*i.e.* deacylated amidantel, aminoamidine, BAY d 9216) [129], whereas tribendimidine rapidly disintegrates in aqueous environments releasing two molecules of dAMD [130,131]. It shows efficacy against the free-living nematode *C. elegans* [132] as well as against parasitic nematodes [133] by itself and *in vivo* dAMD will be further metabolised to acetylated dAMD (AdAMD). Again, AdAMD also exhibits activity against several hookworm species and *C. sinensis* [134].

After several years of extensive experimental studies and clinical investigations, in 2004 tribendimidine has been registered for the treatment against the soil transmitted helminthoses

caused by *A. lumbricoides*, *A. duodenale* and *N. americanus* as well as against *E. vermicularis* in the People's Republic of China [127]. The use of the oral tablet was considered to be safe and only very light and transient adverse effects have been reported [135]. Acute toxicity after oral or intraperitoneal treatment of mice was determined with an LD_{50} of 950 ± 207 mg/kg and 277 ± 27 mg/kg, respectively. In rats, the oral LD_{50} was 2001 ± 79 mg/kg. Moreover, rats had no detectable side effects up to 250 mg/kg given orally on 14 consecutive days indicating absence of long-term toxicity. Also, no toxic effects were observed for dogs orally receiving 30 mg/kg on 14 consecutive days [127].

In two phase IV trials in the People's Republic of China 1292 adolescents and adults (15-70 years of age) and 899 children (4-14 years of age), who were infected with soil-transmitted helminths, received a single enteric-coated tablet containing 400 mg and 200 mg tribendimidine, respectively. The treatment resulted in high cure rates against *A. lumbricoides* (90.1-95.0%) and moderate-to-high cure rates against hookworms (82.0-88.4%) [134]. However, single oral tribendimidine treatment showed only low cure rates against *T. trichiura* [127,134-136], only baseline activity against human intestinal taeniasis [136] and only 70.0%, 40.0% and 36.4% efficacy against *O. viverrini*, *C. sinensis* and *S. stercoralis*, respectively [134].

Investigations on loss-of-function mutants of *C. elegans*, suggest that tribendimidine acts as an agonist of the L-subtype of AChRs and therefore shares its mode of action with levamisole and pyrantel [137]. However, recent data demonstrate substantial differences in the composition of AChRs and their susceptibility to anthelmintics comparing different nematodes species. In the body muscle of *O. dentatum* four subtypes of AChR with distinct pharmacological properties have been identified using electrophysiology. The loss of one of these subtypes (G 35 pS) was associated with levamisole resistance. By heterologous expression of different combinations of AChR subunits in *X. laevis* oocytes, four pharmacologically different types of AChR were reconstituted, which all showed effects in the presence of tribendimidine but not all in the presence of levamisole, indicating that tribendimidine may still be effective on levamisole resistant worms [94]. These findings agree with the synergistic effects of levamisole and tribendimidine that have been reported against *Ancylostoma ceylanicum in vitro* and *in vivo* whereas antagonistic effects of the same combination where observed against *H. bakeri* [133].

Again, this clearly demonstrates that the effects of at least AChR agonists have to be analysed on each nematode species of interest individually and that *C. elegans* might not be a suitable model for screening of new anthelmintics acting on AChRs.

1.5.2 Cyclooctadepsipeptide

In 1992, anthelmintic efficacy of a new *N*-methylated cyclooctadepsipeptide, PF1022A, which was isolated by Meiji Seika Kaisha Ltd, was originally published [124]. PF1022A is a fermentation product of the fungus *Rosellinia* spp. PF1022, which was found on the leaves of the plant *Camellia japonica*. The fungus is of the *Mycelia sterilia* type, characterised by forming neither sexual nor asexual spores under culture conditions [138]. PF1022A is one of eight members (PF1022A-PF1022H) of a family of cyclooctadepsipeptides consisting of four alternating residues of *N*-methyl-(*S*)-amino acids and four 2-hydroxy-carboxylic acids bound via amide and ester linkage [138]. The fermentation products PF1022A-H are synthesised by the nonribosomal *N*-methyl-cyclooctadepsipeptide synthetase or PF1022-synthethase, which was isolated and characterised from *Rosellinia* spp. PF1022 [139].

In comparison to PF1022B-H, PF1022A was significantly higher efficacious against *Ascaridia galli* in chicken [124] and was later successfully tested for nematicidal efficacy against a wide range of nematodes in a variety of hosts, including *Angiostrongylus cantonensis* [140], *N. brasiliensis* [141] and *S. ratti* [142] in rats, *Angiostrongylus costaricensis* [143], *H. bakeri* [144] and intestinal stages of *T. spiralis* [145] in mice, *H. contortus*, *Ostertagia ostertagi* and *T. colubriformis* in jirds [146], *A. caninum* in dogs, cyathostomes in horses, *H. contortus* and *T. colubriformis* in sheep as well as *Dictyocaulus viviparus* in cattle [142]. However, migrating stages of *N. brasiliensis* [141], encysted muscle stages of *T. spiralis* [145] as well as cerebral stages *A. cantonensis* [140] were less or not at all affected by treatments with PF1022A, suggesting that it has a low systemic bioavailability in the host.

To increase solubility and to improve bioavailability a range of derivatives were synthesised, resulting in emodepside, a semi-synthetic derivative of PF1022A, characterised by two additional morpholine rings in the *para*-position of each of the two (*R*)-phenyllactic acids. Based on the improved attributes, emodepside shows, besides the efficacy profile mentioned for PF1022A, higher efficacy against cerebral stages of *A. cantonensis* and muscle stages of *T. spiralis* [138] and was found to be highly effective against *Heterakis spumosa* [147] and *T. muris* [148,149] in mice, *Aelurostrongylus abstrusus* [150], immature and mature *A. tubaeforme* [151] as well as *T. leonina* and *T. cati* [152] in cats, immature and mature *A. caninum* [153], immature and mature *T. leonina* and *T. canis* [154], larval, preadult and mature adult stages of *T. vulpis* [155,156], and immature and mature *U. stenocephala* [153] in dogs. It was also found to be efficacious against a variety of nematodes of reptiles (including

geckos, lizards, snakes and turtles) [148] as well as several nematodes of horses and ruminants (for review see [138]).

Interestingly, in *Mastomys coucha* emodepside was also found to be highly effective against third and fourth stage larvae as well as preadult worms of *Acanthocheilonema viteae*, fairly effective against these stages of *Litomosoides sigmodontis* but not effective against the same stages of *B. malayi*. However, levels of microfilaraemia were generally and significantly reduced when treating against adult worms [157,158]. Similar findings were observed by Townson *et al.* [159], where emodepside was effective against adult *Onchocerca gutturosa in vitro* and against *Onchocerca lienalis* microfilariae in an *in vivo* mouse-model. But the same authors reported that emodepside was much less potent against *Brugia pahangi*, demonstrating that the effect of anthelmintics can differ among even closely related filarial species, for unknown reasons [75].

In general, emodepside was reported to be of low toxicity and exhibits no genotoxic properties. Although some adverse effects were noted in embryotoxicity/teratogenicity studies in rats and rabbits, emodepside is safe in pregnant cats [160].

Fujisawa Pharmaceutical Co. Ltd. filed a patent for emodepside in 1993 [160]. Emodepside in combination with praziquantel was introduced into the market by Bayer HealthCare, division Animal Health as Profender®, the first topical anthelmintic for felines and as an oral canine product in 2005 and 2009, respectively. Furthermore, Procox® (a combination of emodepside and toltrazuril), an oral suspension for puppies, entered the market for the control of nematodes and coccidian in 2011 [138]. Next to its importance in veterinary medicine, emodepside is considered to be one of the most promising candidates for evaluation for treatment of human onchocerciasis and soil-transmitted helminthoses in recent review [70,160,161].

Importantly, PF1022A as well as emodepside showed full efficacy against several nematode isolates being resistant to closantel, fenbantel, fenbendazole, levamisole and ivermectin in sheep and cattle suggesting that cyclooctadepsipeptides exhibit a new mode of action [138,162]. In early ligand-receptor interaction studies using *A. suum* muscle preparations, it was shown that radiolabeled PF1022A bound efficiently and specifically to a distinct membrane fraction of *A. suum* [163]. Shortly afterwards, it was proposed that PF1022A interacts with GABA receptors of *A. suum*, since it displaced radioactive labelled GABA and it also reduced binding of bicuculline in a dose-dependent manner as it is known for other GABAergic agents as well [164]. However, in a different study, it has been shown that bicuculline does not act as a $GABA_A$ antagonist in *A. suum* which reduces the support of the

hypothesis that cyclooctadepsipeptides act on $GABA_A$ channels [165]. However, further evidence for an emodepside $GABA_A$-receptor interaction was given by the analysis of two *C. elegans* mutant strains. In *C. elegans* neuromuscular junction, a single gene, *unc-49*, encodes for $GABA_A$ receptors. *C. elegans unc-49* loss-of-function mutants were shown to be completely resistant to the selective $GABA_A$ receptors agonist muscimol and also significantly less susceptible to emodepside. By reintroducing *T. canis* unc-49 into *unc-49* deficient *C. elegans*, muscimol and emodepside sensitivity were fully rescued [166].

By using a PF1022A ligand immunoscreening of a *H. contortus* cDNA library, a receptor (HC110-R) similar to the mammalian G-protein coupled receptor latrophilin and the *C. elegans* LAT-1 was identified as potential binding partner [167]. Orthologous proteins were also identified *in C. oncophora*, *O. ostertagi* and *A. caninum* [168,169]. Based on the observations that emodepside inhibits pharyngeal pumping, *lat-1* deficient *C. elegans* were analysed for their phenotype in the presence of emodepside. It was demonstrated that these worms showed drastically reduced emodepside induced inhibition of the pharynx, indicating that emodepsides paralytic effects on the pharynx are LAT-1 dependent [114]. However, it has been shown that besides feeding, also locomotion and reproduction of *C. elegans* are strongly affected by emodepside and that these effects still persist in *lat-1* deficient *C. elegans* in the presence of emodepside, clearly demonstrating that LAT-1 is not the only target of emodepside [170].

Finally, in *C. elegans*, the large-conductance calcium- and voltage-gated potassium channel SLO-1 was identified as essential for emodepside susceptibility. Firstly, a mutagenesis screening resulted in nine independent *C. elegans* strains with defects in SLO-1 and secondly the *C. elegans* strain (NM1968) carrying a functional null mutation in SLO-1 (allele *js379*) was found to be highly resistant against emodepside [171]. Importantly, reintroduction of *slo-1* from *A. caninum* and *C. oncophora* reconstitute emodepside sensitivity of the *C. elegans* SLO-1 loss-of-function mutant strain [172]. By using tissue specific promoters it was demonstrated that emodepside sensitivity was rescued by either presynaptic expression of *C. elegans* SLO-1 in neurons or postsynaptic expression of *C. elegans* SLO-1 in muscle cells. However, introduction of the human orthologous gene *kcmna-1* resulted in 10-100 fold less emodepside susceptible *C. elegans* in comparison to the worms expressing *C. elegans* SLO-1 [173]. Concurrently, KCNMA-1 and SLO-1 rescue the phenotype in locomotion of the wild type in a consistent way, suggesting that the affinity of emodepside is highly specific to the nematode SLO-1 channel and indicating that the toxicity is specific to nematodes [173]. Electrophysiological experiments provided further insight in the involvement of SLO-1 in the

mode of action of emodepside. Current clamp studies showed that emodepside caused slow calcium- and potassium-dependent hyperpolarisation [173] and more recently, voltage clamp experiments on *A. suum* muscle flaps showed that emodepside induced slow activation of calcium dependent and voltage-activated SLO-1-like potassium channels. Stimulation of protein kinase C (PKC) and NO pathways enhanced these effects [174]. These results lead to a model presented by Krücken *et al.* [138]: A pathway activated by a G-protein-coupled receptor, such as LAT-1, which involves PKC might modulate emodepside-induced activation of SLO-1 by e.g. changing the voltage- or calcium-dependency or the conductance of the channel due to phosphorylation of SLO-1. A model of the currently suggested complex mode of action of cyclooctadepsipeptides is given in Fig. 1-2.

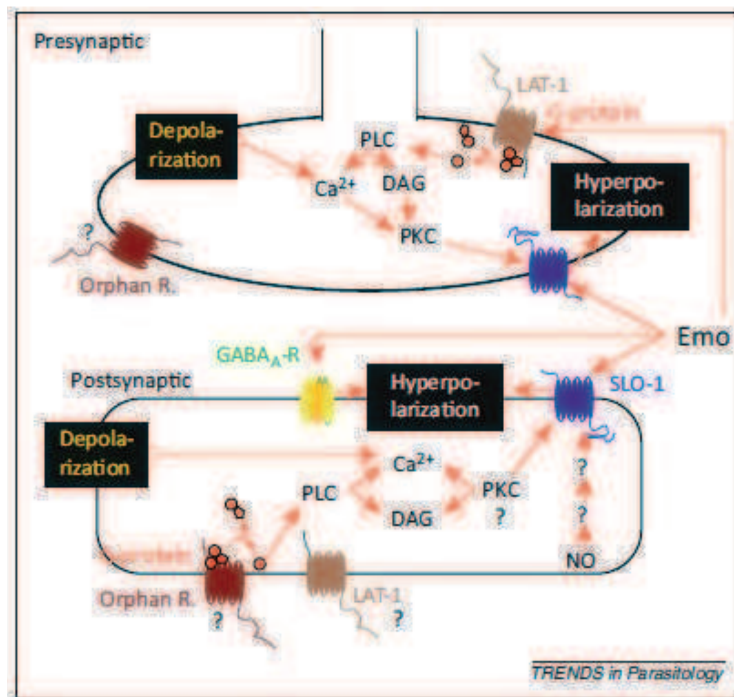

Fig. 1-2. Model for the mode of action of cyclooctadepsipeptides (taken from [138]).

The cartoon highlights the most likely scenario, in which direct activation of SLO-1 and $GABA_A$ by cyclooctadepsipeptides is shown. LAT-1 potentially modulates the inhibitory effect of emodepside on the presynaptic pharyngeal neurons by an activation cascade, activating PKC. In contrast to LAT-1, PKC is also involved in this modulation in the motor neuron/body wall musculature circuit. Additional (orphan) G-protein-coupled receptors, which might be able to modulate the effects of emodepside in particular on the body muscles, are also shown. The pathway leading to facilitated activation of SLO-1 channels in response to NO is currently mysterious and requires further experimental approaches.

However, it has been described previously, that the phylum Nematoda is highly diverse and that orthologous genes of even closely related nematode species not necessarily encode for proteins that show the same physiological properties including the absence or the presence of drug interactions. Before starting this project, SLO-1 has only been identified in the clade V nematodes *C. elegans, A. caninum*, *C. oncophora* and *H. contortus* and the clade III ascarids *A. suum*, *P. equorum* and *T. canis* [138]. Thus, it is of enormous importance to check for the presence of SLO-1 in clade III filariae as well as clade I trichocephalids since both of these groups contain parasitic nematodes of major importance. Assuming that SLO-1 can be identified and isolated from both clades, expressing the individual SLO-1 channels in a system such as mammalian cells would allow investigations on potential drug target interaction in an environment, where other potential nematode targets ($GABA_A$ and LAT-1) are not present and therefore, give further insight in the mode of action of this drug class.

1.5.3 Spiroindoles

Paraherquamide was the first compound of the spiroindole class that was attributed to have anthelmintic efficacy. It is an oxindole alkaloid metabolite of *Penicilium paraherquei*. Single oral treatments with 0.5 mg/kg or higher were found to be highly effective against several gastrointestinal nematodes including macrocyclic lactone and benzimidazole resistant isolates in sheep [126]. However, paraherquamide was found to be very toxic with an LD_{50} value of less than 15 mg/kg in mice and observations of serious side effects including death in dogs, that were administered with 0.5 mg/kg of paraherquamide [175]. Derquantel (2-desoxy-paraherquamide) is a semisynthetic derivative of paraherquamide, originally described to have up to four times higher anthelminthic efficacy against *Haemonchus sp.* and *Trichostrongylus sp.* [176] and being effective against populations of strongyles and *Nematodirus sp*. with resistance to at least one other anthelmintic [177]. Next to the higher efficacy, the safety profile of derquantel was also significantly improved. Treatments with up to 200 mg/kg in mice and 25 mg/kg in dogs resulted in only minor side effects [176]. In 2010 and 2012 Startect®, a combination of derquantel plus abamectin was registered for the treatment of gastrointestinal nematode infections in sheep in New Zealand and England, respectively [121].

It has been demonstrated by Qian *et al.* [178] that derquantel is a selective competitive antagonist, especially of the *B*-subtype, of AChRs in *A. suum*. Recently Buxton *et al.* [94] published data on the heterologous expression of different combinations of AChR subunits of

O. dentatum in *X. laevis* oocytes, showing that derquantel acts as competitive binder in the presence the levamisole on one subtype of AChR (UNC-29–UNC63–UNC38-ACR8), but as a negative allosteric modulator in the presence of pyrantel on another receptor subtype composed of UNC-29, UNC-63 and UNC-38). Since derquantel was found to be a potent antagonist of both subtypes, it is suggested by Buxton *et al.* [94] that derquantel could still be active against levamisole resistant nematodes, associated with reduced ACR-8 expression.

1.5.4 Amino acetonitrile derivatives

Kaminsky *et al.* [122] first reported on the synthetic anthelmintic drug class AADs in 2008. More than 600 derivatives of low molecular mass were synthesised and evaluated concerning *in vitro* potency on larval development of *H. contortus* and *T. colubriformis*. For the best compounds, 0.01-0.032 ppm were sufficient to fully suppress the development of larvae. Interestingly, anthelmintic properties of AADs are strongly enantiomer specific [121]. Next to the promising *in vivo* efficacy against gastrointestinal nematodes using fairly low dosages in sheep, all tested AADs were well tolerated and of low toxicity in rodents and ruminants [122]. The AAD monepantel was registered as Zolvix® in New Zealand in 2009 and nowadays in Australia and several European and South American countries [121].

Using a mutagenesis screen in *C. elegans*, monepantel resistant worms were mapped for mutations in *acr-23*, a gene belonging to a nematode-specific subfamily of AChR subunits, called DEG-3 family. Besides its role in monepantel sensitivity no further biological function is known for ACR-23. A large proportion of *C. elegans* strains resistant to monepantel showed truncations of the predicted ACR-23. However, these worms showed no overt phenotype besides monepantel resistance [122] potentially supporting rapid selection for resistance against monepantel. Two fully resistant and one less susceptible isolate of *H. contortus* were obtained by *in vitro* and *in vivo* selection, respectively. All three isolates, at least partially, lost *H. contortus des-2*, a gene very similar to the *C. elegans des-2*. DES-2 also belongs to the AChR subfamily DEG-3. Interestingly, *C. elegans* lacking DES-2 are not resistant to monepantel. However, next to the truncation/loss of DES-2, both fully resistant isolates also showed truncations in the *H. contortus* homologue of *acr-23* (*Hcacr-23H* or *mptl-1*) [122]. Recently Rufener *et al.* [179] reported on the heterologous expression of *C. elegans* ACR-23 in *X. laevis* oocytes, showing that monepantel acts as an direct agonist of this subtype of AChR. Surprisingly, monepantel was not able to act as a direct agonist of *H. contortus* DEG-3/DES-2 receptors heterologously expressed in *X. laevis* oocytes.

However, activation of these channels by choline was substantially enhanced by monepantel, suggesting that monepantel is a type II positive allosteric modulator of *H. contortus* DEG-3/DES-2 channels [180]. Despite the fact that the introduction of Zolvix® on the market is less than five years ago, first reports of resistant isolates of *T. circumcincta* and *T. colubriformis* have been published [113].

1.5.5 Thiazolides

The class of thiazolides, which essentially contains nitazoxanide derivatives, was originally published in the 1970s [125]. The parent compound nitazoxanide (2-acetyloloxy-*N*-(5-nitro-2-thiazolyl)benzamide) was initially developed as a veterinary drug targeting intestinal cestodes [125]. Nitazoxanide is a broad-spectrum drug for the treatment of gastrointestinal infections including protozoan parasites, nematodes and cestodes, anaerobic bacteria as well as viruses [181]. Nitazoxanide was commercialised for the treatment human protozoan infections (*Cryptosporidium parvum* and *Giardia intestinalis*) [181-183]. Therapeutic activity against helminths was demonstrated for the nematodes *A. duodenale*, *A. lumbricoides, E. vermicularis*, *Strongyloides* spp. and *T. trichiura* as well as for the platyhelminths *F. hepatica H. nana, T. saginata* and metacestodes of *E. multilocularis* and *E. granulosus* (for review see [125,181]).

It has been demonstrated that nitazoxanide inhibits the pyruvate-ferredoxin oxireductase of protozoans and anaerobic bacteria. However, it is not clear if nitazoxanide targets protozoan parasites via further pathways [181]. The mode of action against helminths is not completely understood. Using *C. elegans* mutagenesis screening, it has been shown that Nitazoxanide interacts with GluCls (*avr-14)* [184].

1.5.6 Drug combinations

Drug combinations are powerful tools in human disease control with numerous combinations used in anticancer chemotherapy [185] and the anti-HIV triple therapy combining indinavir, zidovudine and lamivudine [186] as prominent example. However, in veterinary parasitology the use of anthelmintic drug combinations is also considered to be a successful treatment strategy [187-189]. Basically, drug combinations can be used for three proposes (i) to increase the anthelmintic spectrum (*e.g.* nematicidal drug plus cestodicidal drugs) (ii) to improve the

efficacy of the respective drugs (*e.g.* abamectin and derquantel in Startect® [190]) (iii) to decelerate the development of resistance [189]. Combinations of macrocyclic lactones combined with doxycycline were found to have lethal effect on macrofilariae of *D. immitis*, whereas treatments with macrocyclic lactones only result in infecundity of the adult females [191,192]. Mathematical modelling revealed already in the mid 1990s that use of anthelmintic drug combinations might significantly delay the onset of resistance [193].

In 1999, Beach *et al.* [194] published the significantly higher efficacy of albendazole plus ivermectin combination against human *W. bancrofti* infections and soil-transmitted helminthosis including *T. trichiura* in comparison to the respective single drug efficacies in a randomised, placebo controlled trial. Based on these findings, a significant number of anthelmintic drug combinations were evaluated to treat human helminth infections (*e.g.* [195-197]).

Furthermore, drug combinations are also evaluated for their combinatory effects against nematodes in early screening phases (*e.g.* in *in vitro* assays), to identify potential additive or even synergistic effects in a host independent environment (e.g. [133,184,198,199]).

1.6 Aims and outline of this thesis

The urgent demand for new anthelmintics is obvious. In veterinary medicine the situation is dichotomous. Besides the prevention of heartworm disease in dogs, which solely relies on the use of macrocyclic lactones, the currently available range of anthelmintics to treat nematode infections of companion animals is adequate. In contrast, the increasing occurrence and spread of resistant nematode populations of cattle, sheep, goat and horses results in a very difficult situation in these host species.

The situation in human medicine is also challenging. Recommended by the WHO, the list of essential medicines contains only four drugs for the treatment of gastrointestinal nematode infections and only three chemotherapeutical substances for the treatment of filariosis. Since the drugs for the treatment of gastrointestinal nematodes belong to only two anthelmintic classes, namely albendazole and mebendazole to the benzimidazoles and levamisole and pyrantel to the cholinergic drugs, the arsenal is even further reduced. In addition, all of these drugs show insufficient efficacy against hookworms and whipworm, when administered as monotherapy in a single dose.

Therefore, the present cumulative thesis further analyses the potential of the aminophenylamidines amidantel, tribendimidine and dAMD as well as of the cyclooctadepsipeptides PF1022A and emodepside as broad-spectrum anthelmintics in human and veterinary medicine.

The first part (chapter 2) aimed on the investigation of the previously suggested cestodicidal effects of the aminophenylamidines in a controlled laboratory trial. The study was conducted in the thoroughly characterised and well-established *in vivo* model targeting the mouse bile duct tapeworm *Hymenolepis microstoma* in mice. This model is well accepted and considered as a good indicator for the evaluation of cestodicidal effects. Due to the contradictory literature on combinatory effects of levamisole plus tribendimidine, the cestodicidal potencies of aminophenylamidines were initially compared to the potency of levamisole, followed by the examination of combinatory effects of levamisole plus tribendimidine.

The second part (chapter 3) aimed on the evaluation of the nematicidal spectra of the aminophenylamidines and cyclooctadepsipeptides by comparing their potency against nematodes *in vitro*. Again, levamisole served as reference drug. Considering the diversity of the phylum Nematoda, the assays were performed against the clade I nematode *T. spiralis* and the clade V rat hookworm *N. brasiliensis*. Thereby the efficacy against two species with

maximal phylogenetic distance was examined. To further increase the diversity, L1 of *T. spiralis* as well as L3 and adult worms of *N. brasiliensis* were used. Again, next to the evaluation of single drugs, combinations of aminophenylamidines with levamisole and of aminophenylamidines with cyclooctadepsipeptides were tested.

The third part (chapter 4) addressed the transferability from anthelmintic *in vitro* potency to anthelmintic effects observed *in vivo*. Therefore, the largely used *in vivo* model *N. brasiliensis* in rats was used. The same monotherapies as well as drug combinations were evaluated and compared to the previously collected *in vitro* data. Unexpected differences in the potency of the two aminophenylamidines tribendimidine and dAMD, led to an *in vivo* plasma pharmacokinetic analysis of these drugs in rats. Furthermore it was investigated, if scanning electron microscopic analysis of the ultrastructure of worms treated with different classes of anthelmintics allows conclusion on their basic mode of action (*e.g.* spastic or flaccid paralysis).

The fourth part (chapter 5) aimed on the evaluation of the potency of aminophenylamidines and cyclooctadepsipeptides in a trichurosis model. Since *Trichuris* (clade I) is the dose-limiting gastrointestinal nematode genus for almost all currently used anthelmintic drug classes in dogs, swine and humans, assessment of the potency against this genus is of major importance. To investigate and compare the anthelmintic efficacy of compounds against whipworms, the *T. muris* mouse model is highly suitable. Therefore, the *T. muris* life cycle was established in mice. After detailed parasitological characterisation of the parasite host interaction, *in vivo* studies individually targeting each parasitic life cycle stage were performed.

The fifth part (chapter 6) aimed on the identification and characterisation of the calcium-activated and voltage-dependent potassium channel SLO-1, which is proposed to be the direct target of the cyclooctadepsipeptides PF1022A and emodepside. SLO-1 was already described in clade V nematodes. Despite presence of parasitic nematodes of major importance for human and animal health, SLO-1 has not been investigated in clade III and clade I nematodes. SLO-1 was therefore identified and cloned from three filariae (clade III) and *T. muris* (clade I), serving for a phylogenetic analysis of SLO-1. Additionally, SLO-1 channels were heterologously expressed in *X. laevis* oocytes to investigate the effects of cyclooctadepsipeptides on these channels.

1.7 References

1. Hippocrates, 1839, Oeuvres complètes, Lattres E (Transl.) J. B. Baillière, Paris.
2. Trompoukis C, German V, Falagas ME, 2007, From the roots of parasitology: Hippocrates' first scientific observations in helminthology. J Parasitol 93, 970-972.
3. Aristotle, 1907, The history of animals, Thompson DAW (Transl.) John Bell, London.
4. Enigk K, 1986, Geschichte der Helminthologie. Gustav Fischer Verlag, Stuttgart.
5. Hiepe T, Aspöck H, 2006, Historischer Abriss, In: Hiepe T, Lucius R, Gottstein B (Eds.), Allgemeine Parasitologie. MVS Medizinverlage Stuttgart GmbH & Co. KG., Stuttgart.
6. *C. elegans* Sequencing Consortium, 1998, Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science 282, 2012-2018.
7. Gibbons LM, 2002, General Organisation, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.
8. Meldal BH, Debenham NJ, De Ley P, De Ley IT, Vanfleteren JR, Vierstraete AR, Bert W, Borgonie G, Moens T, Tyler PA, Austen MC, Blaxter ML, Rogers AD, Lambshead PJ, 2007, An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. Mol Phylogenet Evol 42, 622-636.
9. Holterman M, van der Wurff A, van den Elsen S, van Megen H, Bongers T, Holovachov O, Bakker J, Helder J, 2006, Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown Clades. Mol Biol Evol 23, 1792-1800.
10. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas WK, 1998, A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71-75.
11. De Ley P, 2006, A quick tour of nematode diversity and the backbone of nematode phylogeny. WormBook: 1-8.
12. Chitwood BG, Chitwood MB, 1933, The characters of a protonematode. J Parasitol 20, 130.
13. Chitwood BG, 1937, A revised classification of the Nematoda. In: Ananymous (Ed.), Papers on helminthology, 30 year jubileum K J Skrjabin. All-Union Lenin Academy of Agricultural Science, Moscow.
14. Chitwood BG, 1958, The designation of official names of higher taxa of vertebrates. Bull Zool Nomencl 15, 860-895.

15. Andrássy I, 1976, Evolution as a basis for the systematization of nematodes. Pitman Publishing, London.
16. Aguinaldo AM, Turbeville JM, Linford LS, Rivera MC, Garey JR, Raff RA, Lake JA, 1997, Evidence for a clade of nematodes, arthropods and other moulting animals. Nature 387, 489-493.
17. Anderson RC, 2000, Nematode parasites of vertebrates: Their delopment and transmission. CABI Publishing, Wallingford.
18. Mehlhorn H, 2001, Encyclopedic reference of parasitology. Springer, Berlin.
19. Jones J, 2002, Nematode sense organs, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.
20. Martin JM, Purcell J, Robertson AP, Valkanov MA, 2002, Neuromuscular organisation and control in nematodes, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.
21. Cook AA, Bhardury P, Dedenham NJ, Meldal BHM, Blaxter ML, Smerdon GR, Austen MC, Lambshead PJD, Rogers AD, 2005, Denaturing gradient gel electrophoresis (DGGE) as a tool for identification of marine nematodes. Mar Ecol Prog Ser 291, 103-113.
22. Neher DA, 2010, Ecology of plant and free-living nematodes in natural and agricultural soil. Annu Rev Phytopathol 48, 371-394.
23. Neher DA, 2001, Role of nematodes in soil health and their use as indicators. J Nematol 33, 161-168.
24. Hope IA, 2002, Embryology, developmental biology and the genome, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.
25. Leung MC, Williams PL, Benedetto A, Au C, Helmcke KJ, Aschner M, Meyer JN, 2008, *Caenorhabditis elegans*: an emerging model in biomedical and environmental toxicology. Toxicol Sci 106, 5-28.
26. Cutter AD, Dey A, Murray RL, 2009, Evolution of the *Caenorhabditis elegans* genome. Mol Biol Evol 26, 1199-1234.
27. Nobelprize.org, 2013, The Nobel Prize in Physiology or Medicine 2002. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2002/: Nobel Media AB.
28. Nobelprize.org, 2013, The Nobel Prize in Physiology or Medicine 2006. http://www.nobelprize.org/nobel_prizes/medicine/laureates/2006/: Nobel Media AB.
29. Nobelprize.org, 2013, The Nobel Prize in Chemistry 2008. http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/: Nobel Media AB.

30. Manzanilla-Lopés RH, Evans K, Bridge J, 2004, Plant diseases caused by nematodes, In: Chen ZX, Chen WY, Chen SY, Dickson DW (Eds.), Nematology: Advances and Perspectives Vol 2: Neamtode Management and Utilization. CABI Publishing, Wallingford.
31. Corwin RM, 1997, Economics of gastrointestinal parasitism of cattle. Vet Parasitol 72, 451-457.
32. Epe C, 2009, Intestinal nematodes: biology and control. Vet Clin North Am Small Anim Pract 39, 1091-1107.
33. Kronefeld M, Kampen H, Sassnau R, Werner D, 2014, Molecular detection of *Dirofilaria immitis*, *Dirofilaria repens* and *Setaria tundra* in mosquitoes from Germany. Parasit Vectors 7, 30.
34. Sassnau R, Kohn M, Demeler J, Kohn B, Muller E, Krücken J, von Samson-Himmelstjerna G, 2013, Is *Dirofilaria repens* endemic in the Havelland district in Brandenburg, Germany? Vector Borne Zoonotic Dis 13, 888-891.
35. Czajka C, Becker N, Jost H, Poppert S, Schmidt-Chanasit J, Kruger A, Tannich E, 2014, Stable transmission of *Dirofilaria repens* nematodes, Northern Germany. Emerg Infect Dis 20, 328-330.
36. Lustigman S, Prichard RK, Gazzinelli A, Grant WN, Boatin BA, McCarthy JS, Basanez MG, 2012, A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 6, e1582.
37. Knopp S, Steinmann P, Keiser J, Utzinger J, 2012, Nematode infections: soil-transmitted helminths and *Trichinella*. Infect Dis Clin North Am 26, 341-358.
38. Hotez PJ, Fenwick A, Savioli L, Molyneux DH, 2009, Rescuing the bottom billion through control of neglected tropical diseases. Lancet 373, 1570-1575.
39. Keiser J, Utzinger J, 2010, The drugs we have and the drugs we need against major helminth infections. Adv Parasit, 73, 197-230.
40. Bundy DA, de Silva NR, 1998, Can we deworm this wormy world? Br Med Bull 54, 421-432.
41. Awasthi S, Bundy D, 2007, Intestinal nematode infection and anaemia in developing countries. BMJ 334, 1065-1066.
42. Stephenson LS, Holland CV, Cooper ES, 2000, The public health significance of *Trichuris trichiura*. Parasitology 121, 73-95.
43. Brooker S, Bethony J, Hotez PJ, 2004, Human hookworm infection in the 21st century. Adv Parasitol 58, 197-288.

44. Stephenson LS, Latham MC, Kinoti SN, Kurz KM, Brigham H, 1990, Improvements in physical fitness of Kenyan schoolboys infected with hookworm, *Trichuris trichiura* and *Ascaris lumbricoides* following a single dose of albendazole. Trans R Soc Trop Med Hyg 84, 277-282.
45. Sakti H, Nokes C, Hertanto WS, Hendratno S, Hall A, Bundy DA, Satoto, 1999, Evidence for an association between hookworm infection and cognitive function in Indonesian school children. Trop Med Int Health 4, 322-334.
46. Flowers RJ, Gray IS, Battersby S, 2011, Philosophy and principles of environmental health, In: Battersby S (Ed.), Clay´s handbook of environmental health. Spon Press, Abingdon.
47. World Health Organization, 2013, Global health estimates summary tables: DALYs by cause, age and sex. In: World Health Organization (Ed.), GHE_DALY_Global_2000_2011.xls World Health Organization, Geneva.
48. World Health Organization, 2013, Lymphatic filariasis, In: World Health Organization (Ed.) Fact sheet No 102, updated March 2013. World Health Organization, Geneva.
49. Hoerauf A, Pfarr K, Mand S, Debrah AY, Specht S, 2011, Filariasis in Africa - treatment challenges and prospects. Clin Microbiol Infect 17, 977-985.
50. Lustigman S, Geldhof P, Grant WN, Osei-Atweneboana MY, Sripa B, Basanez MG, 2012, A research agenda for helminth diseases of humans: basic research and enabling technologies to support control and elimination of helminthiases. PLoS Negl Trop Dis 6, e1445.
51. Little MP, Breitling LP, Basanez MG, Alley ES, Boatin BA, 2004, Association between microfilarial load and excess mortality in onchocerciasis: an epidemiological study. Lancet 363, 1514-1521.
52. Holden-Dye L, Walker RJ, 2007, Anthelmintic drugs, In: WormBook, 1-13.
53. Andrews P, Haberkorn A, Thomas H, 1986, Antiparasitic drugs: Mechanism of action, pharmacokinetics and *in vitro* and *in vivo* assays of drug activity, In: Lorian V (Ed.), Antibiotics in laboratory medicine. Williams and Wilkins, Balitomre.
54. Harder A, 2009, Anthelmintics, Ullmann's Encyclopedia of Industrial Chemistry Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.
55. Harder A, 2002, Chemotherapeutic approaches to trematodes (except schistosomes) and cestodes: current level of knowledge and outlook. Parasitol Res 88, 587-590.

56. Keiser J, Utzinger J, 2009, Food-borne trematodiases. Clin Microbiol Rev 22: 466-483.
57. Craig P, Ito A, 2007, Intestinal cestodes. Curr Opin Infect Dis 20, 524-532.
58. Min DY, 1990, Cestode infections in Korea. Kisaengchunghak Chapchi 28, 123-144.
59. Dorny P, Praet N, Deckers N, Gabriel S, 2009, Emerging food-borne parasites. Vet Parasitol 163, 196-206.
60. Deplazes P, van Knapen F, Schweiger A, Overgaauw PA, 2011, Role of pet dogs and cats in the transmission of helminthic zoonoses in Europe, with a focus on echinococcosis and toxocarosis. Vet Parasitol 182, 41-53.
61. Utzinger J, Keiser J, 2004, Schistosomiasis and soil-transmitted helminthiasis: common drugs for treatment and control. Expert Opin Pharmacother 5: 263-285.
62. Greenberg RM, 2005, Ca^{2+} signalling, voltage-gated Ca^{2+} channels and praziquantel in flatworm neuromusculature. Parasitology 131, 97-108.
63. Doenhoff MJ, Cioli D, Utzinger J, 2008, Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Curr Opin Infect Dis 21, 659-667.
64. Lateef M, Zargar SA, Khan AR, Nazir M, Shoukat A, 2008, Successful treatment of niclosamide- and praziquantel-resistant beef tapeworm infection with nitazoxanide. Int J Infect Dis 12, 80-82.
65. Piedrafita D, Preston S, Kemp J, de Veer M, Sherrard J, Kraska T, Elhay M, Meeusen E, 2013, The effect of different adjuvants on immune parameters and protection following vaccination of sheep with a larval-specific antigen of the gastrointestinal nematode, *Haemonchus contortus*. PLoS One 8, e78357.
66. Hotez P, 2011, Enlarging the "Audacious Goal": elimination of the world's high prevalence neglected tropical diseases. Vaccine, 104-110.
67. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A, 2010, Developing vaccines to combat hookworm infection and intestinal schistosomiasis. Nat Rev Microbiol 8, 814-826.
68. World Health Organization, 2013, WHO Model List of Essential Medicines for children. World Health Organization, Geneva.
69. World Health Organization, 2013, WHO Model List of Essential Medicines. World Health Organization, Geneva.
70. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, Yang GJ, Sripa B, Lustigman S, 2012, A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6, e1549.

71. Ziegelbauer K, Speich B, Mausezahl D, Bos R, Keiser J, Utzinger J, 2012, Effect of sanitation on soil-transmitted helminth infection: systematic review and meta-analysis. PLoS Med 9: e1001162.
72. Taylor MJ, Hoerauf A, Bockarie M, 2010, Lymphatic filariasis and onchocerciasis. Lancet 376, 1175-1185.
73. Padgett JJ, Jacobsen KH, 2008, Loiasis: African eye worm. Trans R Soc Trop Med Hyg 102, 983-989.
74. Simonsen PE, Onapa AW, Asio SM, 2011, *Mansonella perstans* filariasis in Africa. Acta Trop 120, 109-120.
75. Geary TG, Mackenzie CD, 2011, Progress and challenges in the discovery of macrofilaricidal drugs. Expert Rev Anti Infect Ther 9, 681-695.
76. Conder GA, 2002, Chemical control of animal-parasitc nematodes, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.
77. McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L, 2008, Heartworm disease in animals and humans. Adv Parasitol 66, 193-285.
78. Brown DD, Siddiqui SZ, Kaji MD, Forrester SG, 2012, Pharmacological characterization of the *Haemonchus contortus* GABA-gated chloride channel, Hco-UNC-49: modulation by macrocyclic lactone anthelmintics and a receptor for piperazine. Vet Parasitol 185, 201-209.
79. Martin RJ, 1985, Gamma-Aminobutyric acid- and piperazine-activated single-channel currents from *Ascaris suum* body muscle. Br J Pharmacol 84, 445-461.
80. Brown HD, Matzuk AR, Ilves IR, Peterson LH, Harris SA, Sarett LH, Egerton JR, Yakstis JJ, Campbell WC, Cuckler ACC, 1961, Antiparasitic drugs. IV. 2-(4'-thiazolyl)-benzimidazole, a new anthelmintic. J Am Chem Soc. 83, 1764-1765.
81. Townsend LB, Wise DS, 1990, The synthesis and chemistry of certain anthelmintic benzimidazoles. Parasitol Today 6, 107-112.
82. Campbell WC, 1990, Benzimidazoles: veterinary uses. Parasitol Today 6, 130-133.
83. Maisonneuve H, Rossignol JF, Addo A, Mojon M, 1985, Ovicidal effects of albendazole in human ascariasis, ancylostomiasis and trichuriasis. Ann Trop Med Parasitol 79, 79-82.
84. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ, 2006, Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367, 1521-1532.
85. Lacey E, 1990, Mode of action of benzimidazoles. Parasitol Today 6, 112-115.

86. Harder A, 2002, Chemotherapeutic approaches to nematodes: current knowledge and outlook. Parasitol Res 88, 272-277.
87. Aceves J, Erlij D, Martinez-Maranon R, 1970, The mechanism of the paralysing action of tetramisole on *Ascaris* somatic muscle. Br J Pharmacol 38, 602-607.
88. Aubry ML, Cowell P, Davey MJ, Shevde S, 1970, Aspects of the pharmacology of a new anthelmintic: pyrantel. Br J Pharmacol 38, 332-344.
89. Atchison WD, Geary TG, Manning B, VandeWaa EA, Thompson DP, 1992, Comparative neuromuscular blocking actions of levamisole and pyrantel-type anthelmintics on rat and gastrointestinal nematode somatic muscle. Toxicol Appl Pharmacol 112, 133-143.
90. Martin RJ, Robertson AP, Buxton SK, Beech RN, Charvet CL, Neveu C, 2012, Levamisole receptors: a second awakening. Trends Parasitol 28, 289-296.
91. Boulin T, Gielen M, Richmond JE, Williams DC, Paoletti P, Bessereau JL, 2008, Eight genes are required for functional reconstitution of the *Caenorhabditis elegans* levamisole-sensitive acetylcholine receptor. Proc Natl Acad Sci 105, 18590-18595.
92. Neveu C, Charvet CL, Fauvin A, Cortet J, Beech RN, Cabaret J, 2010, Genetic diversity of levamisole receptor subunits in parasitic nematode species and abbreviated transcripts associated with resistance. Pharmacogenet Genomics 20, 414-425.
93. Williamson SM, Robertson AP, Brown L, Williams T, Woods DJ, Martin RJ, Sattelle DB, Wolstenholme AJ, 2009, The nicotinic acetylcholine receptors of the parasitic nematode *Ascaris suum*: formation of two distinct drug targets by varying the relative expression levels of two subunits. PLoS Pathog 5, e1000517.
94. Buxton SK, Charvet CL, Neveu C, Cabaret J, Cortet J, Peineau N, Abongwa M, Courtot E, Robertson AP, Martin RJ, 2014, Investigation of acetylcholine receptor diversity in a nematode parasite leads to characterization of tribendimidine- and derquantel-sensitive nAChRs. PLoS Pathog 10, e1003870.
95. Shoop WL, Mrozik H, Fisher MH, 1995, Structure and activity of avermectins and milbemycins in animal health. Vet Parasitol 59, 139-156.
96. Campbell WC, Fisher MH, Stapley EO, Albers-Schonberg G, Jacob TA, 1983, Ivermectin: a potent new antiparasitic agent. Science 221, 823-828.
97. Molyneux DH, Nantulya V, 2005, Public-private partnerships in blindness prevention: reaching beyond the eye. Eye 19, 1050-1056.

98. Rodriguez-Perez MA, Unnasch TR, Real-Najarro O, 2011, Assessment and monitoring of onchocerciasis in Latin America. Adv Parasitol 77, 175-226.
99. Traore S, Wilson MD, Sima A, Barro T, Diallo A, Ake A, Coulibaly S, Cheke RA, Meyer RR, Mas J, McCall PJ, Post RJ, Zoure H, Noma M, Yameogo L, Seketeli AV, Amazigo UV, 2009, The elimination of the onchocerciasis vector from the island of Bioko as a result of larviciding by the WHO African Programme for Onchocerciasis Control. Acta Trop 111, 211-218.
100. Bockarie MJ, Deb RM, 2010, Elimination of lymphatic filariasis: do we have the drugs to complete the job? Curr Opin Infect Dis 23, 617-620.
101. Dent JA, Smith MM, Vassilatis DK, Avery L, 2000, The genetics of ivermectin resistance in *Caenorhabditis elegans*. Proc Natl Acad Sci 97, 2674-2679.
102. Lynagh T, Lynch JW, 2010, An improved ivermectin-activated chloride channel receptor for inhibiting electrical activity in defined neuronal populations. J Biol Chem 285, 14890-14897.
103. Lynagh T, Lynch JW, 2010, A glycine residue essential for high ivermectin sensitivity in Cys-loop ion channel receptors. Int J Parasitol 40: 1477-1481.
104. Hibbs RE, Gouaux E, 2011, Principles of activation and permeation in an anion-selective Cys-loop receptor. Nature 474, 54-60.
105. Wolstenholme AJ, 2011, Ion channels and receptor as targets for the control of parasitic nematodes. Int J Parasitol Drugs Drug Resist 1, 2-13.
106. Wolstenholme AJ, Kaplan RM, 2012, Resistance to macrocyclic lactones. Curr Pharm Biotechnol 13, 873-887.
107. Sangster NC, Dobson RJ, 2002, Anthelmintic resistance, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.
108. Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC, 2004, Drug resistance in veterinary helminths. Trends Parasitol 20, 469-476.
109. Kaplan RM, 2004, Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol 20, 477-481.
110. Sutherland IA, Leathwick DM, 2011, Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends Parasitol 27, 176-181.
111. Peregrine AS, Molento MB, Kaplan RM, Nielsen MK, 2014, Anthelmintic resistance in important parasites of horses: Does it really matter? Vet Parasitol 17, 1-8.
112. Conway DP, 1964, Variance in the Effectiveness of Thiabendazole against *Haemonchus contortus* in sheep. Am J Vet Res 25, 844-846.

113. Scott I, Pomroy WE, Kenyon PR, Smith G, Adlington B, Moss A, 2013, Lack of efficacy of monepantel against *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*. Vet Parasitol 198, 166-171.
114. Willson J, Amliwala K, Davis A, Cook A, Cuttle MF, Kriek N, Hopper NA, O'Connor V, Harder A, Walker RJ, Holden-Dye L, 2004, Latrotoxin receptor signaling engages the UNC-13-dependent vesicle-priming pathway in *C. elegans*. Curr Biol 14, 1374-1379.
115. Demeler J, Krüger N, Krücken J, von der Heyden VC, Ramünke S, Kuttler U, Miltsch S, Lopez Cepeda M, Knox M, Vercruysse J, Geldhof P, Harder A, von Samson-Himmelstjerna G, 2013, Phylogenetic characterization of beta-tubulins and development of pyrosequencing assays for benzimidazole resistance in cattle nematodes. PLoS One 8, e70212.
116. Bourguinat C, Keller K, Prichard RK, Geary TG, 2011, Genetic polymorphism in *Dirofilaria immitis*. Vet Parasitol 176, 368-373.
117. Diawara A, Drake LJ, Suswillo RR, Kihara J, Bundy DA, Scott ME, Halpenny C, Stothard JR, Prichard RK, 2009, Assays to detect beta-tubulin codon 200 polymorphism in *Trichuris trichiura* and *Ascaris lumbricoides*. PLoS Negl Trop Dis 3, e397.
118. Nana-Djeunga H, Bourguinat C, Pion SD, Kamgno J, Gardon J, Njiokou F, Boussinesq M, Prichard RK ,2012, Single nucleotide polymorphisms in beta-tubulin selected in *Onchocerca volvulus* following repeated ivermectin treatment: possible indication of resistance selection. Mol Biochem Parasitol 185, 10-18.
119. Brennan GP, Fairweather I, Trudgett A, Hoey E, McCoy, McConville M, Meaney M, Robinson M, McFerran N, Ryan L, Lanusse C, Mottier L, Alvarez L, Solana H, Virkel G, Brophy PM, 2007, Understanding triclabendazole resistance. Exp Mol Pathol 82, 104-109.
120. Koul PA, Waheed A, Hayat M, Sofi BA, 1999, Praziquantel in niclosamide-resistant *Taenia saginata* infection. Scan J Infect Dis 31, 603-604.
121. Epe C, Kaminsky R, 2013, New advancement in anthelmintic drugs in veterinary medicine. Trends Parasitol 29, 129-134.
122. Kaminsky R, Ducray P, Jung M, Clover R, Rufener L, Bouvier J, Weber SS, Wenger A, Wieland-Berghausen S, Goebel T, Gauvry N, Pautrat F, Skripsky T, Froelich O, Komoin-Oka C, Westlund B, Sluder A, Maser P, 2008, A new class of anthelmintics effective against drug-resistant nematodes. Nature 452, 176-180.

123. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, Thomas H, 1979, Amidantel, a potent anthelminthic from a new chemical class. Arzneimittelforschung 29, 31-32.
124. Sasaki T, Takagi M, Yaguchi T, Miyadoh S, Okada T, Koyama M, 1992, A new anthelmintic cyclodepsipeptide, PF1022A. J Antibiot 45, 692-697.
125. Hemphill A, Mueller J, Esposito M, 2006, Nitazoxanide, a broad-spectrum thiazolide anti-infective agent for the treatment of gastrointestinal infections. Expert Opin Pharmacother 7, 953-964.
126. Shoop WL, Egerton JR, Eary CH, Suhayda D, 1990, Anthelmintic activity of paraherquamide in sheep. J Parasitol 76, 349-351.
127. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W, 2005, Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94, 1-14.
128. Keiser J, Shu-Hua X, Utzinger J, 2006, Effect of tribendimidine on adult *Echinostoma caproni* harbored in mice, including scanning electron microscopic observations. J Parasitol 92, 858-862.
129. Keiser J, Shu-Hua X, Chollet J, Tanner M, Utzinger J, 2007, Evaluation of the *in vivo* activity of tribendimidine against *Schistosoma mansoni*, *Fasciola hepatica*, *Clonorchis sinensis*, and *Opisthorchis viverrini*. Antimicrob Agents Chemother 51, 1096-1098.
130. Yuan G, Wang B, Wei C, Zhang R, Guo R, 2008, LC-MS Determination of *p*-(1-Dimethylamino ethylimino)aniline: a Metabolite of Tribendimidine in Human Plasma. Chromatographia 68, 139-142.
131. Yuan G, Xu J, Qu T, Wang B, Zhang R, Wei C, Guo R, 2010, Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs R D 10, 83-90.
132. Tomlinson G, Albuquerque CA, Woods RA, 1985, The effects of amidantel (BAY d 8815) and its deacylated derivative (BAY d 9216) on *Caenorhabditis elegans.* Eur J Pharmacol 113, 255-262.
133. Tritten L, Nwosu U, Vargas M, Keiser J, 2012, *In vitro* and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. Acta Trop 122: 101-107.

134. Xiao SH, Utzinger J, Tanner M, Keiser J, Xue J, 2013, Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. Acta Trop 126, 115-126.

135. Xiao SH, Wu ZX, Zhang JH, Wang SQ, Wang SH, Qiu DC, Wang C, 2007, Clinical observation on 899 children infected with intestinal nematodes and treated with tribendimidine enteric coated tablets. Chin J of Parasitol and Parasit Dis 25: 372-375.

136. Steinmann P, Zhou XN, Du ZW, Jiang JY, Xiao SH, Wu ZX, Zhou H, Utzinger J, 2008, Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. PLoS Negl Trop Dis 2, e322.

137. Hu Y, Xiao SH, Aroian RV, 2009, The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3, e499.

138. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, Welz C, von Samson-Himmelstjerna G, 2012, Anthelmintic cyclcooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28, 385-394.

139. Müller J, Feifel SC, Schmiederer T, Zocher R, Süssmuth RD, 2009, *In vitro* synthesis of new cyclodepsipeptides of the PF1022-type: probing the alpha-D-hydroxy acid tolerance of PF1022 synthetase. Chembiochem 10, 323-328.

140. Kachi S, Ishih A, Terada M, 1995, Effects of PF1022A on adult *Angiostrongylus cantonensis* in the pulmonary arteries and larvae migrating into the central nervous system of rats. Parasitol Res 81, 631-637.

141. Wang M, Watanabe N, Shomura T, Ohtomo H, 1995, Effects of PF1022A on *Nippostrongylus brasiliensis* in rats and *Hymenolepis nana* in mice. Jpn J Parasitol 440, 306-310.

142. von Samson-Himmelstjerna G, Harder A, Schnieder T, Kalbe J, Mencke N, 2000, *In vivo* activities of the new anthelmintic depsipeptide PF1022A. Parasitol Res 86, 194-199.

143. Kachi S, Terada M, Hashimoto H, 1998, Effects of amorphous and polymorphs of PF1022A, a new antinematode drug, on *Angistrongylus costaricensis* in mice. Jpn J Pharmacol 77, 235-245.

144. Nwosu U, Vargas M, Harder A, Keiser J, 2011, Efficacy of the cyclooctadepsipeptide PF1022A against *Heligmosoides bakeri in vitro* and *in vivo*. Parasitology 138, 1193-1201.

145. Wang M, Watanabe N, Shomura T, Ohtomo H, 1994, Effects of PF1022A from *Mycelia sterilia* on *Trichinella spiralis*. Jpn J Parasitol 43, 346-350.

146. Conder GA, Johnson SS, Nowakowski DS, Blake TE, Dutton FE, Nelson SJ, Thomas EM, Davis JP, Thompson DP, 1995, Anthelmintic profile of the cyclodepsipeptide PF1022A in *in vitro* and *in vivo* models. J Antibiot 48, 820-823.

147. Nicolay F, Harder A, von Samson-Himmelstjerna G, Mehlhorn H, 2000, Synergistic action of a cyclic depsipeptide and piperazine on nematodes. Parasitol Res 86, 982-992.

148. Mehlhorn H, Schmahl G, Frese M, Mevissen I, Harder A, Krieger K, 2005, Effects of a combinations of emodepside and praziquantel on parasites of reptiles and rodents. Parasitol Res 97, 65-69.

149. Schmahl G, Mehlhorn H, Harder A, Klimpel S, Krieger K, 2007, Efficacy of a combination of emodepside plus praziquantel against larval and adult stages of nematodes (*Trichuris muris*, *Angiostrongylus cantonensis*) in rodents. Parasitol Res 101, 77-84.

150. Traversa D, Milillo P, Di Cesare A, Lohr B, Iorio R, Pampurini F, Schaper R, Bartolini R, Heine J, 2009, Efficacy and safety of emodepside 2.1%/praziquantel 8.6% spot-on formulation in the treatment of feline aelurostrongylosis. Parasitol Res 105, 83-89.

151. Altreuther G, Borgsteede FH, Buch J, Charles SD, Cruthers L, Epe C, Young DR, Krieger KJ, 2005, Efficacy of a topically administered combination of emodepside and praziquantel against mature and immature *Ancylostoma tubaeforme* in domestic cats. Parasitol Res 97, 51-57.

152. Reinemeyer CR, Charles SD, Buch J, Settje T, Altreuther G, Cruthers L, McCall JW, Young DR, Epe C, 2005, Evaluation of the efficacy of emodepside plus praziquantel topical solution against ascarid infections (*Toxocara cati* or *Toxascaris leonina*) in cats. Parasitol Res 97, 41-50.

153. Schimmel A, Altreuther G, Schroeder I, Charles S, Cruthers L, Ketzis J, Kok DJ, Kraemer F, McCall JW, Krieger KJ, 2009, Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature adult *Ancylostoma caninum* and *Uncinaria stenocephala* infections in dogs. Parasitol Res 105, 9-16.

154. Altreuther G, Schimmel A, Schroeder I, Bach T, Charles S, Kok DJ, Kraemer F, Wolken S, Young D, Krieger KJ, 2009, Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature infections with *Toxocara canis* and *Toxascaris leonina* in dogs. Parasitol Res 105, 1-8.

155. Petry G, Altreuther G, Wolken S, Swart P, Kok DJ, 2013, Efficacy of emodepside plus toltrazuril oral suspension for dogs (Procox, Bayer) against *Trichuris vulpis* in naturally infected dogs. Parasitol Res 112, 133-138.

156. Schimmel A, Altreuther G, Schroeder I, Charles S, Cruthers L, Kok DJ, Kraemer F, Krieger KJ, 2009, Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature adult *Trichuris vulpis* infections in dogs. Parasitol Res 105, 17-22.

157. Zahner H, Taubert A, Harder A, von Samson-Himmelstjerna G, 2001, Effects of Bay 44-4400, a new cyclodepsipeptide, on developing stages of filariae (*Acanthochelonema viteae, Brugia malayi*, *Litomosoides sigmodontis*) in the rodent *Mastomys coucha*. Acta Trop 80, 19-28.

158. Zahner H, Taubert A, Harder A, von Samson-Himmelstjerna G, 2001, Filaricidal efficacy of the anthelmintically active cyclodepsipeptides. Int J Parasitol 31, 1515-1522.

159. Townson S, Freeman A, Harris A, Harder A, 2005, Acitivty of the cyclooctadepsipeptide emodepside against *Onchocerca gutturosa*, *Onchocerca lienalis* and *Brugia pahangi*. Am J Trop Med Hyg 73, 93.

160. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, Don R, Keiser J, 2011, Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5, e1138.

161. Geary TG, Woo K, McCarthy JS, Mackenzie CD, Horton J, Prichard RK, de Silva NR, Olliaro PL, Lazdins-Helds JK, Engels DA, Bundy DA, 2010, Unresolved issues in anthelmintic pharmacology for helminthiases of humans. Int J Parasitol 40, 1-13.

162. von Samson-Himmelstjerna G, Harder A, Sangster NC, Coles GC, 2005, Efficacy of two cyclooctadepsipeptides, PF1022A and emodepside, against anthelmintic-resistant nematodes in sheep and cattle. Parasitology 130, 343-347.

163. Pleiss U, Harder A, Turberg A, Londershausen M, Iinuma K, Mencke N, Jeschke P, Bonse G, 1996, Synthesis of a radiolabeled cyclodepsipeptide [3H-methyl] PF1022A. J Labelled Comp Radiopharm 38, 61-69.

164. Chen W, Terada M, Cheng JT, 1996, Characterization of subtypes of gamma-aminobutyric acid receptors in an *Ascaris muscle* preparation by binding assay and binding of PF1022A, a new anthelmintic, on the receptors. Parasitol Res 82, 97-101.
165. Holden-Dye L, Hewitt GM, Wann KT, Krogsgaard-Larsen P, Walker RJ, 1988, Studies involving avermectin and the 4-aminobutyric acid (GABA) receptor of *Ascaris suum* muscle. Pest Manag Sci 24, 231-245.
166. Miltsch SM, Krücken J, Demeler J, Janssen IJ, Krüger N, Harder A, von Samson-Himmelstjerna G, 2012, Decreased emodepside sensitivity in unc-49 gamma-aminobutyric acid (GABA)-receptor-deficient *Caenorhabditis elegans*. Int J Parasitol 42, 761-770.
167. Saeger B, Schmitt-Wrede HP, Dehnhardt M, Benten WP, Krücken J, Harder A, von Samson-Himmelstjerna G, Wiegand H, Wunderlich F, 2001, Latrophilin-like receptor from the parasitic nematode *Haemonchus contortus* as target for the anthelmintic depsipeptide PF1022A. FASEB J 15, 1332-1334.
168. Krüger N, Harder A, von Samson-Himmelstjerna G, 2009, The putative cyclooctadepsipeptide receptor depsiphilin of the canine hookworm *Ancylostoma caninum*. Parasitol Res 105, 91-100.
169. Welz C, Harder A, Schnieder T, Hoglund J, von Samson-Himmelstjerna G, 2005, Putative G protein-coupled receptors in parasitic nematodes - potential targets for the new anthelmintic class cyclooctadepsipeptides? Parasitol Res 97, 22-32.
170. Bull K, Cook A, Hopper NA, Harder A, Holden-Dye L, Walker RJ, 2007, Effects of the novel anthelmintic emodepside on the locomotion, egg-laying behaviour and development of *Caenorhabditis elegans*. Int J Parasitol 37, 627-636.
171. Holden-Dye L, O'Connor V, Hopper NA, Walker RJ, Harder A, Bull K, Guest M, 2007, SLO, SLO, quick, quick, slow: calcium-activated potassium channels as regulators of *Caenorhabditis elegans* behaviour and targets for anthelmintics. Invert Neurosci 7, 199-208.
172. Welz C, Krüger N, Schniederjans M, Miltsch SM, Krücken J, Guest M, Holden-Dye L, Harder A, von Samson-Himmelstjerna G, 2011, SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in *Caenorhabditis elegans* slo-1 loss of function mutants. PLoS Pathog 7, e1001330.

173. Crisford A, Murray C, O'Connor V, Edwards RJ, Krüger N, Welz C, von Samson-Himmelstjerna G, Harder A, Walker RJ, Holden-Dye L, 2011, Selective toxicity of the anthelmintic emodepside revealed by heterologous expression of human KCNMA1 in *Caenorhabditis elegans*. Mol Pharmacol 79, 1031-1043.

174. Buxton SK, Neveu C, Charvet CL, Robertson AP, Martin RJ, 2011, On the mode of action of emodepside: slow effects on membrane potential and voltage-activated currents in *Ascaris suum*. Br J Pharmacol 164, 453-470.

175. Shoop WL, Haines HW, Eary CH, Michael BF, 1992, Acute toxicity of paraherquamide and its potential as an anthelmintic. Am J Vet Res 53, 2032-2034.

176. Lee BH, Clothier MF, Johnson SS, 2001, Semi-synthesis of 2-deoxo- and 3-epi-paraherquamide A. Bioorg Med Chem Lett 11, 553-554.

177. Little PR, Hodges A, Watson TG, Seed JA, Maeder SJ, 2010, Field efficacy and safety of an oral formulation of the novel combination anthelmintic, derquantel-abamectin, in sheep in New Zealand. N Z Vet J 58, 121-129.

178. Qian H, Martin RJ, Robertson AP, 2006, Pharmacology of N-, L-, and B-subtypes of nematode nAChR resolved at the single-channel level in *Ascaris suum*. FASEB J 20, 2606-2608.

179. Rufener L, Bedoni N, Baur R, Rey S, Glauser DA, Bouvier J, Beech R, Sigel E, Puoti A, 2013, acr-23 Encodes a monepantel-sensitive channel in *Caenorhabditis elegans*. PLoS Pathog 9, e1003524.

180. Rufener L, Baur R, Kaminsky R, Maser P, Sigel E, 2010, Monepantel allosterically activates DEG-3/DES-2 channels of the gastrointestinal nematode *Haemonchus contortus*. Mol Pharmacol 78, 895-902.

181. Fox LM, Saravolatz LD, 2005, Nitazoxanide: a new thiazolide antiparasitic agent. Clin Infect Dis 40, 1173-1180.

182. Rossignol JF, Ayoub A, Ayers MS, 2001, Treatment of diarrhea caused by *Giardia intestinalis* and *Entamoeba histolytica* or *E. dispar*: a randomized, double-blind, placebo-controlled study of nitazoxanide. J Infect Dis 184, 381-384.

183. Rossignol JF, Ayoub A, Ayers MS, 2001, Treatment of diarrhea caused by *Cryptosporidium parvum*: a prospective randomized, double-blind, placebo-controlled study of Nitazoxanide. J Infect Dis 184, 103-106.

184. Somvanshi VS, Ellis BL, Hu Y, Aroian RV, 2014, Nitazoxanide: Nematicidal mode of action and drug combination studies. Mol Biochem Parasitol 193, 1-8.

185. Frei E, Eder JP, 2003, Combination chemotherapy, In: Kufe DW, Pollock RE, Weichelbaum RR, Bast RC, S. GT, Holland JF, Frei E (Eds.) Cancer Medicine 6. Decker Publishing, New York.
186. Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, Richman DD, Valentine FT, Jonas L, Meibohm A, Emini EA, Chodakewitz JA, 1997, Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med 337, 734-739.
187. Leathwick DM, Hosking BC, 2009, Managing anthelmintic resistance: modelling strategic use of a new anthelmintic class to slow the development of resistance to existing classes. N Z Vet J 57, 203-207.
188. Leathwick DM, Hosking BC, Bisset SA, McKay CH, 2009, Managing anthelmintic resistance: is it feasible in New Zealand to delay the emergence of resistance to a new anthelmintic class? N Z Vet J 57, 181-192.
189. Geary TG, Hosking BC, Skuce PJ, von Samson-Himmelstjerna G, Maeder S, Holdsworth P, Pomroy W, Vercruysse J, 2012, World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Guideline: Anthelmintic combination products targeting nematode infections of ruminants and horses. Vet Parasitol 190, 306-316.
190. Little PR, Hodge A, Maeder SJ, Wirtherle NC, Nicholas DR, Cox GG, Conder GA, 2011, Efficacy of a combined oral formulation of derquantel-abamectin against the adult and larval stages of nematodes in sheep, including anthelmintic-resistant strains. Vet Parasitol 181, 180-193.
191. Bazzocchi C, Mortarino M, Grandi G, Kramer LH, Genchi C, Bandi C, Genchi M, Sacchi L, McCall JW, 2008, Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against *Dirofilaria immitis* in experimentally infected dogs. Int J Parasitol 38, 1401-1410.
192. Grandi G, Quintavalla C, Mavropoulou A, Genchi M, Gnudi G, Bertoni G, Kramer L, 2010, A combination of doxycycline and ivermectin is adulticidal in dogs with naturally acquired heartworm disease (*Dirofilaria immitis*). Vet Parasitol 169, 347-351.
193. Barnes EH, Dobson RJ, Barger IA, 1995, Worm control and anthelmintic resistance: adventures with a model. Parasitol Today 11, 56-63.

194. Beach MJ, Streit TG, Addiss DG, Prospere R, Roberts JM, Lammie PJ, 1999, Assessment of combined ivermectin and albendazole for treatment of intestinal helminth and *Wuchereria bancrofti* infections in Haitian schoolchildren. Am J Trop Med Hyg 60, 479-486.

195. Knopp S, Mohammed KA, Speich B, Hattendorf J, Khamis IS, Khamis AN, Stothard JR, Rollinson D, Marti H, Utzinger J, 2010, Albendazole and mebendazole administered alone or in combination with ivermectin against *Trichuris trichiura*: a randomized controlled trial. Clin Infect Dis 51, 1420-1428.

196. Speich B, Ame SM, Ali SM, Alles R, Hattendorf J, Utzinger J, Albonico M, Keiser J, 2012, Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against *Trichuris trichiura* infection: a randomized controlled trial. PLoS Negl Trop Dis 6, e1685.

197. Namwanje H, Kabatereine NB, Olsen A, 2011, Efficacy of single and double doses of albendazole and mebendazole alone and in combination in the treatment of *Trichuris trichiura* in school-age children in Uganda. Trans R Soc Trop Med Hyg 105, 586-590.

198. Tritten L, Silbereisen A, Keiser J, 2012, Nitazoxanide: *In vitro* and *in vivo* drug effects against *Trichuris muris* and *Ancylostoma ceylanicum*, alone or in combination. Int J Parasitol Drugs Drug Resist 2, 98-105.

199. Keiser J, Tritten L, Silbereisen A, Speich B, Adelfio R, Vargas M, 2013, Activity of oxantel pamoate monotherapy and combination chemotherapy against *Trichuris muris* and hookworms: revival of an old drug. PLoS Negl Trop Dis 7, e2119.

Chapter 2

In vivo efficacy of the anthelmintic tribendimidine against the cestode *Hymenolepis microstoma* in a controlled laboratory trial

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2 *In vivo* efficacy of the anthelmintic tribendimidine against the cestode *Hymenolepis microstoma* in a controlled laboratory trial

2.1 Abstract

Tribendimidine has been registered for the treatment of human soil transmitted helminthiases in China. In the model nematode *C. elegans* it is an agonist of L-subtype nicotinic acetylcholine receptors and therefore shares its mode of action with levamisole and pyrantel. Besides its broad spectrum of nematicidal efficacy, tribendimidine is efficacious against several trematodes and has been attributed to have anti-cestodal effects. However, there are few published data available for the latter. The efficacy of tribendimidine and its nematicidal metabolite dAMD against *H. microstoma* were examined for their anti-cestodal potential. Doses of 50 and 100 mg/kg body weight dAMD and 10, 25, 50, and 100 mg/kg tribendimidine were administered orally on three consecutive days to mice experimentally infected with eight cysticercoids. Necropsy was performed and the worm burdens were determined one day after the last treatment. Furthermore, levamisole was used in combination with tribendimidine (100 mg/kg levamisole plus 10 and 25 mg/kg tribendimidine, respectively) and alone (50 and 100 mg/kg) to investigate any possible interactions of the partner compounds against cestodes. Tribendimidine showed a very high efficacy at dosages of 50 mg/kg or higher. Surprisingly, dAMD led to no reduction of the worm burden in any of the treatments. Combinations of levamisole with tribendimidine did not augment the effects of tribendimidine alone and as expected levamisole alone also showed no anti-cestodal activity. To our knowledge, this study shows for the first time activity of tribendimidine against a cestode in a controlled laboratory study. Due to the excellent cure rates observed here, multiple tribendimidine treatments might be considered as useful scheme for treatments of cestode, nematode and trematode infections although this would significantly increase both costs and management efforts. Moreover, the differences between tribendimidine and dAMD indicate at least a strong difference in sensitivity of *H. microstoma* or a strong difference in drug availability.

2.2 Introduction

Parasitic helminths cause a huge but frequently underestimated DALYs in particular under poor hygienic conditions [1]. In tropical and subtropical areas, soil transmitted nematodes (in particular the roundworm *A. lumbricoides*, the hookworms *A. duodenale* and *N. americanus*, and the whipworm *T. trichiura*) as well as some vector-borne filariae can result in life-threatening diseases and are therefore increasingly subject of health management and combat strategies mostly relying on systematic treatment of populations with either benzimidazoles or macrocyclic lactones [2-5]. In contrast, food-borne helminths, particularly trematodes and cestodes, are often still considered to be problems of only regional interest. At least tapeworms, however, occur all over the world, with the highest prevalence in developing countries [6]. Though there are an estimated 40 different cestode species affecting humans as definitive host, the four most common cestodes alone – *i.e. T. saginata*, *T. solium*, *H. nana*, and *D. latum* – are estimated to cause 170 – 200 million human infections [7]. Infections with adult tapeworms mostly result in negligible or only mild symptoms such as diarrhoea, abdominal discomfort and weight loss [8,9]. However, infections with larval stages of *Taenia solium* after oral ingestion of infectious eggs can result in life-threatening neurocysticercosis [7]. Furthermore, zoonoses caused by metacestode stages of *E. multilocularis* (alveolar echinococcosis) and *E. granulosus* (cystic echinococcosis) are widespread in the northern hemisphere or even globally, respectively. In particular, alveolar echinococcosis is lethal within 10-15 years if left untreated [10].

Treatment of adult intestinal cestode infections with a single dose of praziquantel, albendazole or niclosamide is usually highly effective [7]. However, the sudden onset and rapid spread of anthelmintic drug resistance have frequently been observed in the veterinary field [11] and should as well be considered realistic scenarios for the treatment of human helminthiases. This becomes increasingly probable with the onset of systematic treatment programs in highly endemic areas. Resistance to praziquantel has already been observed in schistosomes [12], benzimidazole resistance has been frequently reported in liver flukes [13] and both praziquantel and niclosamide resistance have been found in *T. saginata* [14, 15]. Accordingly, there is no reason to believe that drug resistance will not become a major drawback of mass treatment programs against cestodes. It is therefore highly desirable and forward-looking to meet the demand for anthelmintics with new modes of action that could be used as resistance-breaking emergence reserves if therapeutic benefit of current standard drugs might be compromised in the nearer future.

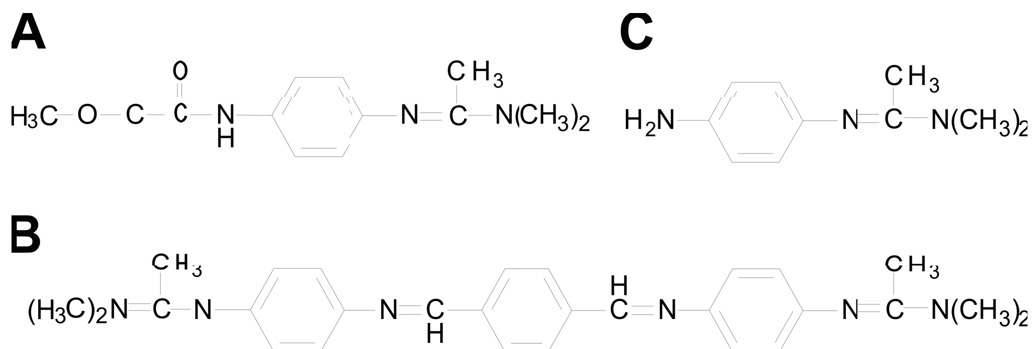

Fig. 2-1. Structure formula of A) amidantel, B) tribendimidine C) and dAMD

At the end of the 1970s, amidantel (Fig. 2-1A) was discovered and developed for treatments against hookworms [16]. In rodents it is highly efficacious against a wide range of filarial and gastrointestinal nematodes, *e.g. D. witei*, *H. bakeri*, *N. brasiliensis*, and *S. ratti* [17]. However, multiple (up to four) treatments with high dosages (up to 500 mg/kg body weight) were necessary to achieve a complete elimination of the worm burdens [17]. In addition, the same authors already reported at least a baseline activity against some platyhelminths. These early results promised a broad-spectrum anthelmintic activity of this drug class. A symmetrical diamidine derivative of amidantel, tribendimidine (Fig. 2-1B), was tested by the Chinese National Institute of Parasitic Diseases during the mid 1980s. Used at comparatively low dosages, tribendimidine showed high efficacy against a broad range of nematodes in rodents (50-200 mg/kg body weight), dogs (6-25 mg/kg body weight) and humans (200 mg for children or 400 mg tablets for adults) [26]. However, both amidantel and tribendimidine are rapidly metabolised to dAMD (Fig. 2-1C) [18, 19], which by itself exerts a strong nematicidal activity against both the model organism *C. elegans* and parasitic nematodes [20, 21]. After several years of further experimental research and clinical investigations, tribendimidine has been registered for the treatment of human soil transmitted helminthiases caused by *A. lumbricoides*, *A. duodenale*, *N. americanus*, and *E. vermicularis* in the People's Republic of China in 2004 [16]. For humans, the use of the oral tablet is considered to be safe and only very light and transient adverse effects if any have been reported [22]. Studies on toxicity have been summarised by Xiao *et al.* [16]. In brief, acute toxicity after orral or intraperitoneal administration to mice was determined with an LD_{50} of 950 ± 207 mg/kg bw and 277 ± 27 mg/kg bw, respectively. In rats, the oral LD_{50} was even 2001 ± 79 mg/kg bw.

Moreover, rats had no detectable side effects up to 250 mg/kg given orally on 14 consecutive days indicating absence of long-term toxicity. Also, no toxic effects were observed for dogs orally receiving 30 mg/kg bw on 14 consecutive days. In nematodes, tribendimidine apparently acts as an agonist of the L-subtype nAChR, as revealed by investigations using loss-of-function mutants in *C. elegans*. This suggests that the mode of action against nematodes is very similar to the one of levamisole and pyrantel [23]. However, synergistic effects of levamisole and tribendimidine have recently been reported against *A. ceylanicum in vitro* and *in vivo* [21] and were also detected by the authors in a *N. brasiliensis* rat model (unpublished data). Synergistic activity suggests that the modes of action are at least not completely identical. Otherwise, only additive effects would be expected.

In addition to its good efficacy against gastrointestinal nematodes, tribendimidine has been described to be efficacious against at least some trematodes (*i.e. E. caproni*, *C. sinensis*, *O. viverrini*) whereas no effects on infections with *F. hepatica* and *S. mansoni* were found [16, 24, 25]. Though there were early reports on moderate activity of amidantel against the cestode *H. diminuta* but not against *H. nana* [17], these reports were somewhat informal without any detailed information on procedures and data except for an ED_{99} value. Efficacy of tribendimidine against unstated cestodes in chicken is also only circumstantially mentioned in a review but detailed data are not available [16]. Moreover, some activity of tribendimidine against human intestinal taeniasis has been found in an open-labelled randomised clinical trial [26].

To further corroborate the potential of tribendimidine as a broad-spectrum anthelmintic in multi-dose treatment schedules, the present study aimed to evaluate the efficacy of tribendimidine against the mouse bile duct tapeworm *H. microstoma*, a well-established rodent model. Adult stages of *H. microstoma* are located in the *Ductus choledochus* (common bile duct) of mice [27]. Besides the evaluation of the anti-cestodal efficacy of tribendimidine against the adult mouse bile duct tapeworm, the study intended to compare tribendimidine with its nematicidal metabolite dAMD.

Despite the fact that levamisole alone has no significant effects on tapeworms, levamisole has been reported to potentiate the effects of mebendazole against *M. corti* [28]. Therefore and because of the synergistic interactions of tribendimidine plus levamisole against *A. ceylanicum in vitro* and *in vivo* [21], activities of tribendimidine-levamisole-combinations and levamisole were evaluated to identify any potential additive or even synergistic interactions between these drugs in cestodes.

Tab. 2-1. Published tribendimidine dosages and cure rates

Parasite	Host	TBD dosage[a]	Cure rate	Reference
Nematodes:				
Ascarids:				
Ascaris lumbricoides	Humans	1× 300 mg	95.0%	[42]
Toxocara canis	Dogs	1× 12 mg/kg bw	100.0%	[16]
Strongylids:				
Ancylostoma caninum	Dogs	1× 12 mg/kg bw	98.8%	[16]
Ancylostoma duodenale	Humans	1× 400 mg	84.2%	[16]
Necator americanus	Humans	1× 400 mg	89.8%	[16]
Necator americanus	Hamster	1× 100 mg	99.7%	[16]
Nippostrongylus brasiliensis	Rats	1× 50 mg	100.0%	[16]
Strongyloides ratti	Rats	1× 50 mg	100.0%	[45]
Strongyloides stercoralis	Humans	1× 400 mg	10.5%	[26]
Oxyurids:				
Enterobius vermicularis	Humans	1× 200 mg	74.1%	[43]
Syphacia mesocriceti	Mouse	1× 200 mg/kg bw	94.5%	[16]
Trichocephalids:				
Trichinella spiralis[b]	Mice	6× 300 mg/kg bw	98.6%	[44]
Trichuris trichiura	Humans	3× 400 mg	33,3%	[43]
Trichuris trichiura	Humans	1× 400 mg	76.8%	[42]
Trematodes:				
Clonorchis sinensis	Rats	1× 150 mg/kg bw	99.1%	[25]
Fasciola hepatica	Rats	1× 800 mg/kg bw	0.0%	[25]
Echinostoma caproni	Mice	1× 125 mg/kg bw	100.0%	[24]
Opisthorchis viverrini	Hamster	1× 400 mg/kg bw	95.7%	[25]
Paragonimus westermani	Dogs	6× 17 mg/kg bw	0.0%	[41]
Schistosoma mansoni	Mice	1× 400 mg/kg bw	0.0%	[25]
Cestodes:				
Hymenolepis microstoma	Mice	3× 50 mg/kg bw	97.8%	
Taenia spp.	Humans	1× 400 mg	66.7%	[26]

If not otherwise indicated, treatments were always against mature stages and oral route of administration was used. [a]Dosages are either given as mg/kg body weight (bw) or as total amount of tribendimidine (TBD) per individual in mg. [b]Encapsulated larvae

2.3 Materials and methods

Drugs

Levamisole was purchased from Sigma-Aldrich (Munich, Germany). Tribendimidine and dAMD were obtained from Shandong Xinhua Pharmaceutical Company Limited (Zibo, People's Republic of China) and Bayer Animal Health GmbH (Leverkusen, Germany), respectively. Drugs were always freshly prepared on the days of treatment as dispersions in Cremophor EL (BASF, Ludwigshafen, Germany) and deionised-water [2:3]. Drug concentrations were adjusted to ensure an application volume of 1 ml Cremophor EL/water per 100 g body weight.

Animal experiments

All presented *in vivo* studies were carried out at the laboratories of Bayer HealthCare AG, Global Drug Discovery Animal Health (Monheim, Germany). The prospective experiments (No. 200/V14, reference number 50.05-230-1-43/01) were registered and approved by the State Office for Nature, Environment, Agriculture, and Consumer Protection, North Rhine-Westphalia, Germany (Landesamt für Natur-, Umwelt- und Verbraucherschutz Nordrhein-Westfalen; LANUV) in accordance with §8a section 1 and 2, German Protection of Animals Act.

Mice were used as definitive hosts for the experimental infections with *H. microstoma* cysticercoids. The lifecycle of *H. microstoma* is maintained at the laboratories of Bayer since the 1960s and the strain is of unknown origin. Female laboratory outbred NMRI mice were purchased from Harlan Netherlands at an age of 10-12 weeks with a body weight of 20-25 g. The mice were housed in Macrolon® cages under environmentally controlled conditions and kept in groups of five animals. Water and SNIFF rodent food pellets were available *ad libitum*. In five consecutive blocks, a total of 100 mice were randomised into 20 groups, consisting of five mice each. For reasons of identification within a cage, mice were individually marked by pen marks on their tails. After seven days of acclimatisation, mice were infected orally with a gavage using 0.5 ml physiological sodium chloride solution containing eight infective cysticercoids of *H. microstoma*. To obtain cysticercoids, five sober adult mealworm beetles (*Tenebrio molitor*) were fed with mature proglottids obtained from freshly isolated adult *H. microstoma*. Twelve days *post infectionem (p.i.)* beetles were dissected in a petri dish containing physiological sodium chloride solution. Cysticercoids were mechanically isolated and drawn up into the gavage.

Treatment-dosages were calculated for each animal depending on individual body weights determined on day 33 *p.i.* On days 34-36 *p.i.* mice were orally treated with Cremophor EL/water dispersions to achieve dosages of 10 mg/kg, 25 mg/kg, 50 mg/kg, or 100 mg/kg tribendimidine, 50 mg/kg or 100 mg/kg dAMD, and 50 mg/kg or 100 mg/kg body weight levamisole. Dosages of levamisole of 100 mg/kg were used since the IPCS INCHEM database of the WHO mentions a LD_{50} of 205-285 mg/kg for acute toxicity of levamisole in mice whereas 100 mg/kg did not result in any signs of toxicity. The negative-control groups received Cremophor EL/water only. In addition, two combinations were administered resulting either in 10 mg/kg tribendimidine plus 100 mg/kg levamisole or in 25 mg/kg tribendimidine plus 100 mg/kg levamisole. On day 37 *p.i.*, mice were euthanised by carbon dioxide suffocation. Subsequently, necropsy was performed and the worm burdens in the common bile ducts and the duodenum were determined.

Efficacy determination and statistical analysis

Numbers of worms found in treatment groups were compared to the controls using the non-parametric Kruskal-Wallis test with Dunn's post hoc test for identification of significant differences between individual groups. No significant differences between controls were observed when the five experimental blocks were compared. Therefore, worm counts from different experiments were pooled and treated as a single block design experiment. Calculations were performed using GraphPad Prism software version 5.03.

2.4 Results

Effects of nematode acetylcholine receptor agonists on H. microstoma worm burden

For all treatment groups and negative-control groups of five consecutive blocks worm counts, mean worm burdens and standard deviations are summarised in Tab. 2-2. In addition, for all treatment groups percentages of efficacy against *H. microstoma* with reference to the respective control-group are given (Tab. 2-2). In the following, all groups which were treated with the same drug dosages were totalled up for further analysis resulting in ten mice for 10 mg/kg, ten mice for 25 mg/kg, ten mice for 50 mg/kg, five mice for 100 mg/kg tribendimidine, five mice for 50 mg/kg, five mice for 100 mg/kg dAMD, five mice for 50 mg/kg, 15 mice for 100 mg/kg levamisole and 25 mice for negative-control.

In control animals, which received only the vehicle, the adult worm burdens were 4.5 ± 2.2 (mean ± SD) (median = four worms) isolated almost exclusively from the *Ductus choledochus*. Thus, about 50% of the cysticercoids reached maturity in the control group and survived until day 37 *p.i.* Only in mice with very high worm burden, posterior ends of one or a few *H. microstoma* protruded into the small intestinal lumen via the major duodenal papilla.

Treatment of infected mice with tribendimidine resulted in a dose-dependent reduction in the number of worms recovered (Fig. 2-2A). While dosages of 10 mg/kg did not result in any apparent effects, 25 mg/kg already reduced the mean worm burden by 57.8% (1.9 ± 2.6; mean ± SD) though this was still not statistically significant due to large variability between individual mice. At 50 mg/kg and 100 mg/kg tribendimidine, however, treatment led to significantly reduced median numbers of cestodes with almost no (one worm in ten mice) or no worms surviving the drug treatment, respectively.

In sharp contrast, the same treatment protocol using the nematicidal tribendimidine metabolite dAMD did not have any effects on cestode burdens (Tab. 2-2 and Fig. 2-2A). After three treatments with either 50 mg or 100 mg dAMD per kg body weight, the number of adult cestodes found in the common bile duct was not significantly different from the negative control (Fig. 2-2A). Instead, mean and median numbers of worms per animal were even slightly, though not significantly, increased when compared to the control. As expected, the nematocide levamisole also did not affect *H. microstoma* at 50 mg/kg or 100 mg/kg (Tab. 2-2 and Fig. 2-2A).

Tab. 2-2. Design and outcome of individual animal experiments

	Worms/mouse	Mean ± SD	% reduction
1st Block:			
Control	2; 4; 4; 5; 6;	4.2 ± 1.5	
50 mg/kg deacylated amidantel	2; 5; 5; 6; 7;	5.0 ± 1.9	0.0
50 mg/kg tribendimidine	0; 0; 0; 0; 1;	0.2 ± 0.5	95.2 ± 11.9
50 mg/kg levamisole	3; 4; 4; 5; 5;	4.2 ± 0.8	0.0
2nd Block:			
Control	1; 1; 4; 5; 7;	3.6 ± 2.6	
50 mg/kg tribendimidine	0; 0; 0; 0; 0;	0.0	100.0
100 mg/kg tribendimidine	0; 0; 0; 0; 0;	0.0	100.0
3rd Block:			
Control	3; 4; 4; 7; 7;	5.0 ± 1.9	
100 mg/kg levamisole	1; 4; 5; 6; 6;	4.4 ± 2.1	12.0 ± 42.0
10 mg/kg tribendimidine	1; 2; 5; 5; 7;	4.0 ± 2.5	20.0 ± 50.0
25 mg/kg tribendimidine	0; 0; 0; 2; 8;	2.0 ± 3.5	60.0 ± 70.0
4th Block:			
Control	1; 1; 2; 5; 7;	3.2 ± 2.7	
100 mg/kg deacylated amidantel	3; 5; 5; 6; 8;	5.4 ± 1.8	0.0
10 mg/kg tribendimidine	2; 4; 6; 7; 7;	5.2 ± 2.2	0.0
100 mg/kg levamisole	2; 4; 4; 5; 5;	4.0 ± 1.2	0.0
100 mg/kg levamisole + 10 mg/kg tribendimidine	1; 2; 2; 3; 7;	3.0 ± 2.4	6.3 ± 75.0
5th Block:			
Control	4; 6; 7; 7; 8;	6.4 ± 1.5	
25 mg/kg tribendimidine	0; 1; 1; 3; 4;	1.8 ± 1.6	71.9 ± 25.0
100 mg/kg levamisole	1; 3; 5; 6; 8;	4.6 ± 2.7	28.1 ± 42.2
100 mg/kg levamisole + 25 mg/kg tribendimidine	0; 0; 0; 5; 6;	2.2 ± 3.0	65.6 ± 46.9

Numbers in the column worms/mouse represent parasite numbers recovered from individual animals. Arithmetic means of worm number per group are shown. The percent reduction was calculated relative to the control from the same experimental block.

Effects of drug combination

Tribendimidine and levamisole were combined at suboptimal dosages of tribendimidine to identify any potential interaction. Again, 10 mg/kg tribendimidine did not show any effects against *H. microstoma* while 25 mg/kg reduced the worm burden by about 71.9% ± 25.0% though the effect was again not statistically significant (Tab. 2-2 and Fig. 2-2B). Combinations of 10 mg/kg or 25 mg/kg tribendimidine with 100 mg/kg levamisole were not more effective than the same dosages of tribendimidine alone (Tab. 2-2 and Fig. 2-2B).

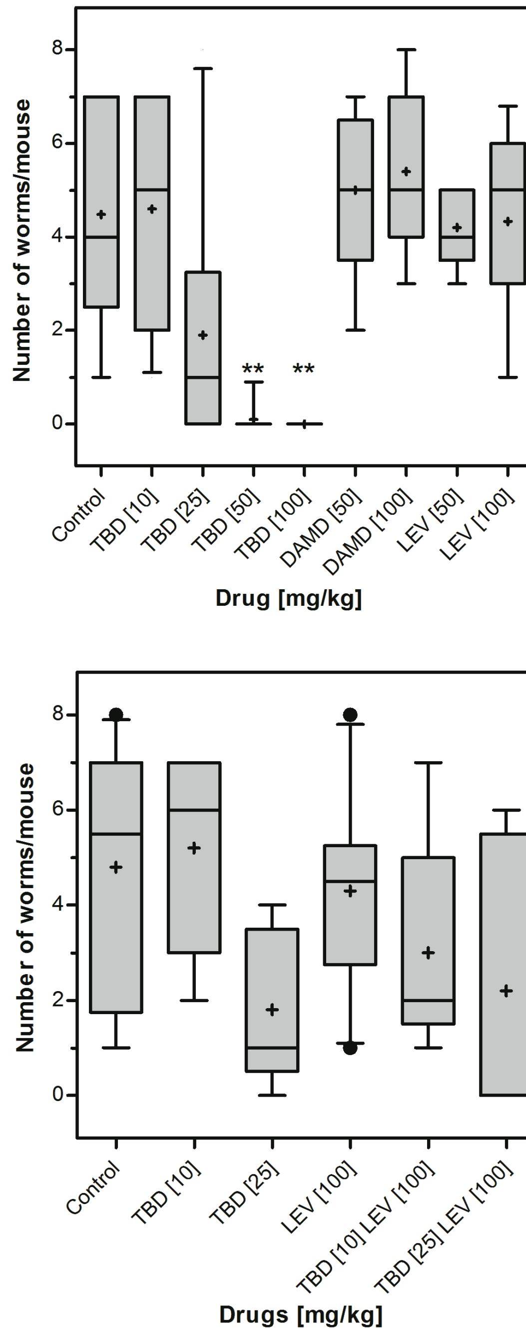

Fig. 2-2. Effects of A) monotherapies and B) drug combinations on the worm burden

Box plots show the median numbers and quartiles of recovered adult *H. microstoma* with whiskers representing 10% and 90% quantiles. Group sizes were between five and 25 animals for every dose. + arithmetic mean; ** *P*<0.01 vs. control; ● outliers. LEV, levamisole; TBD, tribendimidine. Dosages shown in square brackets are given in mg/kg body weight.

2.5 Discussion

Though activity of amidantel against certain cestodes has already been claimed in the original publication [17], detailed results have not been published for any cestode – at least in terms of worm counts. The only publications dealing with cestodicidal activity of tribendimidine are a review, mentioning efficacy against various chicken cestodes [16] and a clinical field trial reporting some effects on *Taenia* spp. [26]. Here we demonstrate a clear dose-dependent anti-cestodal activity of tribendimidine against the mouse bile duct tapeworm *H. microstoma*. Three treatments with dosages of 50 mg/kg and 100 mg/kg body weight achieved nearly complete and complete parasite clearance, respectively. In rodents these dosages are in the middle range of the heterogenic spectrum of dosages that are also effective against nematodes and trematodes, starting with 1× 50 mg/kg against *N. brasiliensis* in rats and ending with 6× 300 mg/kg against *T. spiralis* in mice (Tab. 2-1), indicating that tribendimidine might indeed have a high potential as broad-spectrum anthelmintic also targeting cestodes. Steinmann *et al.* [26] demonstrated a higher efficacy of tribendimidine than of albendazole against *Taenia* spp. in humans after single oral treatment but cure rates for both drugs were too low (66.7% for tribendimidine) for a routine use against human taeniasis. Therefore, they suggested evaluating the efficacy of multiple-dose treatments with tribendimidine to cure human intestinal taeniasis and infections with *S. stercoralis*. Thus, the excellent cure rates observed here with three consecutive treatments corroborate that multiple treatments should be considered as useful scheme for treatments of human helminthiases although this would of course significantly increase both costs and management efforts.

Tribendimidine is rapidly metabolised to dAMD, which is by itself a very potent nematocide [20,21] and flukicide [29]. In *C. elegans*, activation of L-type AChRs has been demonstrated to be crucial for tribendimidine and levamisole activity *in vitro* [23]. Tribendimidine and dAMD have the same mode of action in *C. elegans* as suggested by early electrophysiological evidence [20]. Furthermore, mutations in AChR genes have been implicated in resistance to amidantel and dAMD [30] as well as in resistance to tribendimidine and levamisole [23]. This further substantiates the view that in nematodes both tribendimidine and dAMD are active by sharing the same mode of action with levamisole via AChR.

In the trematode *C. sinensis*, *in vitro* experiments revealed similar activity of dAMD and of tribendimidine suggesting that in this case also both drugs are highly active [29]. This suggests that a substantial part of the anthelmintic action of tribendimidine is in fact exerted by its metabolite dAMD. In contrast, the results of the present study show that dAMD is not

active at all against *H. microstoma* at twice the concentration that is sufficient for full activity of tribendimidine. Two different explanations should be considered to explain these apparently conflicting results. First, the mode of action of tribendimidine might differ between nematodes and trematodes on one side and cestodes on the other side. Secondly, the lower hydrophobicity of dAMD in comparison to tribendimidine might result in a different distribution of the drug among host tissues. This could result in non-sufficient concentrations in the common bile duct. *In vitro* experiments will be required to distinguish between the possibilities that concentrations of dAMD achieved in the bile duct after oral administration are not sufficiently high to kill *H. microstoma* or if dAMD is by itself ineffective against *H. microstoma*.

Remarkably, acetylcholine acts as an inhibitory neurotransmitter on isolated *H. microstoma* muscle preparations [31], but also causes flaccid paralysis in *F. hepatica* [32] suggesting that inhibitory acetylcholine receptors are not unique to cestodes but presumably widely distributed among platyhelminths. Currently, knowledge about platyhelminth acetylcholine receptors is only very sparse. Even for *Schistosoma* spp. only three very atypical acetylcholine receptors have been described [33, 34], and a tBLASTn search in *Echinococcus* genome databases did not identify any obvious homologs in cestodes. Our knowledge has to improve considerably before we can discuss similarities and differences between trematodes and cestodes that might contribute to the differences in the mode of action of tribendimidine and dAMD. However, if differences in acetylcholine signalling would be the reason for the observed differences in efficacy of tribendimidine and dAMD, then it would be expected that nematodes differ from platyhelminths and not that cestodes differ from trematodes and nematodes.

The nematocide levamisole, a nicotinergic agonist, is not known to exhibit any effects against trematodes or cestodes [28, 35-37]. Nevertheless, since synergism between levamisole and mebendazole against *M. corti* has been reported previously [28] and while tribendimidine and levamisole have very similar modes of action against parasitic nematodes, we were interested in a possible synergistic effect against *H. microstoma*. As expected, levamisole alone did not affect *H. microstoma* at 100 mg/kg and also did not show any additional or synergistic effects in combination with suboptimal tribendimidine dosages. The differences between levamisole and tribendimidine can be explained by (i) presence of AChRs in cestodes that are tribendimidine sensitive but levamisole insensitive, (ii) highly ineffective uptake of levamisole by cestodes or (iii) a completely different mode of action of tribendimidine in cestodes than in nematodes. However, since the AChR agonist pyrantel is known to be active

against the equine cestodes *Anoplocephala* spp. [38, 39], combinations of tribendimidine with pyrantel should be evaluated in future experiments.

If a completely different mode of action of tribendimidine in cestodes is considered, tribendimidine metabolism should be taken into account. Tribendimidine is not detectable in plasma samples since it is very rapidly cleaved into dAMD and terephthalaldehyde (TPAL) in humans [19] and tribendimidine is also not detectable in the plasma of rats (Kulke *et al.*, unpublished data). TPAL, however, is also very unstable *in vivo* and is metabolised to terephalic acid (TPAC). In plasma samples, only dAMD and TPAC are detectable using high performance liquid chromatography followed by mass spectrometry [19]. This situation might be different in the gastrointestinal system since small amounts of tribendimidine have been shown to be excreted with the faeces [16]. In contrast to dAMD and tribendimidine, TPAL and TPAC are inactive against nematodes (*N. americanus*, *N. brasiliensis*) and trematodes (*C. sinensis*) [29, 40].

Since tribendimidine is very rapidly metabolised to dAMD, optimisation of application routes and derivatisation of tribendimidine to obtain a more stable cestocide might be important parameters for future development of this drug. In addition, even for very closely related cestodes and trematodes, the optimal drug derivative and formulation might depend on the tissue localisation.

2.6 Conclusions

The present study provides for the first time experimental evidence for a high efficacy of tribendimidine against cestodes in a controlled laboratory system. The excellent cure rates observed here with three consecutive treatments of 50 mg/kg tribendimidine corroborate the suggestion of Steinmann *et al.* [26] that multiple treatments should be considered as useful scheme for treatments of helminthiases.

The fact that only tribendimidine but not its nematicidal metabolite dAMD is active in this model and with the concentrations used is currently difficult to interpret due to our only very limited knowledge about the mode of tribendimidine action in platyhelminths. Since acetylcholine signalling in nematodes and platyhelminths differ substantially, a direct transfer of knowledge obtained from *C. elegans* is not feasible.

2.7 Financial support

The presented study was funded by the Bayer HealthCare AG. The Bayer HealthCare AG sells anthelmintics for the use in veterinary medicine.

2.8 Conflict of interest

A. Harder and C. Welz are employees of Bayer HealthCare AG. D. Kulke was also employee of Bayer HealthCare AG, when the study was conducted. Except of the authors, Bayer HealthCare AG had no influence on the design of the study and the decision to publish the results.

2.9 References

1. Hotez PJ, Bundy DAP, Beegle K, Brooker S, Drake L, de Silva N, Montresor A, Engels D, Jukes M, Chitsulo L, Chow J, Laxminarayan R, Michaud C, Bethony J, Correa-Oliveira R, Shuhua X, Fenwick A, Savioli L, 2006, Helminth Infections: Soil-transmitted Helminth Infections and Schistosomiasis, In: Jamison DT, Breman JG, Measham AR, Alleyne G, Claeson M, Evans DB, Jha P, Mills A, Musgrove P (Eds.), Disease control priorities in developing countries. Oxford University Press, New York, 467-482.
2. Crompton DWT, World Health Organization, 2006, Preventive chemotherapy in human helminthiasis: coordinated use of anthelminthic drugs in control interventions: a manual for health professionals and programme managers. World Health Organization, Geneva, 1-62.
3. Diawara L, Traore MO, Badji A, Bissan Y, Doumbia K, Goita SF, Konate L, Mounkoro K, Sarr MD, Seck AF, Toe L, Touree S, Remme JH, 2009, Feasibility of onchocerciasis elimination with ivermectin treatment in endemic foci in Africa: first evidence from studies in Mali and Senegal. PLoS Negl Trop Dis 3, e497.
4. Moncayo AL, Vaca M, Amorim L, Rodriguez A, Erazo S, Oviedo G, Quinzo I, Padilla M, Chico M, Lovato R, Gomez E, Barreto ML, Cooper PJ, 2008, Impact of long-term treatment with ivermectin on the prevalence and intensity of soil-transmitted helminth infections. PLoS Negl Trop Dis 2, e293.
5. Winnen M, Plaisier AP, Alley ES, Nagelkerke NJ, van Oortmarssen G, Boatin BA, Habbema JD, 2002, Can ivermectin mass treatments eliminate onchocerciasis in Africa? Bull World Health Organ 80, 384-391.
6. Harder A, 2002, Chemotherapeutic approaches to trematodes (except schistosomes) and cestodes: current level of knowledge and outlook. Parasitol Res 88, 587-590.
7. Craig P, Ito A, 2007, Intestinal cestodes. Curr Opin Infect Dis 20, 524-532.
8. Dorny P, Praet N, Deckers N, Gabriel S, 2009, Emerging food-borne parasites. Vet Parasitol 163, 196-206.
9. Min DY, 1990, Cestode infections in Korea. Kisaengchunghak Chapchi 28, 123-144.
10. Deplazes P, van Knapen F, Schweiger A, Overgaauw PA, 2011, Role of pet dogs and cats in the transmission of helminthic zoonoses in Europe, with a focus on echinococcosis and toxocarosis. Vet Parasitol 182, 41-53.
11. Sangster NC, 2001, Managing parasiticide resistance. Vet Parasitol 98, 89-109.

12. Doenhoff MJ, Cioli D, Utzinger J, 2008, Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Curr Opin Infect Dis 21, 659-667.
13. Brennan GP, Fairweather I, Trudgett A, Hoey E, McCoy, McConville M, Meaney M, Robinson M, McFerran N, Ryan L, Lanusse C, Mottier L, Alvarez L, Solana H, Virkel G, Brophy PM, 2007, Understanding triclabendazole resistance. Exp Mol Pathol 82, 104-109.
14. Koul PA, Waheed A, Hayat M, Sofi BA, 1999, Praziquantel in niclosamide-resistant *Taenia saginata* infection. Scand J Infect Dis 31, 603-604.
15. Lateef M, Zargar SA, Khan AR, Nazir M, Shoukat A, 2008, Successful treatment of niclosamide- and praziquantel-resistant beef tapeworm infection with nitazoxanide. Int J Infect Dis 12, 80-82.
16. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W, 2005, Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94, 1-14.
17. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, Thomas H, 1979, Amidantel, a potent anthelminthic from a new chemical class. Arzneim Forsch 29, 31-32.
18. Yuan G, Wang B, Wei C, Zhang R, Guo R, 2008, LC-MS Determination of *p*-(1-Dimethylamino ethylimino)aniline: a Metabolite of Tribendimidine in Human Plasma. Chromatographia 68, 139-142.
19. Yuan G, Xu J, Qu T, Wang B, Zhang R, Wei C, Guo R, 2010, Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs R D 10, 83-90.
20. Tomlinson G, Albuquerque CA, Woods RA, 1985, The effects of amidantel (BAY d 8815) and its deacylated derivative (BAY d 9216) on *Caenorhabditis elegans*. Eur J Pharmacol 113, 255-262.
21. Tritten L, Nwosu U, Vargas M, Keiser J, 2012, *In vitro* and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. Acta Trop 122:101-107.
22. Xiao SH, Wu ZX, Zhang JH, Wang SQ, Wang SH, Qiu DC, Wang C, 2007, Clinical observation on 899 children infected with intestinal nematodes and treated with tribendimidine enteric coated tablets. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 25, 372-375.

23. Hu Y, Xiao SH, Aroian RV, 2009, The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3, e499.
24. Keiser J, Shu-Hua X, Utzinger J, 2006, Effect of tribendimidine on adult *Echinostoma caproni* harbored in mice, including scanning electron microscopic observations. J Parasitol 92, 858-862.
25. Keiser, J, Shu-Hua X, Chollet J, Tanner M, Utzinger J, 2007, Evaluation of the *in vivo* activity of tribendimidine against *Schistosoma mansoni, Fasciola hepatica, Clonorchis sinensis,* and *Opisthorchis viverrini*. Antimicrob Agents Chemother 51, 1096-1098.
26. Steinmann P, Zhou XN, Du ZW, Jiang JY, Xiao SH, Wu ZX, Zhou H, Utzinger J, 2008, Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. PLoS Negl Trop Dis 2, e322.
27. Cunningham LJ, Olson PD, 2010, Description of *Hymenolepis microstoma* (Nottingham strain): a classical tapeworm model for research in the genomic era. Parasit Vectors 3, 123.
28. Bennet EM, Behm C, Bryant C, 1978, Effects of mebendazole and levamisole on tetrathyridia of *Mesocestoides corti* in the mouse. Int J Parasitol 8, 463-466.
29. Xiao SH, Xue J, Xu LL, Zheng Q, Qiang HQ, Zhang YN, 2009, The *in vitro* and *in vivo* effect of tribendimidine and its metabolites against *Clonorchis sinensis*. Parasitol Res 105, 1497-1507.
30. Woods RA, Malone KMB, Albuquerque CA, Tomlinson G, 1986, The Effects of Amidantel (Bay D 8815) and Its Deacylated Derivative (Bay D 9216) on Wild-Type and Resistant Mutants of *Caenorhabditis elegans*. Can J Zool 64, 1310-1316.
31. Thompson CS, Mettrick DF, 1984, Neuromuscular physiology of *Hymenolepis diminuta* and *H. microstoma* (Cestoda). Parasitology 89, 567-578.
32. Chance MR, Mansour TE, 1953, A contribution to the pharmacology of movement in the liver fluke. Br J Pharmacol Chemother 8, 134-138.
33. Bentley GN, Jones AK, Oliveros Parra WG, Agnew A, 2004, ShAR1alpha and ShAR1beta: novel putative nicotinic acetylcholine receptor subunits from the platyhelminth blood fluke *Schistosoma*. Gene 329, 27-38.

34. Bentley GN, Jones AK, Agnew A, 2007, ShAR2beta, a divergent nicotinic acetylcholine receptor subunit from the blood fluke *Schistosoma*. Parasitology 134, 833-840.
35. Rabbani GH, Gilman RH, Kabir I, Mondel G, 1985, The treatment of *Fasciolopsis buski* infection in children: a comparison of thiabendazole, mebendazole, levamisole, pyrantel pamoate, hexylresorcinol and tetrachloroethylene. Trans R Soc Trop Med Hyg 79, 513-515.
36. Smith MC, Sherman DM, 2009, Goat medicine, Wiley-Blackwell, Ames, Iowa, 454-456.
37. Sturchler D, 1982, Chemotherapy of human intestinal helminthiases: a review, with particular reference to community treatment. Adv Pharmacol Chemother 19, 129-154.
38. Marchiondo AA, White GW, Smith LL, Reinemeyer CR, Dascanio JJ, Johnson EG, Shugart JI, 2006, Clinical field efficacy and safety of pyrantel pamoate paste (19.13% w/w pyrantel base) against *Anoplocephala* spp. in naturally infected horses. Vet Parasitol 137, 94-102.
39. Reinemeyer CR, Hutchens DE, Eckblad WP, Marchiondo AA, Shugart JI, 2006, Dose-confirmation studies of the cestocidal activity of pyrantel pamoate paste in horses. Vet Parasitol 138, 234-239.
40. Xue J, Utzinger J, Zhang YN, Tanner M, Keiser J, Xiao SH, 2008, Artemether and tribendimidine lack activity in experimental treatment of *Paragonimus westermani* in the dog. Parasitol Res 102, 537-540.
41. Xue J, Xiao SH, Xu LL, Qiang HQ, 2010, The effect of tribendimidine and its metabolites against *Necator americanus* in golden hamsters and *Nippostrongylus braziliensis* in rats. Parasitol Res 106, 775-781.
42. Zhang JH, Xiao SH, Wu ZX, Qiu DC, Wang SH, Wang SQ, Wang C, 2008, Tribendimidine enteric coated tablet in treatment of 1,292 cases with intestinal nematode infection--a phase IV clinical trial. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 26, 6-9.
43. Wu ZX, Fang YY, Liu YS, 2006, Effect of a novel drug - enteric coated tribendimidine in the treatment of intestinal nematode infections. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 24, 23-26.

44. Li RH, Gao JH, Wang SF, Pei YJ, Shen JY, Yan PM, Yin GR, 2011, Effect of oral administration of tribendimidine at different dosages against *Trichinella spiralis* encapsulated larvae in mice. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 29, 117-121.
45. Keiser J, Thiemann K, Endriss Y, Utzinger J, 2008, *Strongyloides ratti*: *in vitro* and *in vivo* activity of tribendimidine. PLoS Negl Trop Dis 2, e136.

Chapter 3

In vitro efficacy of cyclooctadepsipeptides and aminophenylamidines alone and in combination against third-stage larvae and adult worms of *Nippostrongylus brasiliensis* and first-stage larvae of *Trichinella spiralis*

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3 *In vitro* efficacy of cyclooctadepsipeptides and aminophenylamidines alone and in combination against third-stage larvae and adult worms of *Nippostrongylus brasiliensis* and first-stage larvae of *Trichinella spiralis*

3.1 Abstract

The present study investigates the *in vitro* efficacy of derivatives of the cyclooctadepsipeptides and the aminophenylamidines, which are promising candidates for the evaluation of the treatment of human soil-transmitted helminthiases.

The effects of emodepside and PF1022A as well as of amidantel, dAMD and tribendimidine were evaluated in a concentration range between 0.01 and 100 µg/ml against L3 and adult worms of *N. brasiliensis* and L1 of *T. spiralis.* Furthermore, drug combinations of PF1022A plus dAMD or tribendimidine and of tribendimidine plus levamisole were tested for any potential additive or even synergistic interactions.

Emodepside had a significantly lower EC_{50} value than PF1022A in the *T. spiralis* (0.02788 vs. 0.05862 µg/ml) and the *N. brasiliensis* (0.06188 vs. 0.1485 µg/ml) motility assays but not in the acetylcholine esterase secretion assay with adult *N. brasiliensis* (0.05650 vs. 0.06886 µg/ml). While amidantel showed only minimal or at best partial inhibition of nematode motility and acetylcholine esterase secretion, tribendimidine was nearly as potent as dAMD. Whereas deacylated amidantel had a significantly lower EC_{50} than tribendimidine in the *N. brasiliensis* L3 motility assay (0.05492 vs. 0.2080 µg/ml), differences were not significant in the *T. spiralis* L1 motility assay (0.7766 vs. 1.145 µg/ml). Surprisingly, none of the combinations showed improved efficacy when compared to the individual drugs including levamisole/tribendimidine, which have previously been reported to act synergistically against *A. ceylanicum*.

3.2 Introduction

Soil-transmitted helminthoses (STHs) are among the most prevalent and persistent parasitic infections in poverty-stricken and resource-constrained regions frequently found under poor hygienic conditions in many regions of sub-Saharan Africa, the Americas, China and East Asia [1]. According to estimation by the WHO, more than 2 billion people suffer from STHs [2]. The most prevalent STHs are caused by the roundworm *A. lumbricoides* with 1.2 billion infections, the whipworm *T. trichiura* with 0.8 billion infections and the hookworms *N. americanus* and *A. duodenale* with 0.7 billion infections worldwide [3]. After malaria, STHs cause more morbidity than any other parasitic disease as far as the number of DALYs is concerned [4].

The control of STHs became increasingly important in public health management and combat strategies, resulting in a significant increase in systematic treatments of highly endemic populations (*i.e.* MDAs) during recent years [5]. However, the rapid advent and spread of anthelmintic drug resistance in the veterinary field [6] should as well be considered as a realistic scenario for the treatment of human STHs, even though it might develop slower because of more tightly regulated treatments in human medicine. Since both WHO lists of essential medicines for adults and children contain only four anthelmintics acting only on two different target sides (albendazole, levamisole, mebendazole and pyrantel pamoate) to treat STHs [7, 8], therapeutic benefit might be compromised in foreseeable future.

To significantly delay the onset of anthelmintic resistance, two major tasks are addressed in anthelmintic research and development: (1) the development of safe and efficacious anthelmintics with new modes of action and (2) the evaluation of drug combinations.

Among the most promising candidates for the evaluation of the treatment of human STHs is the semisynthetic cyclooctadepsipeptide emodepside [9]. It has been characterised as a compound with *in vitro* and *in vivo* activity against a broad spectrum of nematodes of canines, felines, reptiles, rodents, and ruminants [10, 11]. Just like the majority of the existing anthelmintics for human use, it was developed for the significantly larger veterinary market (Profender® and Procox®) [5]. The natural precursor of emodepside, PF1022A, has a broad spectrum of anthelmintic activity as well [12-14] and should also be considered for evaluation of human use [9]. The unique, resistance-breaking mode of action of the cyclooctadepsipeptides is not completely understood. In *C. elegans*, the large-conductance calcium-activated, voltage-gated potassium channel SLO-1 was described to be essential for emodepside anthelmintic activity [15] and SLO-1 channels of *A. caninum* and *C. oncophora*

reconstitute emodepside sensitivity in *C. elegans* SLO-1 loss-of-function mutants [16].

Another development candidate as a drug against human STHs is the aminophenylamidine tribendimidine, which was approved for the control of human *A. lumbricoides*, *A. duodenale*, *N. americanus*, and *E. vermicularis* infections in China in 2004 [17]. Tribendimidine is a symmetrical diamidine derivative of amidantel, which was reported to be efficacious, using high and multiple dosages, against several nematodes and platyhelminths in the late 1970s [18]. Tribendimidine is highly effective against a broad range of nematodes and at least some trematodes and cestodes in rodents, chicken, dogs and humans in fairly low dosages [17, 19, 20]. Both amidantel and tribendimidine are rapidly metabolised to dAMD [21], which by itself exerts a strong anthelmintic activity against nematodes [22, 23]. In *C. elegans*, tribendimidine apparently acts as an agonist of the L-subtype nAChRs, suggesting that it shares its mode of action with levamisole and pyrantel [24]. Despite apparently identical targets, synergistic effects of levamisole and tribendimidine have recently been reported against *A. ceylanicum* when exposed to these drugs *in vitro* or treated *in vivo* [23].

To further corroborate the potential of emodepside and PF1022A as well as of amidantel, dAMD and tribendimidine as promising candidates for the evaluation of the treatment of human STHs, the present study aimed to evaluate their efficacy against L3 and adult worms of *N. brasiliensis* and L1 of *T. spiralis* in comparison to levamisole.

To identify any potential additive or even synergistic interactions using drug combinations, the efficacy of PF1022A-dAMD combinations, PF1022A-tribendimidine combinations and tribendimidine-levamisole combinations was also evaluated.

3.3 Materials and methods

Ethical statement

All animal experiments were carried out at the laboratories of Bayer HealthCare AG, Global Drug Discovery Animal Health (Monheim, Germany) and were registered and approved (no. 200/V14, reference number by LANUV, North Rhine-Westphalia, Germany in accordance with §8a section 1 and 2, German Protection of Animals Act.

Drugs

Amidantel, dAMD, emodepside and PF1022A were obtained from Bayer HealthCare AG, Global Drug Discovery Animal Health (Monheim, Germany). Levamisole was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany), and tribendimidine was obtained from Shandong Xinhua Pharmaceutical Company Limited (Zibo, People's Republic of China). All drugs were stored at 4 °C until further use.

For all *in vitro* assays, test compounds were dissolved in dimethyl sulphoxide (DMSO) at a concentration of 20,000 µg/ml and serial dilutions were performed in DMSO resulting in stock solutions of 2,000, 200, 20, and 2 µg/ml. Stock solutions were stored at 4 °C until they were diluted 1:200 with worm medium (20 g/l Bacto Casitone, 10 g/l yeast extract, 5 g/l glucose, 0.8 g/l KH_2PO_4, 0.8 g/l K_2HPO_4, 10 µg/ml sisomycin and 1 µg/ml clotrimazole, pH 7.2). Final drug concentrations were 100, 10, 1, 0.1 and 0.01 µg/ml in 0.5% DMSO. In addition to individual drugs, combinations of PF1022A plus dAMD, PF1022A plus tribendimidine and levamisole plus tribendimidine were tested containing equal concentrations of both drugs in 0.5% DMSO.

Animals

Female laboratory outbred Wistar rats (HsdCpb:WU) were purchased from Harlan Netherlands at an age of 6-10 weeks with a body weight of 125-175 g. Female laboratory outbred mice (HsdWin:CFW-1) were purchased (Harlan, Netherlands) at an age of 4-8 weeks with a body weight of 15-20 g. All rodents were housed in Macrolon® cages under environmentally controlled conditions and kept in groups of 3-5 animals. Water and SNIFF® rodent food pellets were available *ad libitum*. All animals were allowed to acclimate for seven days before starting any experiments.

Parasites and infections

N. brasiliensis in rats

The life cycle of *N. brasiliensis* was maintained as described previously [13]. Briefly, three female HsdCpb:WU rats were subcutaneously infected via the nuchal fold with 0.3 ml Ringer's solution (118.4 mM NaCl, 4.7 mM KCl, 2.52 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25.01 mM $NaHCO_3$, pH 7.4) containing approximately 2500 infective L3. Six days *p.i.,* the bottom of a Macrolon® cage was covered with moistened filter paper, and the animals were placed on a floor grate for 24 h. Faeces containing eggs were collected from the filter paper, mixed with a slurry of charcoal and charcoal-filtered water to achieve a pasty texture. The mixture was placed on watch glasses in a water-filled humid chamber and incubated at 37 °C and 95% relative humidity for five days to allow hatching of L1, migration into the surrounding water and further development. Larvae were collected in an Erlenmeyer flask and further incubated for another two days at room temperature to ensure complete development of infective L3. Larvae were washed and then used for infection of rats and for *in vitro* motility assays.

On day 7 *p.i.,* rats were euthanised by carbon dioxide suffocation. After necropsy, small intestines were dissected and cut open longitudinally. Duodenum and ingesta were transferred into a bag consisting of five gauze layers and hung into a conical flask filled with Ringer's solution (plus 10 µg/ml sisomycin and 1 µg/ml clotrimazole). Recovered adult worms were transferred into worm medium and kept at 37 °C and 95% relative humidity until use in the acetylcholine esterase secretion assay, but for a maximum of 24 h.

T. spiralis in mice

Passage of *T. spiralis* was performed as described previously [25]. Briefly, three female HsdWin:CFW-1 mice were orally infected with 0.5 ml Ringer's solution (plus 10 µg/ml sisomycin and 1 µg/ml clotrimazole) containing 300 freshly isolated larvae. After three weeks to six months *p.i.,* mice were euthanised by carbon dioxide suffocation. Carcasses were homogenised using a mincer. The musculature was treated with artificial digestive juice (7.1 g/l pepsin in 300 ml 0.6% hydrochloric acid) at 37 °C for 40 min to encyst larvae from their host cells. Larvae were separated from remaining debris by baermanisation through a stack of 200-, 100- and 80-µm sieves and collected from the surface of the latter. After three wash cycles using Ringer's solution, larvae could be used for infection of mice. For usage in

the motility assay, larvae were transferred into worm medium and kept at 37 °C and 95% relative humidity until further use, but for a maximum of 24 h.

In vitro assays

T. spiralis L1 and N. brasiliensis L3 motility assays

Assays were carried out in 24-well plates containing 2 ml prewarmed (37 °C) worm medium per well. Two drugs were tested per plate with each concentration in duplicate. Six negative controls (worm medium with 0.5% DMSO) were always run in parallel to the assays on an extra plate. Exactly 20 larvae were added into each well and then incubated at 37 °C and 95% relative humidity for 72 h (*T. spiralis* L1) and 24 h (*N. brasiliensis* L3), respectively.

Motility was then evaluated using an inverse microscope with a temperature-controlled object stage (37 °C). Larvae were categorised as paralysed when they showed only minimal movement of the head and/or tail or no movement and as motile when they displayed similar activity as in negative controls. Assays were considered as valid only if the mean of the motile larvae in the negative controls was higher than 90%. Assays were repeated three times. For statistics, data from these repeats were co-analysed in one large dataset.

N. brasiliensis acetylcholine esterase secretion assay

Acetylcholine esterase (AChE) is known to be secreted by many parasitic nematodes, *e.g.* *N. brasiliensis*, *Oesophagostomum* spp. and *Trichostrongylus* spp. [26, 27]. It has also been shown that anthelmintics are able to inhibit secretion of AChE by *N. brasiliensis* [28]. This has been used to set up a *N. brasiliensis* AChE assay [29] using colourimetric determination of AChE activity [30] in the culture medium. Thus, reduction of AChE activity was measured in the presence of anthelmintic drugs to quantify their activity.

Drug concentrations and controls were identical to those used in the motility assays. Since secretion of AChE is gender and body weight specific [31], two female and three male adult worms were placed in each well containing 1 ml of pre-warmed medium with drugs plus vehicle and incubated at 37 °C and 95% relative humidity for five days. All drug concentrations were performed in duplicate. From each well 25 µl medium were transferred into a 96 well plate. Then, 250 µl 5,5′-dithio-bis (2-nitrobenzoic acid) (0.25 µM) and 25 µl acetylthiocholine (4 mM) were added. AChE cleaves acetylthiocholine into acetate and thiocholine [30]. In a consecutive reaction, thiocholine reacts with 5,5′-dithio-bis(2-nitrobenzoic acid) to thionitrobenzoate. Thionitrobenzoate is a yellow dye and its

concentration can be determined by measuring the absorption at 405 nm. The A_{405} was measured after two and seven minutes of incubation at RT using an Expert 96 plate reader (Asys-Hitech, Salzburg, Austria) and the software MikroWin 2000 (Mikrotek, Overrath, Germany). The difference in absorption between both time points was taken as measure of AChE activity. The arithmetic mean of 12 no drug control wells was set to 100% activity, and reduction of AChE activity in percentage relative to the negative control was calculated for all test compound concentrations. Within an assay, every drug concentration was performed in duplicate, and the software reported the mean of these duplicates. Assays were repeated three times, and data from these repeats were merged for statistical analysis.

Calculation of dose-response curves and statistical analysis

For all assays, inhibition of nematode activity (*i.e.* motility or secretion of AChE) was plotted against the $\log_{10}$ of the drug concentrations. Data were fit by a four-parameter logistic model using GraphPad Prism 5.03. Top and bottom were constrained to values between 0 and 100%. EC_{50} values were compared using the extra sum of squares F test implemented as default for this type of analysis in GraphPad Prism. In cases where multiple tests were performed (comparison of three aminophenylamidines and comparison of drug combinations with both individual drugs), P values were corrected using the Bonferroni–Holmes approach.

3.4 Results

T. spiralis L1 motility assay

After incubation for 72 h, motility of *T. spiralis* L1 in negative controls ranged between 95.00% ± 4.47% and 96.67% ± 4.08%. The effects of levamisole, the cyclooctadepsipeptides emodepside and PF1022A, the aminophenylamidines amidantel, dAMD and tribendimidine as well as the combinations of PF1022A plus dAMD, PF1022A plus tribendimidine and levamisole plus tribendimidine on the motility of *T. spiralis* L1 are shown in Fig. 3-1. Tab. 3-1 summarises EC_{50} (with 95% confidence intervals (CI)) and coefficients of determination (R^2) values and reports P values for comparisons between EC_{50} values. All tested compounds suppressed larval motility in a dose-dependent manner.

The comparison of the EC_{50} values of the cyclooctadepsipeptides PF1022A and emodepside revealed a significant difference with emodepside showing a half-maximal effect at a 2.1-fold lower concentration than PF1022A (Fig. 3-1a, Tab. 3-1). Among the aminophenylamidine derivatives, amidantel was by far the least effective with only about 18% inhibition of motility even at the highest concentration (Fig. 3-1b). Therefore, no dose-response curve was calculated for amidantel in this assay. No significant difference in EC_{50} values was observed between tribendimidine and dAMD.

When drug combinations were compared with individual drugs, none of the combinations was significantly more efficacious in terms of a lower EC_{50} value than at least one of the individual drugs in the combination. In particular, the combinations of PF1022A with dAMD or tribendimidine were not superior to PF1022A alone, and the combination of tribendimidine and levamisole had no significantly lower EC_{50} than tribendimidine alone (Fig. 3-1c-e).

Tab. 3-1. Comparison of tested drugs and drug combinations in the *T. spiralis* L1 motility assay

Compounds	**EC_{50} with 95% CI (in µg/ml)**	***P* value**[a]	**R^2**
Emodepside	0.02788 (0.01890-0.04112)	0.0038 (vs. PF1022A)	0.9716
PF1022A	0.05862 (0.03860-0.08903)	0.0038 (vs. emodepside)	0.9654
Levamisole	2.154 (1.586-2.926)		0.9762
Amidantel	nd[b]	nd[b]	nd[b]
dAMD	0.7766 (0.4132-1.460)	0.1256 (vs. tribendimidine)	0.9476
Tribendimidine	1.145 (0.8410-1.558)	0.1256 (vs. dAMD)	0.9618
PF1022A+ dAMD	0.05259 (0.03831-0.07221)	<0.0002 (vs. dAMD) 0.6415 (vs. PF1022A)	0.9773
PF1022A+ Tribendimidine	0.1025 (0.08404-0.1249)	<0.0002 (vs. tribendimidine) 0.0072 (vs. PF1022A)	0.9799
Levamisole+ tribendimidine	1.042 (0.7160-1.516)	0.0006 (vs. levamisole) 0.6253 (vs. tribendimidine)	0.9700

Presented are the mean EC_{50} values with the 95% CI and R^2 as well as *P* values, for determination of significant differences. [a]Significant difference in EC_{50} to drug in brackets. *P* values were corrected for multiple testing using the Bonferroni–Holmes method. [b]Not determined due to ambiguous curve fit for amidantel since only partial inhibition was obtained at high amidantel concentrations.

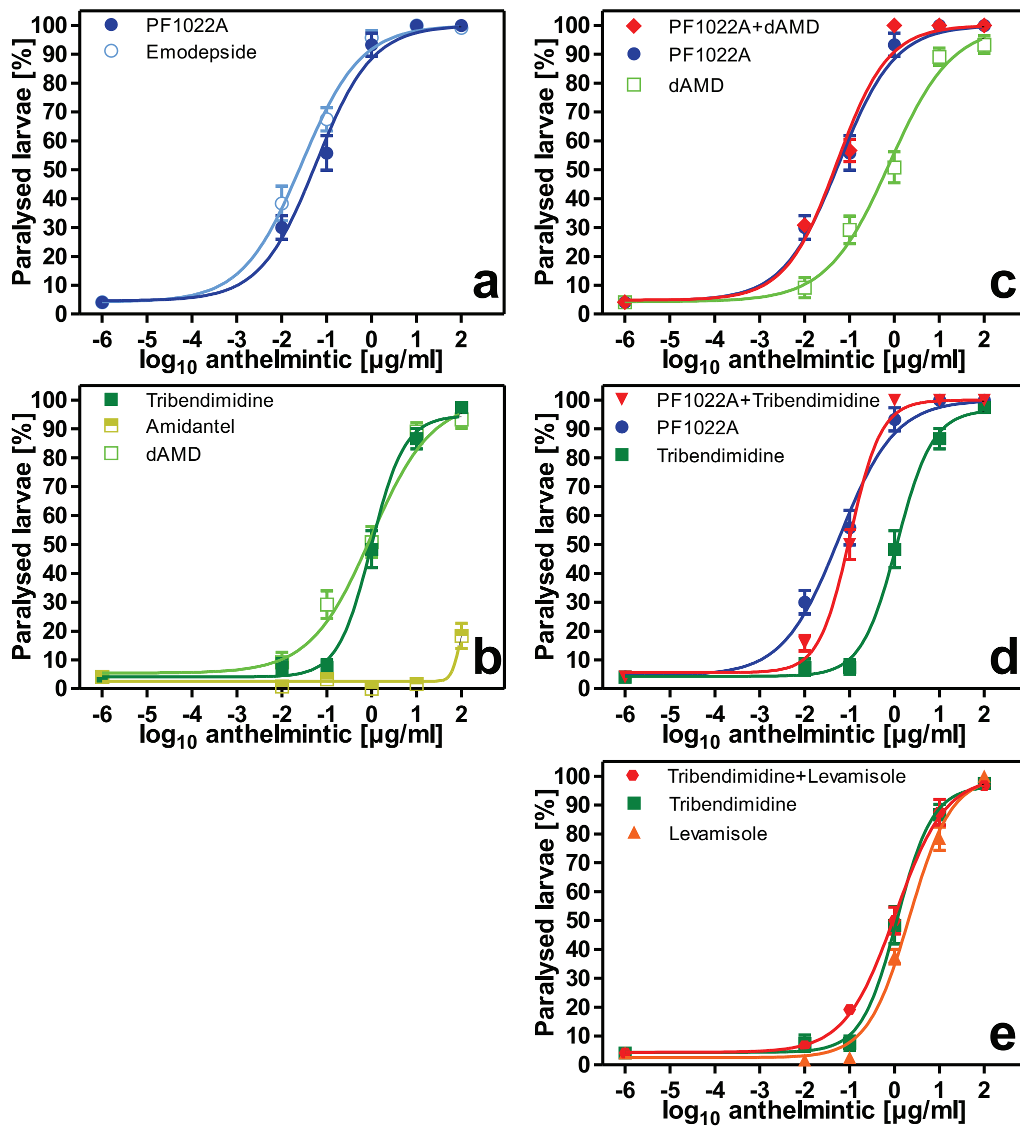

Fig. 3-1. *T. spiralis* L1 motility assay

Data were expressed as inhibition of the number of motile larvae in drugs and controls relative to the mean of the vehicle controls. Dose-response curves are shown for a) the cyclooctadepsipeptides PF1022A and emodepside; b) the aminophenylamidine derivatives tribendimidine, amidantel and dAMD; c) a combination of PF1022A and dAMD in comparison with the individual drugs; d) a combination of PF1022A and tribendimidine in comparison with the individual drugs; and e) a combination of levamisole and tribendimidine in comparison with the individual drugs. Data points are means ± standard error of the mean (SEM), and top and bottom values in the four-parameter logistic regression were constrained to values between 0% and 100%.

N. brasiliensis L3 motility assays

Motility in negative controls in the *N. brasiliensis* L3 motility assay ranged between 92.50% ± 5.24% and 92.50% ± 8.22%. The effects of amidantel, dAMD, tribendimidine, levamisole, emodepside, PF1022A and the drug combinations on the *N. brasiliensis* L3 motility are resumed in Tab. 3-2 and Fig. 3-2. All drugs and drug combinations inhibited larval motility in a dose-dependent manner.

Similar to the results of the *T. spiralis* motility assay, the EC_{50} for emodepside was about 2.4-fold lower than the EC_{50} for PF1022A (*P* <0.0001). Amidantel was only effective in the highest concentration; however, even at 100 µg/ml inhibition of *N. brasiliensis* movement was still slightly below 50%. Therefore, dose-response curves had to be calculated by extrapolation resulting in a very wide 95% CI for the EC_{50} value. In comparison to tribendimidine and dAMD, the calculated EC_{50} value was about 680- and 2500-fold higher, respectively (*P* <0.0003). The roughly 3.3-fold difference between tribendimidine and dAMD was also highly significant (*P* <0.0009).

Again, as observed in the *T. spiralis* L1 motility assay, combinations of drugs were not significantly superior in efficacy to at least one of the single drugs. The combination of PF1022A and dAMD was not significantly better than dAMD alone. The effects of PF1022A plus tribendimidine were not significantly different in EC_{50} from PF1022A alone, and the combination of levamisole and tribendimidine even had a significantly higher EC_{50} value than tribendimidine alone (*P*=0.0022).

Tab. 3-2. Comparison of tested drugs and drug combinations in the *N. brasiliensis* L3 motility assay

Compounds	**EC_{50} with 95% CI (in µg/ml)**	***P* value[a]**	***R^2***
Emodepside	0.06188 (0.05011-0.07641)	<0.0001 (vs. PF1022A)	0.9822
PF1022A	0.1485 (0.0983-0.2244)	<0.0001 (vs. emodepside)	0.9695
Levamisole	2.059 (1.434-2.956)		0.9591
Amidantel[b]	141.5 (2.17×10^{-5} – 9.25×10^{8})	<0.0003 (vs. dAMD) <0.0003 (vs. tribendimidine)	0.7989
dAMD	0.05492 0.03946-0.07646)	<0.0003 (vs. amidantel) 0.0009 (vs. tribendimidine)	0.9619
Tribendimidine	0.2080 (0.1147-0.3771)	<0.0003 (vs. amidantel) 0.0009 (vs. dAMD)	0.9550
PF1022A+ dAMD	0.06389 (0.04100-0.09958)	0.0418 (vs. PF1022A) 0.5589 (vs. dAMD)	0.9595
PF1022A+ Tribendimidine	0.07908 (0.06518-0.09595)	<0.0002 (vs. tribendimidine) 0.0899 (vs. PF1022A)	0.9812
Levamisole+ Tribendimidine	0.5559 (0.3004-1.029)	<0.0002 (vs. levamisole) 0.0022 (vs. tribendimidine)	0.9509

Presented are the mean EC_{50} values with the 95% CI and R^2 as well as *P* values, for determination of significant differences. [a] Significant difference in EC_{50} to drug in brackets. *P* values were corrected for multiple testing using the Bonferroni-Holmes method. [b] The 95% confidence interval for amidantel is very wide and the R^2 quite low since only partial inhibition was obtained at high amidantel concentrations.

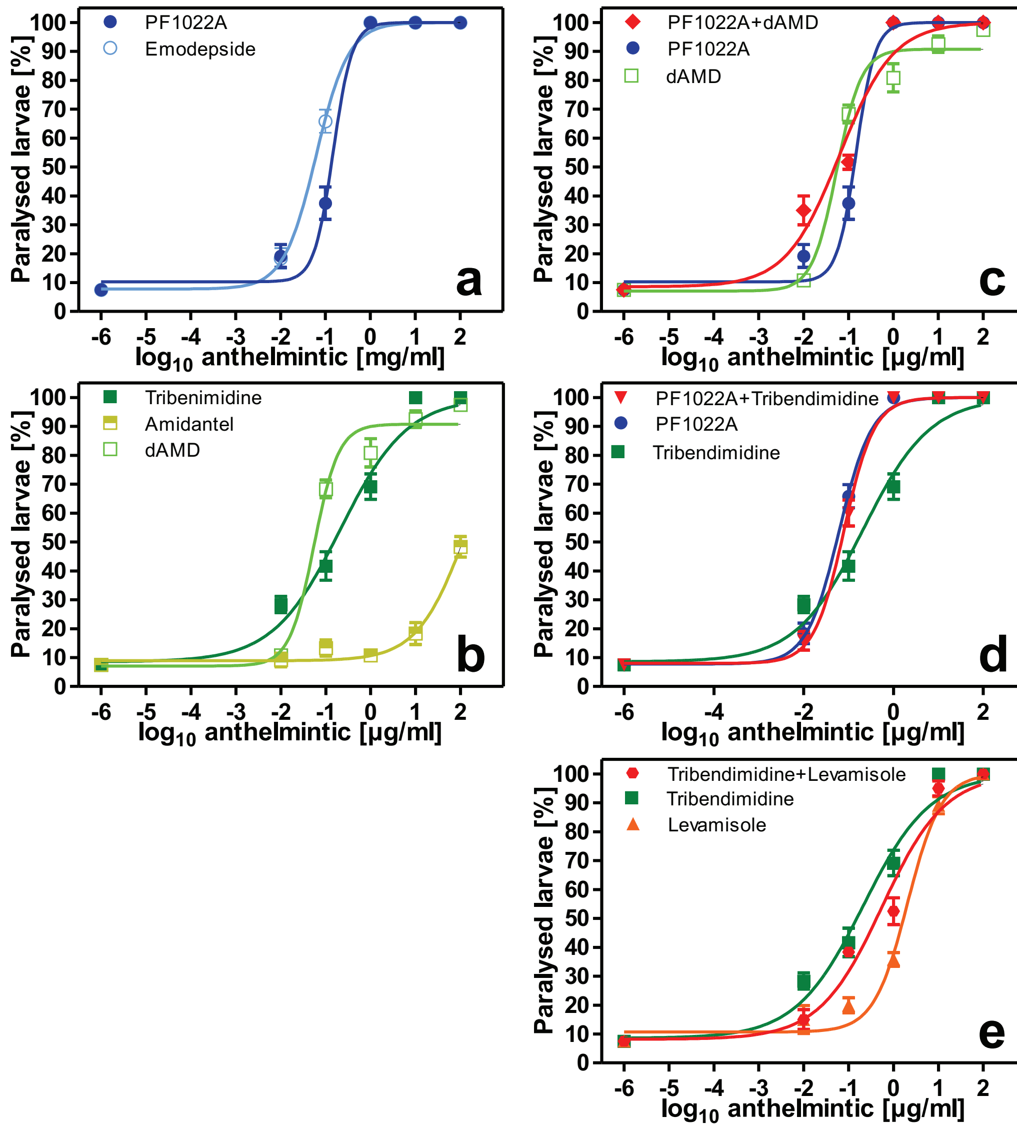

Fig. 3-2. *N. brasiliensis* motility assay

Data were expressed as inhibition of the number of motile larvae in drugs and controls relative to the mean of the vehicle controls. Dose-response curves are shown for a) the cyclooctadepsipeptides PF1022A and emodepside; b) the aminophenylamidine derivatives tribendimidine, amidantel and dAMD; c) a combination of PF1022A and dAMD in comparison with the individual drugs; d) a combination of PF1022A and tribendimidine in comparison with the individual drugs; and e) a combination of levamisole and tribendimidine in comparison with the individual drugs. Means ± SEM are presented and top and bottom values were constrained between 0% and 100%.

N. brasiliensis acetylcholine esterase secretion assay

The effects of amidantel, dAMD, tribendimidine, levamisole, emodepside and PF1022A as well as of the drug combinations on the AChE secretion by adult *N. brasiliensis* are shown in Fig. 3-3 and summarised in Tab. 3-3. All compounds and combinations inhibited AChE secretion in a concentration-dependent manner.

In general, maximal inhibition in the acetylcholine esterase secretion assay was much lower than in the motility assays. In contrast to the results of both motility assays, no significant difference in EC_{50} values for emodepside and PF1022A was detected. Surprisingly, maximal inhibition observed for amidantel was about 56%, which was only slightly lower than maximal inhibition for tribendimidine (63%). Thus, amidantel showed the strongest maximal effect only in the assay, which revealed the lowest maximal effects for all other drugs. Among the aminophenylamidine derivatives, the EC_{50} value is significantly lower for dAMD than for tribendimidine and amidantel. The difference between tribendimidine and amidantel was not significant in this assay.

Regarding drug combinations, PF1022A plus tribendimidine had no significantly lower EC_{50} value than tribendimidine alone and PF1022A plus dAMD was even less efficient than PF1022A alone. Moreover, the combination of levamisole plus tribendimidine had a significantly higher EC_{50} value than levamisole alone.

Tab. 3-3. Comparison of tested drugs and drug-combinations in the *N. brasiliensis* acetylcholine esterase secretion assay

Compounds	**EC_{50} with 95% CI [µg/ml]**	***P* value[a]**	**R^2**
Emodepside	0.05650 (0.03975-0.08031)	0.5306 (vs. PF1022A)	0.9847
PF1022A	0.06886 (0.03914-0.1211)	0.5306 (vs. emodepside)	0.9667
Levamisole	0.2091 (0.1293-0.3384)		0.9663
Amidantel[b]	66.91 (0.1120-39958)	<0.0003 (vs. dAMD) 0.3302 (vs. tribendimidine)	0.9215
dAMD	1.074 (0.8306-1.388)	<0.0003 (vs. amidantel) 0.3302 (vs. tribendimidine)	0.9737
Tribendimidine	3.693 (0.3303-41.28)	0.3302 (vs. amidantel) 0.3302 (vs. dAMD)	0.9123
PF1022A+ dAMD	0.1482 (0.1032-0.2128)	<0.0002 (vs. dAMD) 0.0221 (vs. PF1022A)	0.9878
PF1022A+ Tribendimidine	0.05342 (0.02760-0.1034)	<0.0002 (vs. tribendimidine) 0.8730 (vs. PF1022A)	0.9743
Levamisole+ Tribendimidine	0.5284 (0.3191-0.8752)	0.0174 (vs. levamisole) 0.0175 (vs. tribendimidine)	0.9539

Presented are the mean EC_{50} values with the 95% CI and R^2 as well as *P* values, for determination of significant differences. [a] Significant difference in EC_{50} to drug in brackets. *P* values were corrected for multiple testing using the Bonferroni–Holmes method. [b] The 95% confidence interval for amidantel is very wide since only partial inhibition was obtained at high amidantel concentrations.

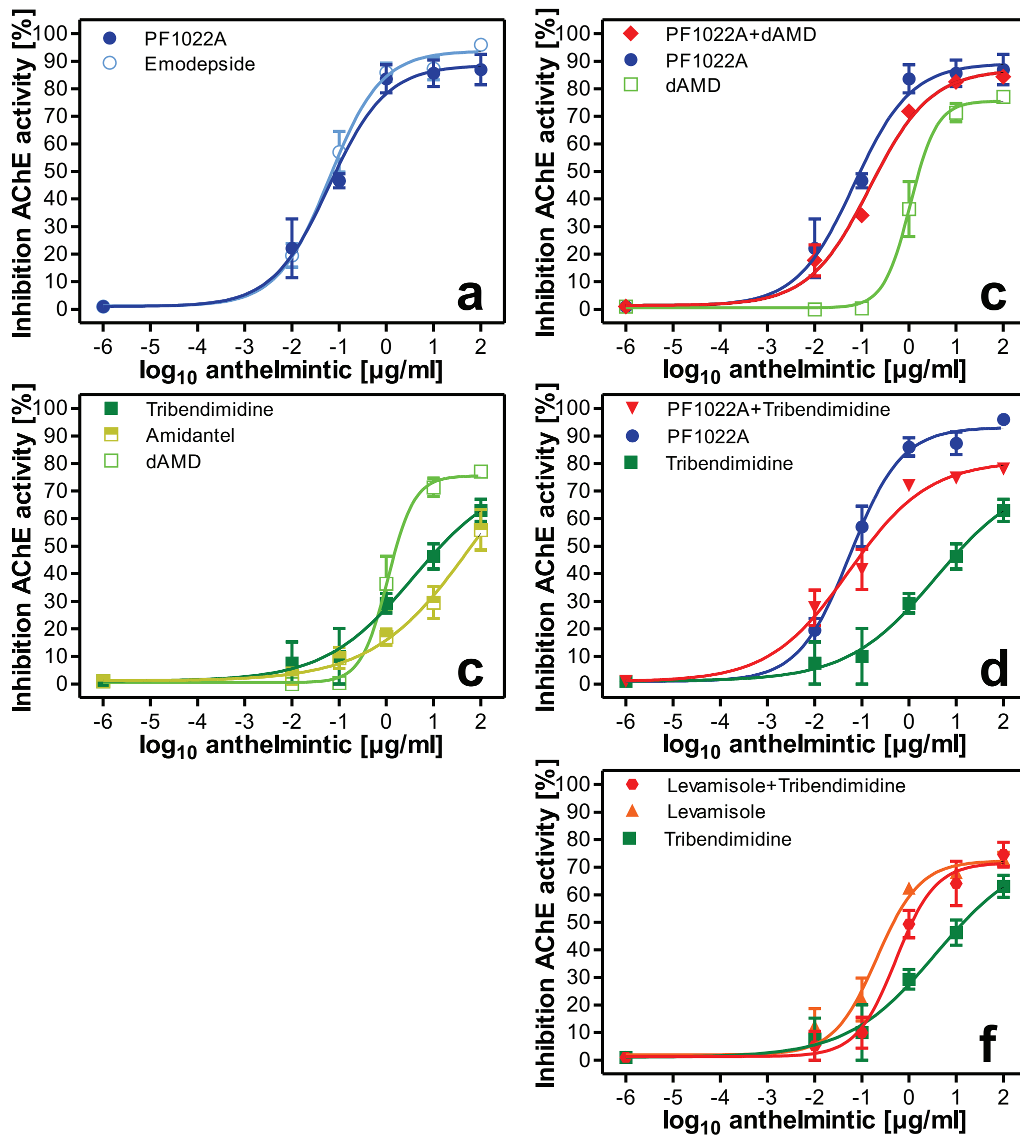

Fig. 3-3. *N. brasiliensis* acetylcholine esterase secretion assay

Data were expressed as inhibition of secreted acetylcholine esterase in drugs and controls relative to the mean of the vehicle controls. Dose-response curves are shown for a) the cyclooctadepsipeptides PF1022A and emodepside; b) the aminophenylamidine derivatives tribendimidine, amidantel and dAMD; c) a combination of PF1022A and dAMD in comparison with the individual drugs; d) a combination of PF1022A and tribendimidine in comparison with the individual drugs; e) and a combination of levamisole and tribendimidine in comparison with the individual drugs. Top and bottom values of all dose-response curves were constrained between 0% and 100% efficacy. Data are shown as means ± SEM.

3.5 Discussion

The recently increased utilisation of MDA against STHs resulted in significant reductions of prevalence, intensity of infections and morbidity. However, also long-term sustainability of the benefits of the MDAs needs to be ensured. Extensive use of those four drugs listed on the WHO list of essential medicines to treat human STHs is expected to strongly select for drug-resistant genotypes, and therapeutic benefits might be compromised soon [5]. Thus, there is an urgent need for the development of safe and efficacious anthelmintics with new modes of action. Prominent candidates for the evaluation to treat STHs and for the admission on the WHO list of essential medicines are the cyclooctadepsipeptides emodepside and PF1022A [9, 14] and the aminophenylamidines amidantel, tribendimidine and dAMD [17], since they are highly efficacious broad-spectrum anthelmintics.

The present study evaluated and compared the *in vitro* efficacy of emodepside and PF1022A as well as amidantel, dAMD and tribendimidine against L3 and adult worms of *N. brasiliensis* and L1 of *T. spiralis in vitro*.

The two cyclooctadepsipeptides showed high efficacy in all three *in vitro* assays, corroborating results obtained in an *in vitro* motility assay using adult *H. contortus* [32]. In both motility assays but not in the AChE secretion assay, emodepside was significantly more potent than PF1022A. These *in vitro* results confirm a number of previously published *in vivo* observations showing that emodepside and PF1022A are highly effective against different stages of *N. brasiliensis* and *T. spiralis* [13, 33-35]. Whereas a single oral treatment of rats with 2.5 mg/kg emodepside resulted in >99.0% efficacy against adult worms in the intestine [13], a 40-fold higher dosage of PF1022A was necessary to achieve similar results [33]. Whether the approximately 2.4-fold difference in EC_{50} values in the *N. brasiliensis* motility assay is sufficient to explain the 40-fold difference in the LD_{99} *in vivo* remains unclear. Differences in drug efficacies between developmental stages and differences in bioavailability of emodepside compared to PF1022A in the host blood system presumably also contribute to the differences in potency *in vivo* [13].

In comparison to the cyclooctadepsipeptides, the efficacy of the aminophenylamidines was more heterogeneous. The wide range of dosages reported to be required of amidantel, dAMD and tribendimidine for elimination of different stages of *N. brasiliensis* and *T. spiralis in vivo* supports these findings [18, 36-38]. *In vivo* both, amidantel and tribendimidine, are rapidly metabolised to the anthelmintically active dAMD [39]. While amidantel showed only minimal or at best partial inhibition of nematode activity even at high concentrations *in vitro*,

tribendimidine was nearly as potent as dAMD. The only significant difference between these drugs was detected in the *N. brasiliensis* motility assay. The most parsimonious explanation for this effect is that amidantel acts predominantly as a pro-drug, whereas tribendimidine is not only active through its metabolite dAMD but has also significant anthelmintic activity by itself. Residual *in vitro* activity of amidantel might be explained by (i) spontaneous decomposition to dAMD in an aqueous environment as known for tribendimidine [21], (ii) metabolisation by the parasite to dAMD and/or (iii) minimal anthelmintic activity of amidantel itself.

Drug combinations are powerful tools not only in human medicine with the anti-HIV triple therapy as a prominent example [40] but also in the field of veterinary parasitology [5, 41]. Combinations are able to decelerate the development of drug resistance but also to increase the spectrum and efficacy of drugs. Since cure rates of single-treatment regimens using albendazole against human *T. trichiura* infections were substantially increased when albendazole plus ivermectin combination were used [42], investigations regarding drug combinations to treatment STHs were intensified recently [43, 44]. Thus, combinations of PF1022A plus dAMD, PF1022A plus tribendimidine and levamisole plus tribendimidine were evaluated in the same assays, to check for any potential additive or even synergistic interactions between drugs.

Unfortunately, none of the combinations showed improved performance when compared to the individual drugs although synergistic effects of tribendimidine and levamisole have been reported previously [23]. Indeed, in some cases, even significantly antagonistic effects have previously been observed such as slightly antagonistic interaction of levamisole–tribendimidine combinations on the survival of third-stage larvae of *H. bakeri in vitro* [23]. Worm burden reduction after oral treatment of mice harbouring adult *H. bakeri* also revealed no significant difference between levamisole/tribendimidine combination and tribendimidine alone [23]. However, the same authors [23] reported synergistic effects of levamisole and tribendimidine on inhibition of motility of *A. ceylanicum* L3 *in vitro* with a combination index of 0.5 and additive effects tending towards synergism against adult *A. ceylanicum in vivo.* Tribendimidine apparently shares its mode of action with levamisole and pyrantel in *C. elegans* [24], and therefore, additive effects for the combined treatment with levamisole and tribendimidine are expected. Since the composition of ionotropic AChRs varies between different nematode species, different sets of subunits might explain different sensitivities to individual cholinergic anthelmintics. For instance, *H. contortus* L-AChR-1 is highly sensitive to levamisole but almost insensitive to pyrantel whereas *H. contortus* L-AChR-2 is less

sensitive to levamisole but highly responsive to pyrantel [45]. Furthermore, it has been shown for *A. caninum* that the presence of different AChR subunit populations varies during different lifecycle stages, resulting in different susceptibilities to cholinergic drugs [46]. Combinations of levamisole plus a aminophenylamidine should therefore be investigated in the species and lifecycle stages of interest, namely L3, L4 and adult worms of *A. lumbricoides*, *T. trichiura, N. americanus* and *A. duodenale* as well as in the respective host species to draw conclusions for a potential use in MDAs against human STHs in terms of efficacy and safety.

Effects of combinations of cyclooctadepsipeptides with aminophenylamidines have not been reported before. Whereas the aminophenylamidines activate nAChRs resulting in muscle contraction [24], the cyclooctadepsipeptides cause muscle relaxation via a mechanism involving SLO-1 potassium channels [14]. Since both drugs have entirely different modes of action, additive effects of drugs from both drug classes might be expected. However, the fact that these drug classes act in influence muscle tonus in opposite directions might prevent additive effects as observed in all *in vitro* assays in this study.

To conclude, the tested cyclooctadepsipeptides and aminophenylamidines all showed a high degree of efficacy against all stages and species and should therefore be evaluated and compared in the *N. brasiliensis in vivo* model to prove if the intrinsic efficacy *in vitro* is a good marker for the *in vivo* efficacy in this model. The same drug combinations should be evaluated in the same *in vivo* model to unequivocally demonstrate the absence or presence of combinatory effects.

3.6 Acknowledgements

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3.7 Conflict of interest

A. Harder and D. Kulke were employees of Bayer HealthCare AG when the study was conducted. Bayer HealthCare had no influence on the design of the study.

3.8 References

1. Lustigman S, Prichard RK, Gazzinelli A, Grant WN, Boatin BA, McCarthy JS, Basanez MG, 2012, A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 6, e1582.
2. Crompton DWT, World Health Organization, 2006, Preventive chemotherapy in human helminthiasis: coordinated use of anthelminthic drugs in control interventions: a manual for health professionals and programme managers. World Health Organization, Geneva.
3. Crompton DWT, Savioli L, 2007, Handbook of helminthiasis for public health. CRC/Taylor & Francis, Boca Raton.
4. Weaver HJ, Hawdon JM, Hoberg EP, 2010, Soil-transmitted helminthiases: implications of climate change and human behavior. Trends Parasitol 26, 574-581.
5. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, Yang GJ, Sripa B, Lustigman S, 2012, A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6, e1549.
6. Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC, 2004, Drug resistance in veterinary helminths. Trends Parasitol 20, 469-476.
7. World Health Organization, 2011, WHO model list of essential medicines : 17th list, March 2011. World Health Organization, Geneva.
8. World Health Organization, 2011, WHO model list of essential medicines for children: 3rd list, March 2011. World Health Organization, Geneva.
9. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, Don R, Keiser J, 2011, Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5, e1138.
10. Harder A, Schmitt-Wrede HP, Krücken J, Marinovski P, Wunderlich F, Willson J, Amliwala K, Holden-Dye L, Walker R, 2003, Cyclooctadepsipeptides--an anthelmintically active class of compounds exhibiting a novel mode of action. Int J Antimicrob Ag 22, 318-331.
11. Mehlhorn H, Schmahl G, Frese M, Mevissen I, Harder A, Krieger K, 2005, Effects of a combinations of emodepside and praziquantel on parasites of reptiles and rodents. Parasitol Res 97, 65-69.
12. Geary TG, Mackenzie CD, 2011, Progress and challenges in the discovery of macrofilaricidal drugs. Expert Rev Anti Infect Ther 9, 681-695.

13. Harder A, von Samson-Himmelstjerna G, 2002, Cyclooctadepsipeptides - a new class of anthelmintically active compounds. Parasitol Res 88, 481-488.
14. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, Welz C, von Samson-Himmelstjerna G, 2012, Anthelmintic cyclcooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28, 385-394.
15. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, Harder A, Holden-Dye L, Hopper NA, 2007, The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. Int J Parasitol 37, 1577-1588.
16. Welz C, Krüger N, Schniederjans M, Miltsch SM, Krücken J, Guest M, Holden-Dye L, Harder A, von Samson-Himmelstjerna G, 2011, SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in *Caenorhabditis elegans* slo-1 loss of function mutants. PLoS Pathog 7, e1001330.
17. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W, 2005, Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94, 1-14.
18. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, Thomas H, 1979, Amidantel, a potent anthelminthic from a new chemical class. Arzneim Forsch 29, 31-32.
19. Kulke D, Krücken J, Welz C, von Samson-Himmelstjerna G, Harder A, 2012, *In vivo* efficacy of the anthelmintic tribendimidine against the cestode *Hymenolepis microstoma* in a controlled laboratory trial. Acta Trop 123, 78-84.
20. Steinmann P, Zhou XN, Du ZW, Jiang JY, Xiao SH, Wu ZX, Zhou H, Utzinger J, 2008, Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. PLoS Negl Trop Dis 2, e322.
21. Yuan G, Xu J, Qu T, Wang B, Zhang R, Wei C, Guo R, 2010, Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs R D 10, 83-90.
22. Tomlinson G, Albuquerque CA, Woods RA (1985) The effects of amidantel (BAY d 8815) and its deacylated derivative (BAY d 9216) on *Caenorhabditis elegans*. Eur J Pharmacol 113, 255-262.

23. Tritten L, Nwosu U, Vargas M, Keiser J, 2012, *In vitro* and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. Acta Trop 122, 101-107.
24. Hu Y, Xiao SH, Aroian RV, 2009, The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3, e499.
25. Nicolay F, Harder A, von Samson-Himmelstjerna G, Mehlhorn H, 2000, Synergistic action of a cyclic depsipeptide and piperazine on nematodes. Parasitol Res 86, 982-992.
26. Ogilvie BM, Rothwell TL, Bremner KC, Schnitzerling HJ, Nolan J, Keith RK, 1973, Acetylcholinesterase secretion by parasitic nematodes. I. Evidence for secretion of the enzyme by a number of species. Int J Parasitol 3, 589-597.
27. Rothwell TL, Ogilvie BM, Love RJ, 1973, Acetylcholinesterase secretion by parasitic nematodes. II. *Trichostrongylus* spp. Int J Parasitol 3, 599-608.
28. Watts SD, Rapson EB, Atkins AM, Lee DL, 1982, Inhibition of acetylcholinesterase secretion from *Nippostrongylus brasiliensis* by benzimidazole anthelmintics. Biochem Pharmacol 31, 3035-3040.
29. Rapson EB, Lee DL, Watts SD, 1981, Changes in the acetylcholinesterase activity of the nematode *Nippostrongylus brasiliensis* following treatment with benzimidazoles *in vivo*. Mol Biochem Parasitol 4, 9-15.
30. Ellman GL, Courtney KD, Andres V, Jr., Feather-Stone RM, 1961, A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88-95.
31. Lumley AM, Lee DL, 1981, *Nippostrongylus brasiliensis* and *Nematodirus battus*: changes in numbers and weight during the course of infection. Exp Parasitol 52:183-190.
32. Conder GA, Johnson SS, Nowakowski DS, Blake TE, Dutton FE, Nelson SJ, Thomas EM, Davis JP, Thompson DP, 1995, Anthelmintic profile of the cyclodepsipeptide PF1022A in *in vitro* and *in vivo* models. J Antibiot 48, 820-823.
33. von Samson-Himmelstjerna G, Harder A, Schnieder T, Kalbe J, Mencke N, 2000, *In vivo* activities of the new anthelmintic depsipeptide PF 1022A. Parasitol Res 86, 194-199.
34. Wang M, Watanabe N, Shomura T, Ohtomo H, 1994, Effects of PF1022A from *Mycelia sterilia* on *Trichinella spiralis* in mice. Jpn J Parasitol 43, 346-350.

35. Wang M, Watanabe N, Shomura T, Ohtomo H, 1995, Effects of PF1022A on *Nippostrongylus brasiliensis* in rats and *Hymenolepis nana* in mice. Jpn J Parasitol 44, 306-310.
36. Li RH, Gao JH, Wang SF, Pei YJ, Shen JY, Yan PM, Yin GR, 2011, Effect of oral administration of tribendimidine at different dosages against *Trichinella spiralis* encapsulated larvae in mice. Chin J Parasitol Parasit Dis 29, 117-121.
37. Xue J, Xiao SH, Xu LL, Qiang HQ, 2010, The effect of tribendimidine and its metabolites against *Necator americanus* in golden hamsters and *Nippostrongylus braziliensis* in rats. Parasitol Res 106, 775-781.
38. Xue J, Xiao SH, Xu LL, Zhang YN, Qiang HQ, 2010, Efficacy of tribendimidine and albendazole in treating mice infected with *Trichinella spiralis*. Chin J Parasitol Parasit Dis 28, 8-11.
39. Woods RA, Malone KMB, 1985, The effects of amidantel (BAY d 8815) and its deacylated derivative (BAY d 9216) on wild-type and resistant mutants of *Caenorhabditis elegans*. Can J Zool 64, 1310-1316.
40. Gulick RM, Mellors JW, Havlir D, Eron JJ, Gonzalez C, McMahon D, Richman DD, Valentine FT, Jonas L, Meibohm A, Emini EA, Chodakewitz JA, Deutsch P, Holder D, Schleif WA, Condra JH, 1997, Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med 337, 734-739.
41. Leathwick DM, Hosking BC, Bisset SA, McKay CH, 2009, Managing anthelmintic resistance: is it feasible in New Zealand to delay the emergence of resistance to a new anthelmintic class? New Zeal Vet J 57, 181-192.
42. Olsen A, 2007, Efficacy and safety of drug combinations in the treatment of schistosomiasis, soil-transmitted helminthiasis, lymphatic filariasis and onchocerciasis. T Roy Soc Trop Med H 101, 747-758.
43. Knopp S, Mohammed KA, Speich B, Hattendorf J, Khamis IS, Khamis AN, Stothard JR, Rollinson D, Marti H, Utzinger J, 2010, Albendazole and mebendazole administered alone or in combination with ivermectin against *Trichuris trichiura*: a randomized controlled trial. Clin Infect Dis 51, 1420-1428.
44. Speich B, Ame SM, Ali SM, Alles R, Hattendorf J, Utzinger J, Albonico M, Keiser J, 2012, Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against *Trichuris trichiura* infection: a randomized controlled trial. PLoS Negl Trop Dis 6:e1685.

45. Martin RJ, Robertson AP, Buxton SK, Beech RN, Charvet CL, Neveu C, 2012, Levamisole receptors: a second awakening. Trends Parasitol 28, 289-296.

46. Kotze AC, Lowe A, O'Grady J, Kopp SR, Behnke JM, 2009, Dose-response assay templates for *in vitro* assessment of resistance to benzimidazole and nicotinic acetylcholine receptor agonist drugs in human hookworms. Am J Trop Med Hyg 81, 163-170.

Chapter 4

In vivo efficacy of PF1022A and nicotinic acetylcholine receptor agonists alone and in combination against *Nippostrongylus brasiliensis*

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4 *In vivo* efficacy of PF1022A and nicotinic acetylcholine receptor agonists alone and in combination against *Nippostrongylus brasiliensis*

4.1 Abstract

The cyclooctadepsipeptide PF1022A and the aminophenylamidines amidantel, dAMD and tribendimidine were tested as examples for drug classes potentially interesting for development as anthelmintics against human helminthoses.

These compounds and levamisole were tested alone and in combination to determine their efficacy against the rat hookworm *N. brasiliensis*. After three oral treatments, intestinal worms were counted. Drug effects on parasite morphology were studied using scanning electron microscopy. Plasma pharmacokinetics were determined for tribendimidine and dAMD.

All drugs reduced worm burden in a dose-dependent manner, however amidantel was significantly less active than the other aminophenylamidines. Combinations of tribendimidine and dAMD with levamisole or PF1022A at suboptimal doses revealed additive effects. While PF1022A caused virtually no changes in morphology, levamisole, dAMD and tribendimidine caused severe contraction, particularly in the hind body region. Worms exposed to combinations of PF1022A and aminophenylamidines were indistinguishable from worms exposed only to aminophenylamidines. After oral treatment with tribendimidine, only the active metabolite dAMD was detectable in plasma and concentrations were not significantly different for oral treatment with dAMD.

The results support further evaluation of cyclooctadepsipeptides alone and in combination with cholinergic drugs to improve efficacy. Combining these with registered drugs may help to prevent development of resistance.

4.2 Introduction

In highly endemic countries, the largest part of morbidity-control regarding STHs relies on chemotherapy using anthelmintics in MDAs, since access to health services and education, epidemiological monitoring and effective sanitation are often unavailable [1,2]. Promising results in vaccination trials against *N. americanus* suggest that less chemotherapy-dependent control strategies might be feasible, at least for hookworms [3,4]. However, since no vaccine has successfully passed any phase 1 clinical trial, the use of anthelmintics remains the gold standard to control STHs in the foreseeable future.

Increased MDAs to highly parasite-exposed populations resulted in considerable declines as far as prevalence, intensity of infection and morbidity are concerned. However, long-term sustainability may be jeopardised, since only albendazole, levamisole, mebendazole and pyrantel pamoate are listed by the WHO as essential medicines to treat human STHs [5] and strong selection pressures caused by the usage of four drugs belonging to only two drug classes may affect genetic diversity and favour spread of drug-resistant parasites [1]. Thus, the urgent need for the development of safe and efficacious new anthelmintics against STHs with new modes of action is apparent. Additionally, the conclusive identification of potent combinations of drugs to counteract selection pressure might ensure an extended long-term sustainability of already approved and putative new drugs [6].

Cyclooctadepsipeptides seem to be an interesting class for the development of drugs to treat human STHs, since they show a high degree of nematicidal efficacy in a broad range of hosts [7,8]. The cyclooctadepsipeptide emodepside, an essential component of the recently registered veterinary products Profender® (Bayer Animal Health, Leverkusen, Germany) and Procox® (Bayer Animal Health, Leverkusen, Germany), was shown to have a unique mode of action involving the large-conductance calcium-activated and voltage-gated potassium channel SLO-1 [9,10] and therefore has resistance breaking properties [11].

Another interesting class is represented by the aminophenylamidines – including tribendimidine, which was approved for the control of human STHs only in China [8,12]. In the late 1970s the aminophenylamidine amidantel was reported to be efficacious against several nematodes and platyhelminths [13]. While studying aminophenylamidine structure-activity relationship, tribendimidine was developed in 1983 [12]. Requiring lower dosages than amidantel, tribendimidine has a broad spectrum of anthelmintic efficacy in a variety of hosts, including humans [12]. The major metabolite of amidantel and tribendimidine is dAMD [14], which also exerts a strong anthelmintic efficacy in nematodes [15], although

apparently not in cestodes – at least not in *H. microstoma* [16]. In *C. elegans*, the mode of action of tribendimidine is very similar to that of levamisole since both drugs act as agonists through at least very similar nAChRs [17]. Despite similar targets, synergistic effects of levamisole and tribendimidine against *A. ceylanicum in vitro* and *in vivo* and antagonistic effects against *H. bakeri in vitro* and *in vivo* [15] have been reported recently. We also observed antagonistic effects of both drug classes against *N. brasiliensis* and *T. spiralis in vitro* [18].

Since the cyclooctadepsipeptides and aminophenylamidines showed a significant *in vitro* efficacy against L3 and adult worms of *N. brasiliensis* [18], the present study aimed to evaluate and compare the *in vivo* potency of both drug classes in the *N. brasiliensis* model. The efficacy of combinations of cyclooctadepsipeptides with aminophenylamidines has not been investigated *in vivo* prior to this study. While the effects of levamisole/tribendimidine combinations were very heterogeneous when comparing different nematode species [15], drug combinations of PF1022A and dAMD, PF1022A and tribendimidine, dAMD and levamisole, as well as tribendimidine and levamisole, were analysed against *N. brasiliensis in vivo* to investigate the absence or presence of combinatory effects in this model.

Furthermore, scanning electron microscopy of *in vivo* treated *N. brasiliensis* was performed to further investigate the potential combinatory effects of the drug combinations on the morphology of the worms in comparison to the effects of the respective drugs alone. Finally, explorative plasma pharmacokinetics of dAMD and tribendimidine were analysed in rats, since the data in the literature is controversial in terms of the passage of tribendimidine into the blood after oral application in different mammalian hosts [12].

4.3 Materials and methods

Ethical statement

All animal studies were conducted at the laboratories of Bayer HealthCare AG, Global Drug Discovery Animal Health (Monheim, Germany). The experiments were approved by LANUV (North Rhine-Westphalia, Germany) in accordance with §8a section 1 and 2, German Protection of Animals Act.

Drugs

Amidantel, dAMD, and PF1022A were accessible at Bayer HealthCare AG, Global Drug Discovery Animal Health (Monheim, Germany). Levamisole was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany) and tribendimidine was obtained from Shandong Xinhua Pharmaceutical Company Limited (Zibo, People's Republic of China). All drugs were stored at 4 °C until further use. Drugs were always freshly prepared as dispersions in Cremophor EL (BASF, Ludwigshafen, Germany) and deionised water [1:3] on days of oral treatment.

Animals

Female laboratory outbred Wistar rats (HsdCpb:WU) were purchased from Harlan Netherlands at an age of 6-10 weeks with a body weight of 125-175 g. Rats were housed in Macrolon® cages under environmentally controlled conditions and kept in groups of 3-5 animals. Water and Sniff® rodent food pellets were available ad libitum. Rats were allowed to acclimate for seven days before starting any experiment.

Parasites and infections

The life cycle of *N. brasiliensis* was maintained as described previously [18]. Briefly, rats were subcutaneously infected with 0.3 ml Ringer's solution (118.4 mM NaCl, 4.7 mM KCl, 2.52 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25.01 mM $NaHCO_3$, pH 7.4) containing routinely 2500 infective L3. On day six *p.i.*, faeces were collected, mixed with charcoal and charcoal-filtered water and incubated at 37 °C and 95 % relative humidity for five days. Larvae were collected and incubated for another two days at room temperature to ensure complete development to infective L3. Larvae were washed and then used for infection of rats.

In vivo efficacy of drugs against N. brasiliensis in rats

In a series of 24 consecutive experiments (blocks), a total of 500 rats were randomised into 100 groups, each consisting of five animals. The rats were infected orally with a gavage using 0.5 ml physiological sodium chloride solution containing infective L3 of *N. brasiliensis*. In initial experiments, different infection doses ranging between approximately 600 and 5000 L3 were used to analyse the relationship between the infective dose and recovered adult worms. Within each block all animals received the same number of L3. For each dose of a drug at least five rats were used. In each block, five vehicle-treated rats were used as infection controls. To achieve exactly the desired drug dosages, amounts of drugs were calculated for individual animals depending on body weights determined on day 3 *p.i.* On days 4-6 *p.i.*, rats were orally treated with drugs in Cremophor EL/water dispersions. Dosages of 50, 75, 100, 125, 200, 250, and 500 mg/kg amidantel, 1.0, 2.5, 3.75, 4.25, 5.0, 10, 25, 50, 100, and 250 mg/kg dAMD, 0.5, 1.0, 1.75, 2.5, 3.75, 5.0, 10, 25, 50, 100, and 250 mg/kg tribendimidine, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5.0, 10, 25, 50, 100, and 250 mg/kg PF1022A, and 1.0, 2.5, 3.75, 5.0, 6.25, 7.5, 50, 100, and 250 mg/kg levamisole were used. The negative control groups received only Cremophor EL/water. Additionally, twelve combinations were administered (Tab. 4-1). On day 7 *p.i.,* rats were euthanised by carbon dioxide suffocation. Subsequently, necropsy was performed and worm burden in the duodenum was determined by counting the parasites under a dissecting microscope.

Tab. 4-1. Doses of drug combinations administered in the *N. brasiliensis in vivo* assay

	Tribendimidine (mg/kg)	**dAMD** (mg/kg)
PF1022A (mg/kg)	1.0/2.5	1.0/2.5
	1.5/2.5	1.5/2.5
	1.5/3.75	1.5/3.75
Levamisole (mg/kg)	2.5/2.5	2.5/2.5
	5.0/2.5	5.0/2.5

Drug in row over drug in column

Morphological investigation of adult N. brasiliensis after drug exposure

For determination of drug effects on parasite morphology, suboptimal dosages of 100 mg/kg amidantel, 2.5 mg/kg dAMD, 2.5 mg/kg tribendimidine, 2.5 mg/kg levamisole, and 1.0 mg/kg PF1022A, as well as combinations of 1.0 mg/kg PF1022A plus 2.5 mg/kg dAMD, 1.0 mg/kg

PF1022A plus 2.5 mg/kg tribendimidine and 2.5 mg/kg levamisole plus 2.5 mg/kg tribendimidine were used. The negative control group received only the vehicle (Cremophor EL/water). A total of 27 rats were randomised into nine groups, consisting of three rats each. Otherwise, the infection- and treatment-protocol was identical to that used for drug efficacy trials.

Immediately after necropsy and resection of the guts, small intestines were opened longitudinally and worms in the intestinal mucosa were carefully isolated using fine surgical forceps. For each treatment group, worms were collected and prefixed in snap-cap vials containing 5% glutaraldehyde (SERVA Electrophoresis GmbH, Heidelberg, Germany) in 0.1 M cacodylate buffer (pH 7.3) at 4 °C overnight. Then, worms were washed six times using 0.1 M cacodylate buffer, followed by dehydration of the samples using an ascending ethanol series. Worms were incubated in 70%, 80%, 90%, 96%, and 100% ethanol for 15 min each followed by incubation in fresh 100% ethanol at 4 °C for 24 h. Critical point drying was performed with the CPD 030 Critical Point Dryer (BAL-TEC GmbH, Schalksmühle, Germany) and carbon dioxide as transition fluid. Dried samples were gold-sputtered for 2 min at 15 mA using the sputter coater Emitech K550 (Emitech Ltd., Ashford, England). Scanning electron microscopy analyses were performed using a Supra 40VP (Carl Zeiss SMT AG, Oberkochen, Germany).

Plasma pharmacokinetics

Orally applied dose rates of 10 mg/kg dAMD or 10 mg/kg tribendimidine led to an almost complete elimination of the worm burden in the *N. brasiliensis in vivo* assays (96.2% and 99.5%, respectively). Since these dose rates did not cause any clinical side effects, they were chosen for explorative plasma pharmacokinetic analyses. To derive plasma concentration-time curves, dAMD and tribendimidine plasma levels were recorded before treatment and between 0.5 and 504 h past treatment.

Two groups of 18 rats each were used for explorative plasma pharmacokinetic analyses. After seven days of acclimatisation, the pre-treatment blood samples were drawn from all rats to ensure drug absence prior to treatment. The body weight of all rats was determined three days prior to treatment to ensure exact dosing. Administrations were performed by gavage at single oral dose rates of 10 mg/kg body weight using 0.1% oral suspensions. Dose volume applied was 1 ml/100 g body weight. Following a sparse sampling schedule, each animal was used to draw three to four blood samples by sublingual bleeding or heart puncture (only for final samples). Samples were drawn in triplicates.

Concentrations of dAMD and tribendimidine were determined by turbulent flow chromatography coupled with a tandem mass spectrometry detector. Plasma was injected directly into the turbulent flow chromatography system while it was in the loading position. Matrix components contained in the injected sample were separated from the retained analytes on an extraction column suited for pre-treatment of samples at high flow rates. After switching to the eluting position, the sample was transferred to an analytical column. The quantitative determination was performed with a tandem mass spectrometric detector. The limit of quantitation was 25 µg/l. Pharmacokinetic evaluation was performed using Phoenix®, version 6.3 (Pharsight Corp., Mountain View, USA). Non-compartmental analysis of extravascular dosing and sparse sampling was used to derive the pharmacokinetic profiles of dAMD in plasma.

Calculation of dose-response curves and statistical analysis

For single drug treatments, reduction of worm burden in comparison to the mean worm burden of the respective placebo-treated control (expressed in per cent) was plotted against the $\log_{10}$ of the drug dosages. Then, four-parameter-logistic curves were fit using GraphPad Prism 5.03. ED_{50} values were compared using the extra sum of squares *F* test. In cases where multiple tests were performed, *P* values were corrected using the Bonferroni-Holmes procedure.

The combinations were compared to respective single drug treatments using the non-parametric Kruskal-Wallis test with Dunn's post hoc test for identification of significant differences between individual groups. In order to identify any additive or synergistic drug effects, efficacy of the combinations was compared in a Wilcoxon signed rank-sum test to a theoretical expected median, which was set to the sum of the median efficacies of the individual drugs. These calculations were also performed using GraphPad Prism software version 5.03.

4.4 Results

Reproducibility of worm recovery for different infective doses

Since the 24 no-drug control groups (each consisting of five animals) varied in the infective doses (600-5000 larvae per rat), preliminary statistical analyses were performed. A positive correlation between the absolute number of larvae used for the oral infection and the absolute number of adult worms recovered from the small intestines after necropsy was identified by linear regression (Fig. 4-1A). There was a significant positive correlation between the number of adult worms recovered from the intestines and the infective dose ($P<0.0001$). The slope of the regression curve was 0.3097 ± 0.01849 and the coefficient of determination was $R^2=0.7056$.

Comparison of control groups from eight independent experiments with a consistent infective dose of 2000 larvae revealed considerable variation in recovery rates (as a percentage of the infective dose) between individual rats but no significant differences in the recovery rates of adult worms between experiments (Fig. 4-1B). When recovery rates between different infective doses were compared using a Kruskal-Wallis followed by a Dunn's post hoc test, the lowest dose of 600 larvae and the two highest doses of 3000 and 5000 larvae resulted in significantly lower or higher worm recovery rates, respectively (Fig. 4-1C). No significant difference in the relative recovery rate of adult worms was identified between any of the infective doses between 1000 and 2500 larvae (Fig. 4-1C). Therefore, only experiments with an infective dose between 1000 and 2500 larvae were included in the analyses of the *in vivo* efficacy of the test compounds against *N. brasiliensis* (n=400 rats).

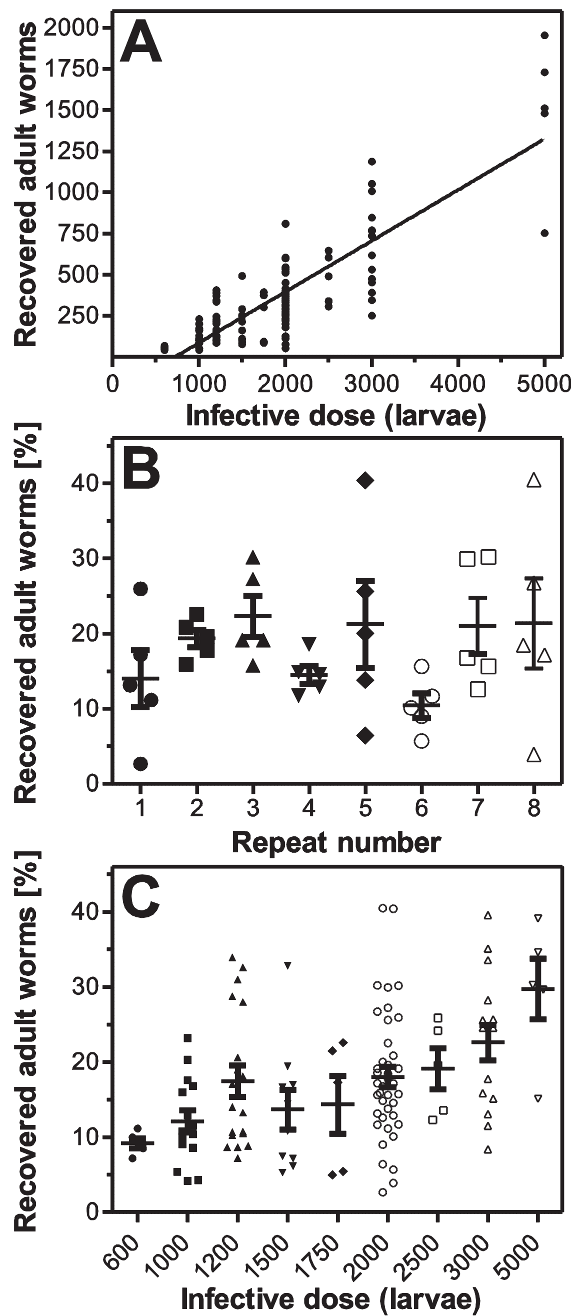

Fig. 4-1. Reproducibility of worm recovery in placebo-treated rats

A) Using infective doses between 600 and 5000 *N. brasiliensis* L3, recovery of adult worms showed significant correlation between infective dose and worm burden ($P<0.0001$). B) When recovery was calculated in percentage of the infective dose, comparison between eight experiments with rats infected with 2000 larvae revealed high variability between individual animals but no significant differences between experiments as determined in a Kruskal-Wallis test. C) Significant differences in recovery rates were observed between experimental groups infected with different numbers of larvae. However, when recovery rates were compared between infective doses ranging from 1000 to 2500 larvae, no significant differences were detectable.

In vivo efficacy of drugs against N. brasiliensis in rats

Levamisole, PF1022A and the aminophenylamidines amidantel, dAMD and tribendimidine diminished the worm burden in the small intestines in a dose-dependent manner. The calculated dose-response curves of all tested drugs are shown in Fig. 4-2, whereas Tab. 4-2 summarises the ED_{50} values with 95% CI and R^2 values. Furthermore, *P*-values for comparisons between ED_{50} values of different aminophenylamidines are indicated (Tab. 4-2). Due to the large variability in worm recovery between individual rats as described above, R^2 for these *in vivo* dose-response curves are relatively low (0.45-0.87).

The ED_{50} value for amidantel was approximately 30 times higher than the ED_{50} values of the other aminophenylamidine derivatives dAMD and tribendimidine (Tab. 4-2). In contrast to the highly significant differences between amidantel and its derivatives dAMD and tribendimidine, the ED_{50} values of dAMD and tribendimidine were not significantly different, illustrated by their very similar dose-response curves (Fig. 4-2B). The cyclooctadepsipeptide PF1022A and the reference drug levamisole also showed high *in vivo* efficacy against *N. brasiliensis*. The ED_{50} value for PF1022A (1.2 mg/kg) was approximately three to four times lower than that of levamisole (4.8 mg/kg), dAMD (3.8 mg/kg) and tribendimidine (3.4 mg/kg).

Tab. 4-2. Comparison of tested drugs in the *N. brasiliensis in vivo* assay

Drug	ED_{50} with 95% CI (in µg/ml)	*P* value[a]	R^2
PF1022A	1.213 (0.9119-1.612)		0.6201
Levamisole	4.817 (3.846-6.032)		0.5294
Amidantel	109.5 (95.58–125.5)	< 0.0003 (*vs.* dAMD) < 0.0003 (*vs.* tribendimidine)	0.4490
dAMD	3.846 (2.829-5.228)	< 0.0003 (*vs.* amidantel) 0.5408 (*vs.* tribendimidine)	0.5573
Tribendimidine	3.387 (2.912-3.940)	< 0.0003 (*vs.* amidantel) 0.5408 (*vs.* dAMD)	0.8677

[a]Significant difference in ED_{50} to drug in brackets. *P* values were corrected for multiple testing using the Bonferroni-Holmes method. Presented are the ED_{50} values with the 95% CI and R^2 as well as *P* values, for determination of significant differences.

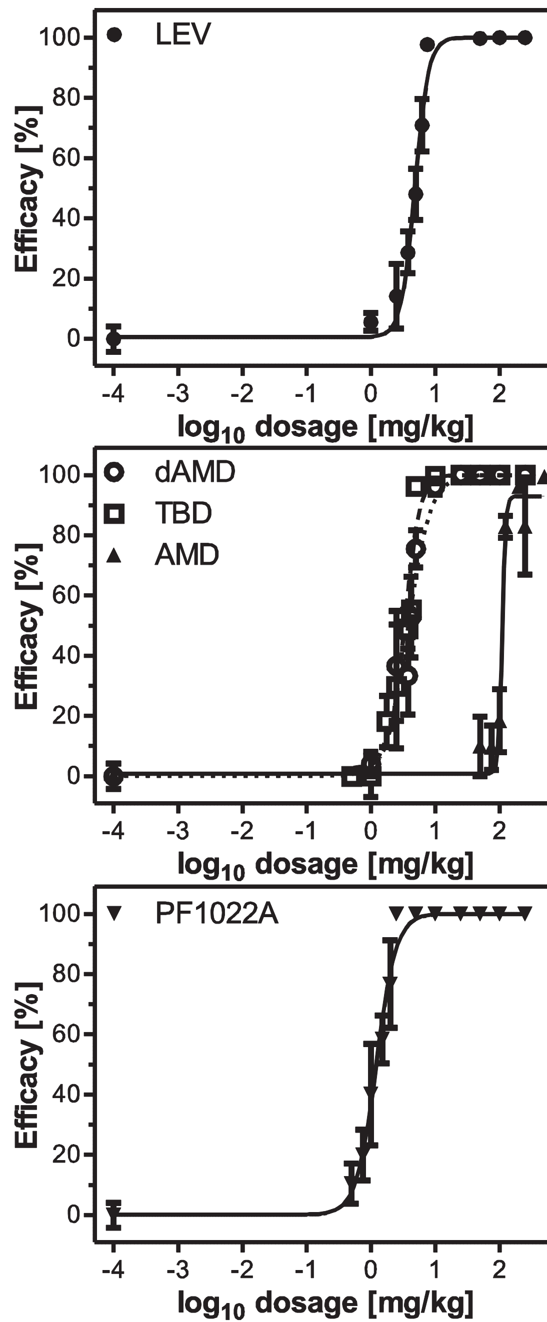

Fig. 4-2. *In vivo* dose-response curves for efficacy of monotherapies against adult *N. brasiliensis*

Efficacy was calculated as relative number of worms recovered from the intestines compared to the vehicle control in percentage. Data shown are means ± SEM. The drug dosages in the negative controls were set to 10^{-4} mg/kg to allow $\log_{10}$ transformation of dosages. Logistic regressions were calculated with top and bottom values constrained between 0% and 100%.

In vivo effects of drug combinations

Combinations of PF1022A and dAMD, PF1022A and tribendimidine, dAMD and levamisole as well as tribendimidine and levamisole were investigated against *N. brasiliensis in vivo* at suboptimal dosages (≤50% reduction in worm burden) of the single drugs (Fig. 4-3). In a series of preliminary statistics all drug combinations and single drug dosages were compared to the no-drug control by using the non-parametric Kruskal-Wallis test with Dunn's post hoc test for identification of significant differences between individual groups. Significant differences were identified only for the drug combinations but not for the single drugs. Combinations of 5.0 mg/kg levamisole plus 3.75 mg/kg dAMD and 5.0 mg/kg levamisole plus 2.5 mg/kg dAMD, but not 2.5 mg/kg levamisole plus 2.5 mg/kg dAMD led to a significant reduction of the worm burden ($P<0.0001$). The same combined dosages of levamisole plus tribendimidine reduced the worm burden in a highly significant manner, but also the combination of 2.5 mg/kg levamisole plus 2.5 mg/kg tribendimidine was significantly more efficacious against *N. brasiliensis* compared with the effects of the respective single drugs ($P<0.0001$).

While the three tested dAMD/PF1022A combinations (1.5 mg/kg PF1022A plus 3.75 mg/kg dAMD, 1.5 mg/kg PF1022A plus 2.5 mg/kg dAMD and 1.0 mg/kg PF1022A plus 2.5 mg/kg dAMD) led to a significant reduction of the worm burden, only two PF1022A-tribendimidine combinations were significantly efficacious (1.5 mg/kg PF1022A plus 3.75 mg/kg tribendimidine and 1.5 mg/kg PF1022A plus 2.5 mg/kg tribendimidine) ($P<0.0001$). The third combination (1.0 mg/kg PF1022A plus 2.5 mg/kg tribendimidine) was not significantly better than the single drugs.

Comparisons of the three different dosages tested for each combination detected no significant differences. There were also no significant differences between combinations of tribendimidine and levamisole and the equivalent dosages of dAMD and levamisole. In addition, combinations of PF1022A with either tribendimidine or dAMD showed no significant differences in efficacy.

Next, all drug combinations were compared to the respective single drug dosages by using the Kruskal-Wallis test with Dunn's post hoc test. Combinations of dAMD plus levamisole and of dAMD plus PF1022A were only significantly better than dAMD alone but not significantly more efficacious than levamisole (Fig. 4-3A) and PF1022A (Fig. 4-3C), respectively. However, combinations of tribendimidine plus levamisole and of tribendimidine plus PF1022A led to a significantly higher elimination of the worm burden than tribendimidine, levamisole or PF1022A alone (Fig. 4-3B and 4-3D).

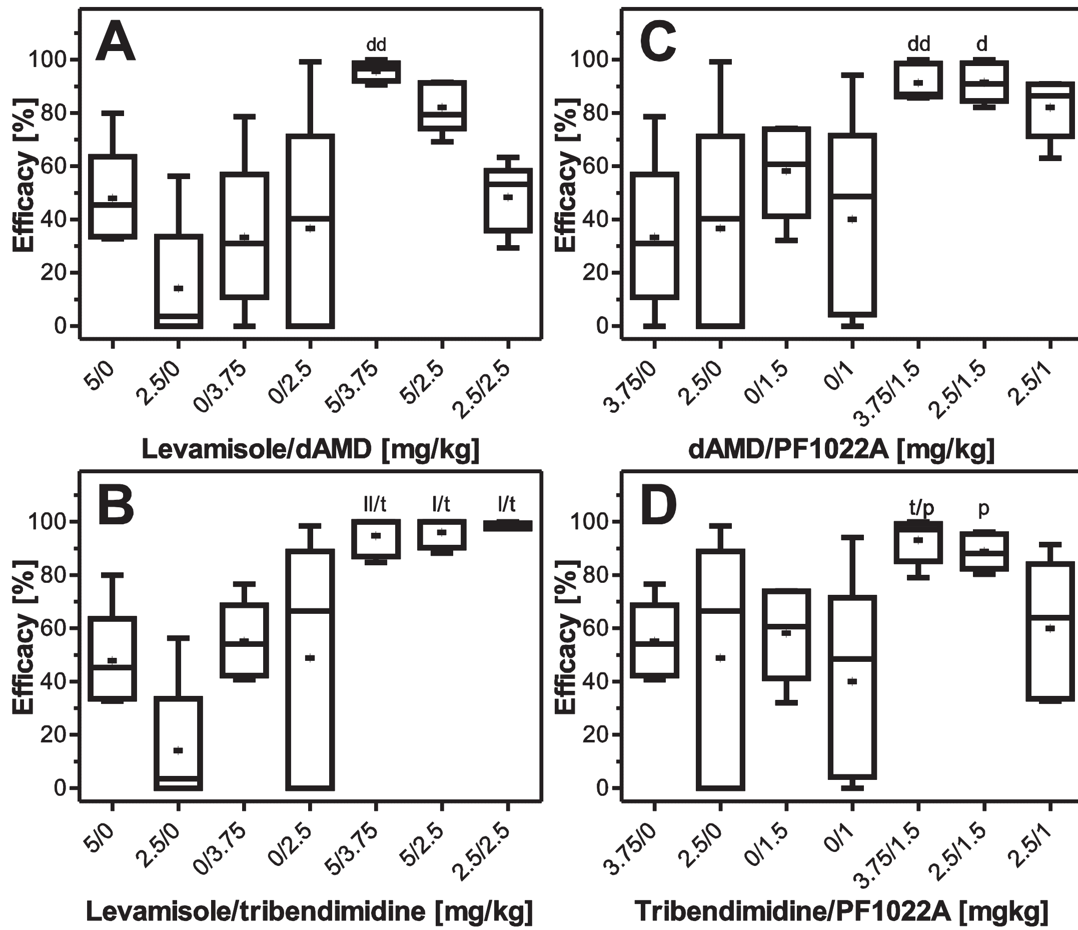

Fig. 4-3. Effects of drug combinations in comparison to treatments with individual drugs at the same dosages

Combinations of A) levamisole and dAMD, B) levamisole and tribendimidine, C) dAMD and PF1022A and D) tribendimidine and PF1022A were administered. Combinations were compared with single drug treatments with the same dosages using the Kruskal-Wallis test followed by the Dunn's *post hoc* test. Arithmetic means are indicated in the box plots by a cross. Whiskers range from minimal to maximal values. Indices at the combinations indicate: d, $P<0.05$ *vs.* dAMD; dd, $P<0.01$ *vs.* dAMD; l, $P<0.05$ *vs.* levamisole; ll, $P<0.01$ *vs.* levamisole; p, $P<0.05$ *vs.* PF1022A; t, $P<0.05$ *vs.* tribendimidine.

Since a number of combinations were significantly more effective than the single drugs, a Wilcoxon rank-sum test against the expected values of the sum of the single drug medians was performed to differentiate additive and synergistic effects. All drug combinations were not significantly different from the expected sum of the single drug efficacies, suggesting that drug combinations acted additively.

Morphological effects of drug treatments on N. brasiliensis by electron microscopy

Scanning electron microscopy studies of *N. brasiliensis* treated *in vivo* with (i) 1.0 mg/kg PF1022A, (ii) 100 mg/kg amidantel, (iii) 2.5 mg/kg dAMD, (iv) 2.5 mg/kg tribendimidine, (v) 2.5 mg/kg levamisole or (vi) placebo (Cremophor EL/water) were performed to investigate the effects of the different drug classes on the overall morphology of the worms. Furthermore, treatments with combinations of (i) 1.0 mg/kg PF1022A and 2.5 mg/kg dAMD, (ii) 1.0 mg/kg PF1022A and 2.5 mg/kg tribendimidine, (iii) 2.5 mg/kg dAMD and 2.5 mg/kg levamisole as well as (iv) 2.5 mg/kg tribendimidine and 2.5 mg/kg levamisole were performed to detect any potential combinatory effects of the drug combinations on the morphology of the worms in comparison to the respective drugs alone.

The typical morphology of vehicle-treated *N. brasiliensis* is shown in Fig. 4-4. The overall condition of the untreated worms was very homogeneous as far as the body surface, attributed to the high hydrostatic pressure in the fluid-filled pseudocoel, acting as a hydroskeleton against the rigid cuticule (Fig. 4-4A), the typical amphids located in the apical region (Fig 4-4B), the occurrence of longitudinal ridges of the cuticule along the whole body (Fig. 4-4C, D) as well as the well-developed cephalic cap with the triradiate mouth opening (Fig. 4-4D) are concerned. This indicates that the worms were intact and in an unscathed condition before fixation. Gender specific characteristics such as the copulatory bursa at the posterior end of male worms (Fig. 4-4A), as well as the vulva aperture surrounded by thick lips and the anus at the base (Fig. 4-4E) and the pair of phasmids at the tip of the tail of females (Fig. 4-4F) are illustrated. The wrinkle formation of the cuticule in Fig. 4-4F is due to the fixation.

The overall shape of the PF1022A-treated worms was indistinguishable from the placebo-treated *N. brasiliensis* (Fig. 4-5A, B). Only at a very high magnification, several small cuticular knobs were observed next to the longitudinal ridges after three treatments with 1 mg PF1022A/kg body weight (Fig. 4-5C). However, treatments with levamisole had significant effects on the total body shape of both male and female worms (Fig. 4-5D, E). Whereas the whole body of male worms was severely contracted (Fig . 4-5D), the morphological changes

of the females were limited to the posterior part (Fig. 4-5E), which appeared to be strongly swollen, presumably due to the completely retracted caudal end (Fig. 4-5E, F). Similar effects were observed when *N. brasiliensis* were treated with aminophenylamidines (Fig. 4-6). Since treatments with amidantel, dAMD and tribendimidine had identical effects on *N. brasiliensis* morphology, only exemplary specimens are shown. Whereas male worms looked indistinguishable from the levamisole treated specimens (Fig. 4-6D), females were also swollen and contracted along the whole body (Fig. 4-6A) except for the cephalic cap, which corresponded to the phenotype of vehicle-treated specimens (Fig. 4-6B). Just like levamisole, the aminophenylamidines induced a complete retraction of the tail region, so that the vulva aperture was no longer visible (Fig. 4-6C). Only tribendimidine caused extrusion of the two twisted spicules (Fig. 4-6E, F). Since none of the combinations altered the effects of the aminophenylamidines alone, these images are not shown.

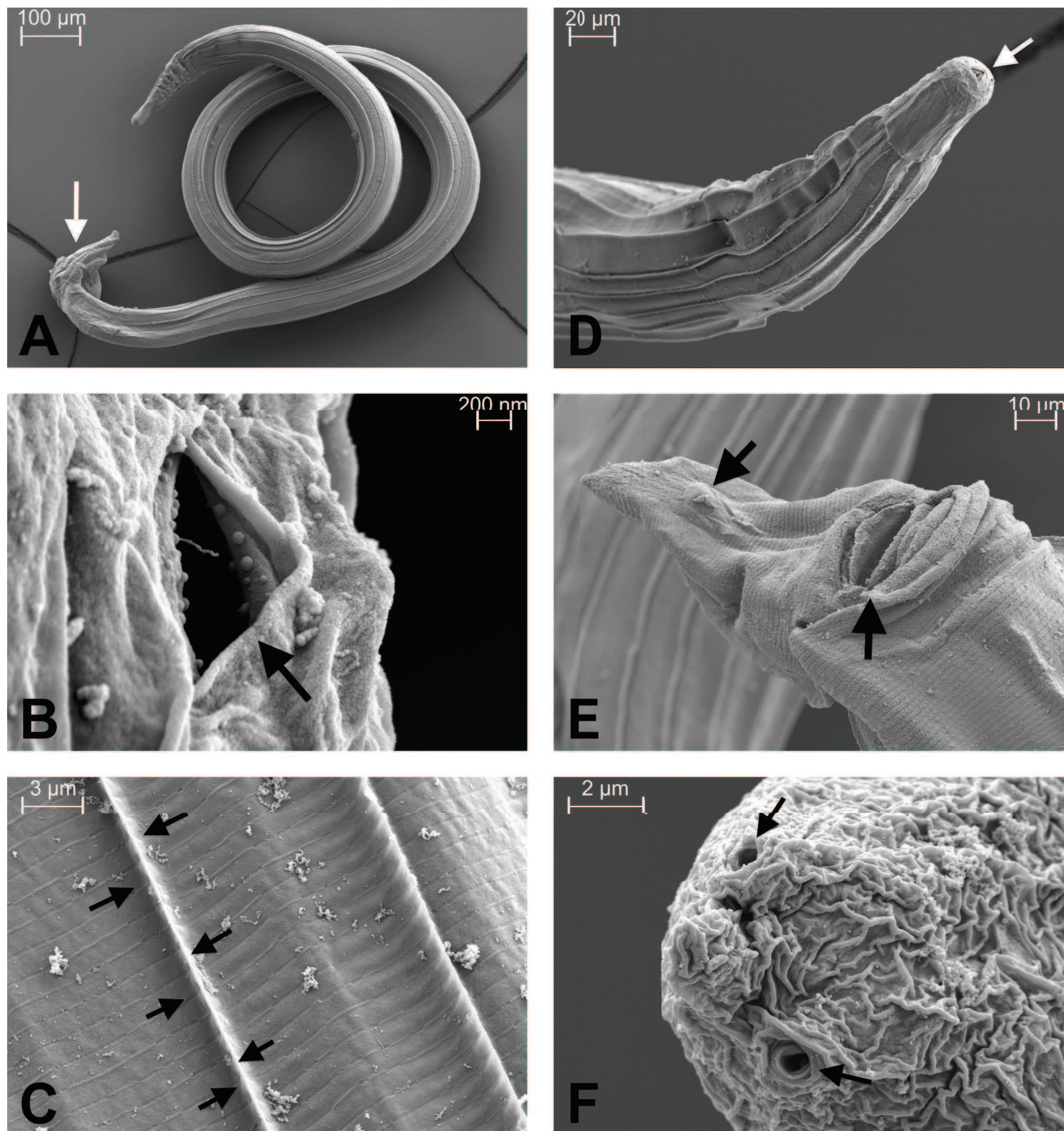

Fig. 4-4. Overall appearance of untreated adult *N. brasiliensis* by scanning electron microscopy

A) Male worm with the copulatory bursa at the posterior end of the tail (arrow). The worm is approximately 2400 µm long and 85 µm wide at midbody (widest part). B) Detailed view of an amphid (see arrow) of a male worm, a secretory organ secreting *e.g.* AChE. C) Detailed view of the cuticule of the midbody of a male worm. Two longitudinal ridges (one is marked by an arrow) and unspecified debris are present. D) Anterior region of a female worm. The well-developed cephalic cap with the triradiate mouth opening (arrow) does not show any longitudinal ridges. E) Ventral view of the caudal end of a female worm showing the vulva aperture (arrow) surrounded by thick lips and the anus (arrow) at the base of the tail. F) Detailed view of a pair of phasmids (see arrows) at the tip of the tail of an adult female worm.

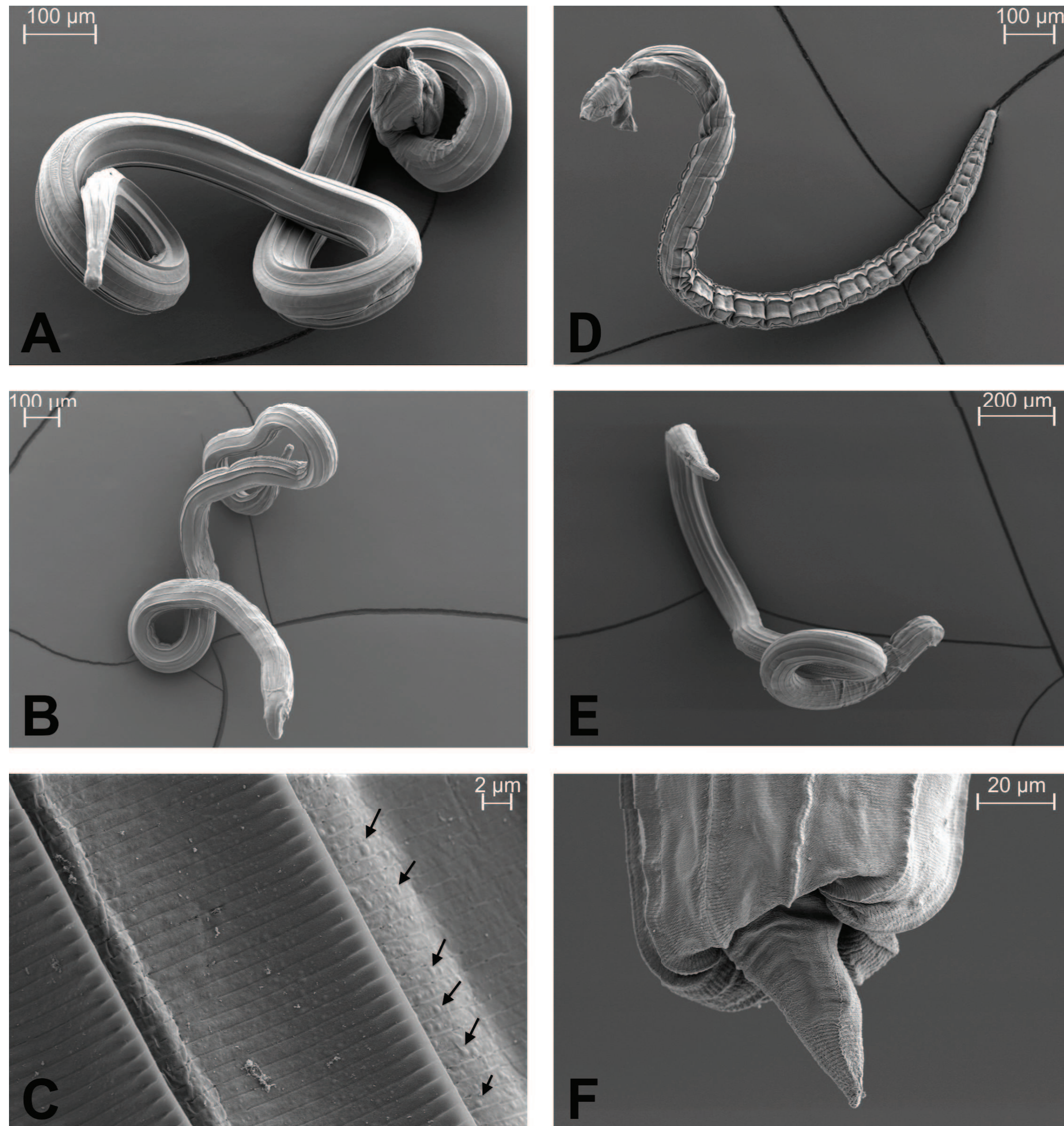

Fig. 4-5. Overall appearance of anthelmintic-treated adult *N. brasiliensis* by scanning electron microscopy

Worms in (A-C) were treated with PF1022A and in (D-F) with levamisole. A) Isolated adult male worm after three treatments with 1 mg PF1022A/kg body weight. B) Female worm after three PF1022A-treatments. The overall appearance is indistinguishable from untreated males and females. C) Detailed view of the cuticule after treatments with PF1022A. Next to the longitudinal ridges, several small tegumental knobs (see arrows) are present. D) Male worm after three treatments with 2.5 mg levamisole/kg body weight. The whole body is strongly contracted and swollen. E) Adult female worm after treatments with levamisole. Similar to the male worm in D), the posterior end appears to be contracted and swollen. F) Posterior end of a female worm after three levamisole-treatments. The caudal end is almost completely retracted.

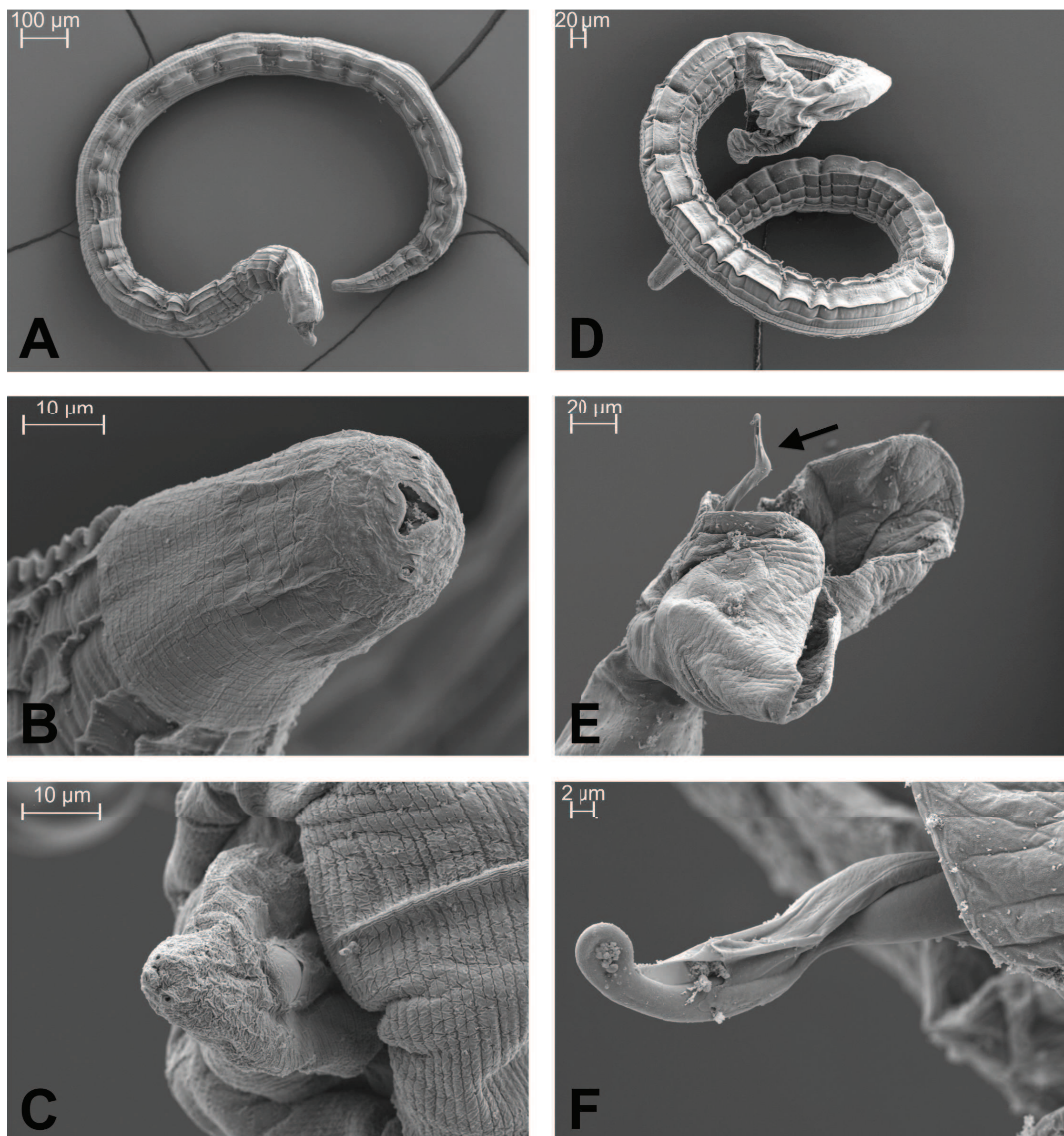

Fig. 4-6. Morphology of adult *N. brasiliensis* treated with aminophenylamidines by scanning electron microscopy

A) Adult female worm after three doses of 100 mg amidantel/kg body, showing a strongly contracted and swollen body. B) The cephalic cap of a dAMD treated female worm corresponds with the phenotype of untreated specimens. The anterior part of the cap consists of approx. eight wider annuli, followed by 17 narrower annuli and the cephalic boundary. C) Tail region of a female worm after three treatments with 2.5 mg tribendimidine/kg body weight. The caudal end is retracted up to the anus aperture. The vulva aperture became completely invisible. D) Male worm after three dAMD-treatments. The whole body is strongly contracted and swollen. E) Copulatory bursa from a male worm treated with tribendimidine. Two twisted spicules (see arrow) are extruded from the here not shown cloaca. A small dorsal and two large lateral lobes surround the cloaca. F) Detailed view of extruded copulatory spicules of a tribendimidine-treated adult male worm.

Plasma concentration-time-profiles and derived pharmacokinetics

In contrast to its metabolite dAMD, tribendimidine was not detectable above its limit of quantification in plasma after single oral dosing at a rate of 10 mg/kg body weight to rats. Tribendimidine rapidly degraded to dAMD in an aqueous environment and was not detectable even in plasma samples spiked with tribendimidine. Plasma pharmacokinetic profiles of dAMD derived after dosing dAMD or tribendimidine were comparable (Fig. 4-7). Since only a small number of data points were available during the elimination phase, half-life could not be calculated reliably. Means (± SEM) of plasma pharmacokinetic parameters of dAMD are presented in Tab. 4-3.

Tab. 4-3. Plasma pharmacokinetic parameters of deacylated amidantel after oral treatment with deacylated amidantel or tribendimidine

Parameter	Unit	dAMD (dAMD)[a]	dAMD (tribendimidine)[b]
C_{max}	µg/L	1382 ± 131	1445 ± 94
AUC_{last}	µg×h/L	4256 ± 145	3927 ± 161

[a]After treatment with dAMD; [b]After treatment with tribendimidine; C_{max}, maximal plasma concentration; AUC_{last}, area under the curve to the last measurement time point; ± SEM; No significant differences were observed between treatment with tribendimidine and dAMD.

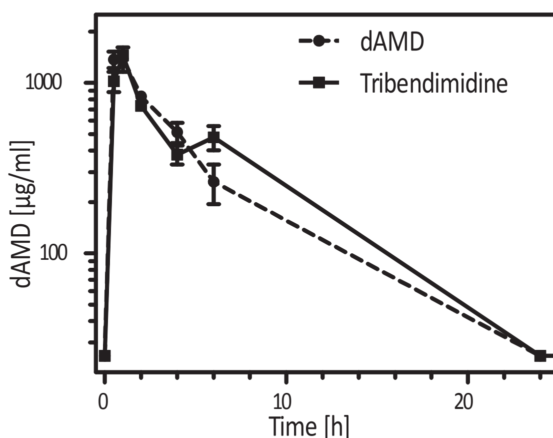

Fig. 4-7. Plasma pharmacokinetic analyses of dAMD after oral treatment with dAMD or tribendimidine

Mean plasma-concentrations (± SD) of dAMD after single oral administration of dAMD (triangles) or of tribendimidine (circles) were recorded over time. For all time points where no dAMD was detected, the value was set to 25 µg/ml, *i.e.* the limit of quantification. No dAMD was detected at time points more than 24 h after drug application.

4.5 Discussion

Within the initial experiments presented in this manuscript, the reproducibility of inoculum size and worm recovery was investigated. Data show relatively high variability between individual animals within each block but differences between blocks were not statistically significant. This underlines the requirement of relatively high numbers of animals when testing anthelmintic drugs to obtain meaningful data. High variability was also observed for intermediate efficacy rates in the drug treated groups, highlighting the fact that the number of animals essentially contribute to the goodness of fit (dose-response curve) and the related determination of the ED_{50}. If only intermediate efficacy rates are available, interpretation of data will remain vague and therefore increasing the risk of misinterpretation.

The study presented here aimed at the evaluation and comparison of the *in vivo* nematocidal efficacy of the cyclooctadepsipeptide PF1022A, the aminophenylamidines amidantel, dAMD and tribendimidine and the reference drug levamisole in rats experimentally infected with the rat hookworm *N. brasiliensis*. The utilisation of drug combinations is considered to be a powerful tool in the field of veterinary parasitology [1,6,19]. In principle, drug combinations can be used for three purposes; (i) to increase the spectrum (*e.g.* emodepside and praziquantel in Profender®), (ii) to improve the efficacy of drugs (*e.g.* abamectin and derquantel in Startect®) and (iii) to decelerate the development of drug resistance (*e.g.* abamectin and derquantel in Startect®) [6]. Since Beach *et al.* [20] found in a randomised, placebo-controlled trial that albendazole plus ivermectin treatments had a significantly higher efficacy against human *W. bancrofti* infections and soil-transmitted helminthiases including *T. trichiura* in comparison to the respective single drug efficacies, investigations regarding drug combinations to treat human helminth infections were also intensified (e.g.[21,22]). Accordingly, in the present study combinations of PF1022A plus dAMD, PF1022A plus tribendimidine, levamisole plus dAMD and levamisole plus tribendimidine were evaluated in a *N. brasiliensis in vivo* assay to detect any potential combinatory effects in comparison to the effects of the individual drugs alone.

PF1022A showed a high degree of efficacy against *N. brasiliensis in vivo*, illustrated by an ED_{50} value of 1.213 mg/kg and by complete elimination of worm burdens using three dosages of at least 2.5 mg/kg PF1022A each. This corroborates a number of previously published *in vitro* and *in vivo* studies showing that PF1022A is highly effective against gastrointestinal nematodes including the rat hookworm (for review see [7]). In *in vitro* assays it has been shown that PF1022A is anthelmintically active against the infective L3 as well as the adult

stage of *N. brasiliensis* [18]. The *in vivo* results presented here agree with a previous study, which employed oral treatments on three consecutive days starting at day five p.i. Dosages of 2.5 mg/kg PF1022A resulted in an almost complete elimination of *N. brasiliensis* [23]. Therefore, the efficacy of PF1022A against *N. brasiliensis* appears to be in the same dose range as the required emodepside dosage (2.5 mg/kg, but in a single oral treatment regime) [24].

The morphology of *N. brasiliensis* after PF1022A treatments was largely unchanged as shown by scanning electron microscopy, except for several small cuticular knobs next to the longitudinal ridges. Since the cyclooctadepsipeptides are known to cause flaccid paralysis, it was not unexpected to find only minimal changes due to PF1022A treatment. However, the cause of the cuticular knobs remains unknown.

In the present study, the *in vivo* EC_{50} for amidantel was about 30-fold higher than that of tribendimidine and dAMD. The *in vivo* efficacies of the aminophenylamidines against *N. brasiliensis* have also been investigated previously. Using the same protocol as in the present publication, three treatments using 250 mg/kg amidantel resulted in complete elimination of the worm burden [13]. This result corresponds to the present findings of an ED_{50} value of 109.5 mg/kg and an almost complete elimination of the worm burden starting at 200 mg/kg. Regarding the *in vivo* efficacy of dAMD and tribendimidine against *N. brasiliensis* using a single-treatment regimen against adult stages, ED_{90} values of 5.104 mg/kg and 8.435 mg/kg were reported, respectively [25]. In contrast, the ED_{50} values of the present study were 3.8 mg/kg and 3.4 mg/kg for dAMD and tribendimidine, respectively and an almost complete elimination of worm burden was achieved using three dosages of 10 mg/kg dAMD or 5 mg/kg tribendimidine, which is in agreement with the results published by Xiao *et al.* [12]. It has been shown, that tribendimidine is rapidly metabolised to dAMD [14], which by itself exerts a strong anthelmintic activity against nematodes [26]. Indeed, our pharmacokinetic analysis could not detect any tribendimidine in the plasma and even in plasma samples spiked with tribendimidine only dAMD was detectable. This strongly supports the report that tribendimidine spontaneously disintegrates in an aqueous environment to two molecules dAMD plus one molecule terephthalaldehyde [25]. However, our results are in marked contrast to Xiao *et al.* [12], summarising in their review unpublished data with *e.g.* peak plasma concentrations of 318 ng/ml tribendimidine at 1.78 h after treatment and AUC of 1674 ng×h/ml after oral dosage of 150 mg/kg tribendimidine to rats. Reason for these striking differences remains unclear, in particular since Xiao *et al.* [12] did not provide any experimental details. The very similar time course of dAMD plasma levels observed in the

present study after treatment with dAMD or tribendimidine presumably corresponds to the very similar anthelmintic activity observed for both drugs in terms of ED_{50} values. Since 1 mg dAMD contains approximately 25% more dAMD than what could be released from 1 mg tribendimidine (approximately 0.8 mg dAMD) and tribendimidine is spontaneously and rapidly converted to dAMD, this high similarity is slightly unexpected. This relatively small increase in available dAMD drug might either be too small to cause a significant difference or the availability of tribendimidine in the Cremophor/water dispersion might be higher than that of dAMD.

To our knowledge, this is the first study describing the effects of nAChR agonists on the overall surface of a parasitic nematode using scanning electron microscopy techniques. At least in *C. elegans* the aminophenylamidines share their mode of action with levamisole, since all drugs are agonists of nAChRs [17]. Therefore, the homogenous effects on the morphology of *N. brasiliensis* are comprehensive. Since activation of nAChRs results in spastic paralysis, significant effects on the total body shape of the worms are expectable. Differences in the intensity of contractions and swellings of the worms might be due to different intrinsic drug concentrations/availability. The reason for extrusion of the two twisted spicules only after tribendimidine but not dAMD exposure remains unclear.

The efficacy of all tested combinations was consistently improved in comparison to the individual drugs. Since a Wilcoxon rank sum test against the expected values of the sum of the single drug medians was not significantly different from the efficacy of the combinations, additive effects can be presumed.

As far as combinations of levamisole and tribendimidine are concerned, the literature is somewhat controversial. Whereas synergistic effects of tribendimidine and levamisole have been reported against *A. ceylanicum* L3 *in vitro* and against adult *A. ceylanicum in vivo*, significantly antagonistic effects have been observed against L3 of *H. bakeri in vitro* and adult *H. bakeri in vivo* [15]. In a previous study, we did not find significantly higher efficacies of the levamisole plus tribendimidine combinations in comparison to at least one drug against *N. brasiliensis* L3 and adult worms as well as against L3 of *T. spiralis* [18]. As mentioned above, tribendimidine apparently shares its mode of action with levamisole and therefore strong changes in overall efficacy can be expected if drug concentrations are in the range where the dose-response curve are steep and synergistic effects of such doses might be expected. Since tribendimidine is unstable in water and not present in human plasma [14], the general combinatory effects of either dAMD and levamisole or tribendimidine and levamisole should be identical. However, there was a slight trend that combinations containing

tribendimidine were more efficacious than combinations containing dAMD, although this was not significant. Since tribendimidine directly disintegrates into two molecules dAMD and one molecule TPAL in aqueous environments and TPAL exerts no efficacy against nematodes [25], the reason for higher efficacies using combinations of tribendimidine/levamisole in comparison to dAMD/levamisole remains unclear as outlined above. However, synergistic effects were observed for none of the drug combinations tested. In addition to the shared mode of action, possible reasons for synergistic effects of levamisole/tribendimidine combinations, as observed in *A. ceylanicum* [15], might be allosteric interactions of both drugs on the same receptor. Differences in the composition and sets of the AChR subunits between nematode species as well as between life cycle stages might result in different sensitivities to individual cholinergic anthelmintics [27,28] but could also influence the interaction of drugs.

The effects of combinations of cyclooctadepsipeptides with aminophenylamidines have only been investigated *in vitro* against *N. brasiliensis* and *T. spiralis* and they were not significantly superior in comparison to either of the respective single drugs [18]. Cyclooctadepsipeptides boost the inhibitory system and lead to hyperpolarisation of cells whereas aminophenylamidines activate the excitatory system, leading to membrane depolarisation. Since both drug classes have opposite effects on the membrane potential of excitable cells, it was unclear whether the combinations might result in additive/synergistic or even antagonistic effects. The results presented here show that additive effects are possible although both drug classes act on neurological systems that antagonise each other.

In conclusion, cyclooctadepsipeptides might be an interesting class for the treatment of human STHs, since they might achieve a complete elimination of the worm burden in a single-treatment regimen [8]. Due to a significant reduction of costs and management efforts, single dosages should be the preferred treatment-scheme, independently of the question: single drug or combination?

Because of their increased efficacy, broader activity spectrum and decreased risk to select for drug resistance, combinations of cholinergic drugs with cyclooctadepsipeptides should be further investigated. However, before further testing of potential drug combinations including the aminophenylamidines, there is in urgent need to obtain more data on the safety of this drug class [29].

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4.7 Financial support

The Bayer HealthCare AG funded the presented study. The Bayer HealthCare AG sells anthelmintics for the use in veterinary medicine.

4.8 Conflict of interest

D. Kulke and K. Fraatz are employees of Bayer HealthCare AG. R. Krebber is employee of Bayer CropScience AG and A. Harder was employee of Bayer HealthCare AG, when the study was conducted.

4.9 References

1. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, Yang GJ, Sripa B, Lustigman S, 2012, A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6, e1549.
2. Ziegelbauer K, Speich B, Mausezahl D, Bos R, Keiser J, Utzinger J, 2012, Effect of sanitation on soil-transmitted helminth infection: systematic review and meta-analysis. PLoS Med 9, e1001162.
3. Hotez P, 2011, Enlarging the "Audacious Goal": elimination of the world's high prevalence neglected tropical diseases. Vaccine 29, 104-110.
4. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A, 2010, Developing vaccines to combat hookworm infection and intestinal schistosomiasis. Nat Rev Microbiol 8, 814-826.
5. World Health Organization, 2011, WHO model list of essential medicines: 17th list, March 2011, World Health Organization, Geneva.
6. Geary TG, Hosking BC, Skuce PJ, von Samson-Himmelstjerna G, Maeder S, Holdsworth P, Pomroy W, Vercruysse J, 2012, World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Guideline: Anthelmintic combination products targeting nematode infections of ruminants and horses. Vet Parasitol 190, 306-316.
7. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, Welz C, von Samson-Himmelstjerna G, 2012, Anthelmintic cyclcooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28, 385-394.
8. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, Don R, Keiser J, 2011, Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5, e1138.
9. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, Harder A, Holden-Dye L, Hopper NA, 2007, The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. Int J Parasitol 37, 1577-1588.
10. Welz C, Krüger N, Schniederjans M, Miltsch SM, Krücken J, Guest M, Holden-Dye L, Harder A, von Samson-Himmelstjerna G, 2011, SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in *Caenorhabditis elegans* slo-1 loss of function mutants. PLoS Pathog 7, e1001330.

11. von Samson-Himmelstjerna G, Harder A, Sangster NC, Coles GC, 2005, Efficacy of two cyclooctadepsipeptides, PF1022A and emodepside, against anthelmintic-resistant nematodes in sheep and cattle. Parasitol 130, 343-347.
12. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W, 2005, Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94, 1-14.
13. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, Thomas H, 1979, Amidantel, a potent anthelminthic from a new chemical class. Arzneim Forsch 29, 31-32.
14. Yuan G, Xu J, Qu T, Wang B, Zhang R, Wei C, Guo R, 2010, Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs R D 10, 83-90.
15. Tritten L, Nwosu U, Vargas M, Keiser J, 2012, *In vitro* and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. Acta Trop 122, 101-107.
16. Kulke D, Krücken J, Welz C, von Samson-Himmelstjerna G, Harder A, 2012, *In vivo* efficacy of the anthelmintic tribendimidine against the cestode *Hymenolepis microstoma* in a controlled laboratory trial. Acta Trop 123, 78-84.
17. Hu Y, Xiao SH, Aroian RV, 2009, The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3, e499.
18. Kulke D, Krücken J, Demeler J, Harder A, Mehlhorn H, von Samson-Himmelstjerna G, 2013, *In vitro* efficacy of cyclooctadepsipepdtides and aminophenylamidines alone and in combination against third-stage larvae and adult worms of *Nippostrongylus brasiliensis* and first-stage larvae of *Trichinella spiralis*. Parasitol Res 112, 335-345.
19. Leathwick DM, Hosking BC, Bisset SA, McKay CH, 2009, Managing anthelmintic resistance: is it feasible in New Zealand to delay the emergence of resistance to a new anthelmintic class? N Z Vet J 57, 181-192.
20. Beach MJ, Streit TG, Addiss DG, Prospere R, Roberts JM, Lammie PJ, 1999, Assessment of combined ivermectin and albendazole for treatment of intestinal helminth and *Wuchereria bancrofti* infections in Haitian schoolchildren. Am J Trop Med Hyg 60, 479-486.

21. Knopp S, Mohammed KA, Speich B, Hattendorf J, Khamis IS, Khamis AN, Stothard JR, Rollinson D, Marti H, Utzinger J, 2010, Albendazole and mebendazole administered alone or in combination with ivermectin against *Trichuris trichiura*: a randomized controlled trial. Clin Infect Dis 51, 1420-1428.
22. Speich B, Ame SM, Ali SM, Alles R, Hattendorf J, Utzinger J, Albonico M, Keiser, J, 2012, Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against *Trichuris trichiura* infection: a randomized controlled trial. PLoS Negl Trop Dis 6, e1685.
23. Wang M, Watanabe N, Shomura T, Ohtomo H, 1995, Effects of PF1022A on *Nippostrongylus brasiliensis* in rats and *Hymenolepis nana* in mice. Jpn. J. Parasitol 44, 306-310.
24. Harder A, von Samson-Himmelstjerna G, 2002, Cyclooctadepsipeptides - a new class of anthelmintically active compounds. Parasitol Res 88, 481-488.
25. Xue J, Xiao SH, Xu LL, Qiang HQ, 2010, The effect of tribendimidine and its metabolites against *Necator americanus* in golden hamsters and *Nippostrongylus braziliensis* in rats. Parasitol Res 106, 775-781.
26. Tomlinson G, Albuquerque CA, Woods RA, 1985, The effects of amidantel (BAY d 8815) and its deacylated derivative (BAY d 9216) on *Caenorhabditis elegans*. Eur J Pharmacol 113, 255-262.
27. Kotze AC, Lowe A, O'Grady J, Kopp SR, Behnke, JM, 2009, Dose-response assay templates for *in vitro* assessment of resistance to benzimidazole and nicotinic acetylcholine receptor agonist drugs in human hookworms. Am J Trop Med Hyg 81, 163-170.
28. Martin RJ, Robertson AP, Buxton SK, Beech RN, Charvet CL, Neveu C, 2012, Levamisole receptors: a second awakening. Trends Parasitol 28, 289-296.
29. Epe C, Kaminsky R, 2013, New advancement in anthelmintic drugs in veterinary medicine. Trends Parasitol 29, 129-34.

Chapter 5

Efficacy of cyclooctadepsipeptides and aminophenylamidines against larval, immature and mature adult stages of a parasitologically characterised trichurosis model in mice

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5 Efficacy of cyclooctadepsipeptides and aminophenylamidines against larval, immature and mature adult stages of a parasitologically characterised trichurosis model in mice

5.1 Abstract

The genus *Trichuris* includes parasites of major relevance in veterinary and human medicine. Despite serious economic losses and enormous impact on public health, treatment options against whipworms are very limited. Additionally, there is an obvious lack of appropriately characterised experimental infection models. Therefore, a detailed parasitological characterization of a *Trichuris muris* isolate was performed in C57BL/10 mice. Subsequently, the *in vivo* efficacies of the aminophenylamidines amidantel, dAMD and tribendimidine as well as the cyclooctadepsipeptides emodepside and in particular PF1022A were analysed. This was performed using various administration routes and treatment schemes targeting histotropic and further developed larval as well as immature and mature adult stages.

Duration of prepatent period, time-dependent localisation of larvae during period of prepatency as well as the duration of patency of the infection were determined before drugs were tested in the characterised trichurosis model. Amidantel showed no effect against mature adult *T. muris*. Tribendimidine showed significantly higher potency than dAMD after oral treatments (ED_{50} values of 6.5 vs. 15.1 mg/kg). However, the opposite was found for intraperitoneal treatments (ED_{50} values of 15.3 vs. 8.3 mg/kg). When emodepside and PF1022A were compared, the latter was significantly less effective against mature adults following intraperitoneal (ED_{50} values of 6.1 vs. 55.7 mg/kg) or subcutaneous (ED_{50} values of 15.2 vs. 225.7 mg/kg) administration. Only minimal differences were observed following oral administration (ED_{50} values of 2.7 vs. 5.2 mg/kg). Triple and most single oral doses with moderate to high dosages of PF1022A showed complete efficacy against histotropic second stage larvae (3× 100 mg/kg or 1× 250 mg/kg), further developed larvae (3× 10 mg/kg or 1× 100 mg/kg) and immature adults (3× 10 mg/kg or 1× 100 mg/kg). Histotropic first stage larvae were only eliminated after three doses of PF1022A (3× 100 mg/kg) but not after a single dose.

These results indicate that the cyclooctadepsipeptides are a drug class with promising candidates for further evaluation for the treatment of trichurosis of humans and livestock animals in single dose regimens.

5.2 Author summary

Treatment options against whipworm infections of humans and livestock are very limited and even anthelmintics recently introduced into the market do not significantly improve the situation. Here, we evaluated members of two relatively new drug classes, the aminophenylamidines (amidantel, dAMD, tribendimidine) and the cyclooctadepsipeptides (PF1022A, emodepside) in a murine trichurosis model. While tribendimidine is licensed for the treatment of human helminthosis caused by hookworms, pinworms and roundworms in China, emodepside is the nematocidal component of dewormers for cats and dogs. With the exception of amidantel, all drugs showed good efficacies against adult whipworms using three consecutive doses. Due to considerations regarding drug safety and price, PF1022A was further evaluated against histotropic L1 and L2, further developed larvae, immature and mature adults using a single or three consecutive doses. Three doses eliminated all stages while a single dose was inefficient against histotropic first stage larvae. In general, higher doses were required for early stages in comparison to stages protruding into the gut lumen. Since only a very basic formulation of drugs was tested, further improvement can be expected from optimised formulations. Cyclooctadepsipeptides should therefore be considered as candidates for evaluation to treat *Trichuris* spp. infections in livestock and humans.

5.3 Introduction

About 20 major human helminthoses have a significant impact on global public health [1]. Since a highly disproportionate share of the burden occurs in developing areas of sub-Saharan Africa, Asia and the Americas, helminth infections belong to both, the "neglected tropical diseases" and the "neglected infections of poverty" [2,3]. In these regions more than a billion people are infected with one or more worm species [2]. An important part of human helminth infections worldwide is caused by soil-transmitted nematodes, including the roundworm *A. lumbricoides* with 800 million infections, the whipworm *T. trichiura* with 600 million infections, and the hookworms *A. duodenale* and *N. americanus* with 600 million infections [4]. An estimated 1.6-6.4 million DALYs are a direct result of trichurosis [4]. In 2010 an estimated 5023 million people lived in areas stable for transmission of *T. trichiura*, plus another 284 million lived in areas of unstable transmission of whipworms, globally [5]. High prevalence often comes along with high abundance of protein energy malnutrition and anaemia as well as limited access to medical care and educational opportunities [6]. Mild *T. trichiura* infections are often asymptomatic, but severe and chronic infections can result in the *Trichuris* dysentery syndrome including chronic inflammation of the intestine, rectal prolapse, anaemia, poor growth, and clubbing of the fingers [6].

Despite the strong impact of helminthoses on public health, only four anthelmintics (albendazole, mebendazole, levamisole, and pyrantel) with only two different modes of action are listed on the WHO list of essential medicines to treat soil-transmitted nematode infections [7] with mebendazole and albendazole being by far the most commonly used drugs [8]. Whereas both drugs are highly effective against adult *A. lumbricoides* in a single dose, only albendazole is used for the treatment against tissue migrating larvae – Mebendazole is poorly absorbed from the gastrointestinal tract thus its therapeutic activity is largely confined to adult/luminal worms [8]. Furthermore, the efficacy of both drugs is unsatisfactory against hookworms and *T. trichiura* in single dose regimen [9]. Higher efficacies against whipworms and hookworms were observed when albendazole or mebendazole were administered using multiple drug administration [10]. However, treatments using multiple doses significantly increase costs and management efforts in particular in poor communities lacking efficient public health infrastructure. Moreover, persistent underdosing of *A. duodenale, N. americanus* and *T. trichiura* within recently increased large-scaled mass drug administration campaigns against filariosis and STHs may favour selection of highly resistant genotypes [9] as already described for *T. trichiura* [11].

In addition to its relevance in human medicine, the genus *Trichuris* also has an enormous impact on veterinary medicine. For instance, *T. vulpis*, the dog whipworm, causes an intestinal parasitosis of clinical relevance and is also suspected to be zoonotic [12]. However, several anthelmintics registered for use in dogs such as diethylcarbamazine, piperazine, ivermectin and pyrantel lack efficacy against *T. vulpis* severely limiting the choice of drug for deworming [12]. In swine, infections with *T. suis,* the dose-limiting nematode for all relevant anthelmintic drug classes, lead to reduced growth rates and therefore result in significant economic losses [13]. Finally, due to the long period of prepatency of *Trichuris* spp. and the lack of efficacy of most drugs against histotropic larval forms, two blocks with one to three doses each are usually necessary to completely eliminate the parasites [12].

It is therefore obvious, that the development of new, safe and highly efficacious drugs to treat soil-transmitted nematode infections is urgently required. In particular, new drugs for the treatment of *Trichuris* spp. using a single dose would significantly increase treatment options in both, human and veterinary medicine. Therefore, the evaluation of the efficacy of promising drug candidates against whipworms is an essential step towards improvement of anthelmintic treatment opportunities.

To investigate and compare the anthelmintic profiles of new drug candidates against whipworm infections, the *T. muris* mouse model is highly suitable [14]. *Trichuris* L1 hatch in the small intestine of their host and migrate rapidly to the caecum and colon [15], where they invade the epithelium [16] and undergo a histotropic phase with two moults lasting several days (duration depends on the particular species and isolate). Then, larvae migrate to the surface of the epithelium extruding their caudal ends freely into the lumen of the intestine (further developed larvae or free larvae) [16]. In general anthelmintics have been reported to be less effective against histotropic larvae, which might be attributed to the poor accessibility of drugs to these larvae within the tissue [12].

In order to eliminate parasites using a single dose or at least a single treatment block, it is desirable to evaluate drug candidates not only against mature adult worms but also against histotropic larvae and further developed immature stages. Since duration of development and timespan of infection depend on both, the host strain [17] and whipworm isolate [18], a detailed characterisation of the respective host-parasite relationship is essential. Thus, localisation of larvae in the course of the prepatent period and onset of patency of the infection have to be analysed carefully before *in vivo* assays against specific stages of *T. muris* can be conducted meaningfully with the respective isolate.

The cyclooctadepsipeptides [19] and the aminophenylamidines [20] are promising anthelmintic classes for further development of broad-spectrum drugs to treat intestinal nematode infections. The semi-synthetic cyclooctadepsipeptide emodepside has been shown to have an almost complete efficacy against immature and mature stages of *T. vulpis* in dogs [21] and *T. muris* in mice [22,23] while the aminophenylamidines amidantel and tribendimidine showed only low to moderate efficacy against *T. muris* in mice [24] and *T. trichiura* in humans [25-27].

Both drug classes have completely different target molecules. It is clear that the aminophenylamidines are agonists of AChRs and have a very similar mode of action as levamisole [28,29] whereas several targets have been suggested for the cyclooctadepsipeptides with the voltage-gated, calcium-activated potassium channel SLO-1 as most important candidate [19,30,31]. However, the G-protein coupled receptor LAT-1 [32] and ionotropic $GABA_A$ receptors [33,34] might also contribute to susceptibility to cyclooctadepsipeptides.

Therefore, the present study investigated and compared the *in vivo* anthelmintic properties of the semi-synthetic cyclooctadepsipeptide emodepside, its parental natural fermentation product PF1022A and the aminophenylamidines amidantel, dAMD and tribendimidine against *T. muris*. Since tribendimidine has previously been reported to have insufficient activity after oral administration in humans [25-27], drugs were also administered intraperitoneally and subcutaneously. In addition to the evaluation of adulticidal efficacy, PF1022A was further tested against histotropic larvae and further developed immature stages of whipworms, using single and three consecutive doses.

5.4 Materials and methods

Ethical statement

All studies presented were conducted at the laboratories of Bayer HealthCare, Global Drug Discovery, Animal Health in Monheim, Germany. The experiments were registered and approved by LANUV, North Rhine-Westphalia, Germany (reference number 200/V14), in accordance with §8a section 1 and 2 of the German Protection of Animals Act and the European Union directive 2010/63/EU.

Drugs

Amidantel, dAMD, emodepside and PF1022A were available at Bayer HealthCare AG, Global Drug Discovery Animal Health in Monheim, Germany. Tribendimidine was obtained from Shandong Xinhua Pharmaceutical Company Limited (Zibo, People's Republic of China). All drugs were stored at 4 °C until further use. Individual drug concentrations were prepared separately as dispersions in Cremophor EL (BASF, Ludwigshafen, Germany) and deionised-water [1:3] on the days of treatment.

Animals and parasites

Female SPF inbred mice of the strain C57BL/10 ScSnOlaHsd (C57BL/10) were purchased from Harlan UK Limited, at four weeks of age. They were housed in Macrolon® cages under environmentally controlled conditions and kept in groups of five animals unless otherwise indicated. Water and Sniff® rodent food pellets were available *ad libitum*. Mice were allowed to acclimate for exactly seven days before starting any experiments. The *T. muris* isolate was kindly provided by Heinz Mehlhorn (Düsseldorf, Germany). A detailed history regarding isolation and passage is not available. Mice were orally infected with a gavage using 0.2 ml fresh tap water with 200 eggs containing fully developed L1 of *T. muris*. Murine faeces were collected on days 49, 56 and 63 *p.i.,* euthanasia was performed by carbon dioxide suffocation. Isolation of the eggs was performed as described later on. The development of L1 in the eggs was performed in stender dishes in an incubator at 27 °C and 95% humidity for approximately eight weeks. Progress of embryonation was controlled weekly. After development of L1 in >90% of the eggs was completed, eggs were stored at 4 °C until further usage for a maximum of six months. Before infection of mice, the egg suspension was washed with fresh tap water at room temperature.

Parasitological characterisation of a T. muris life cycle in C57BL/10 mice

Determination of the periods of prepatency and patency

To assess the duration of prepatent period, ten mice were infected. Starting from day 7 *p.i.*, all ten mice were housed on grids to collect faeces for 24 h once a week. During these periods, the bottom of the cage was covered with 300 ml tap water. Faeces and water were collected in a 1 l beaker and homogenised with a hand-held blender. Using a wooden spatula, fine components of the faeces were separated from remaining debris by filtration through a 200 µm sieve and collected in a clean 1 l beaker. The residues were rinsed with tap water until the filtrate reached a volume of 600 ml. After sedimentation for 1 h, the supernatants (approximately 500 ml) were removed. The sediment was centrifuged at 2000 ×g and room temperature for 10 min. The pellet was resuspended in 200 ml tap water and centrifuged under the same conditions. After another washing step, the pellet was resuspended in 200 ml saturated sodium chloride solution. Then, samples were centrifuged at 2000×g and room temperature for 5 min, the top 25 ml were filled into a 300-ml beaker and 225 ml tap water were added. After at least 2 h of sedimentation the supernatant was decanted and the sediment was washed in tap water another four times. After decanting the supernatant, the sediment (approximately 20 ml) was examined for the presence of eggs. Examination of faeces was continued until three consecutive samples were found to be negative. Three independent experiments with ten mice each were performed.

Variation in egg output in the course of patency of the infection

To determine the variation in egg output in the course of patency of the infection, a faecal egg count method was adapted from Stoll [35]. In brief, ten mice were infected. Only animals, positive for eggs in their faeces on day 35 *p.i.*, were included in the study. Starting from day 35 *p.i.*, mice were housed individually on grids in Macrolon® cages to collect individual faeces for 12 h periods once a week. Faecal samples (0.5 g) were weighed from each mouse, 7.0 ml water were added and incubated for 15 min. Faeces were roughly macerated with a wooden spatula followed by an extensive homogenisation using a magnetic stirrer at low speed until samples were analysed. For each sample, three 75 µl aliquots were pipetted on microscope slides and eggs were counted. To obtain the number of eggs per gram (epg) faeces, the arithmetic mean of the three counts was multiplied by 200 to calculate the number epg faeces. Faeces were analysed until 15 weeks *p.i.*, since status of patency of the infection became quite variable afterwards.

Time course of localisation of larvae in the course of prepatent period

To analyse the time course of the localisation of larvae during prepatent period the following experiment was adapted from Panesar [36]. For this experiment 120 mice were infected. During the first 40 days *p.i.,* three mice were euthanised daily and their duodena, caeca and colons were removed and split open. The luminal content was removed and inspected for any stages of *T. muris*. Then, the mucosa of the guts was examined for the presence of worms extruding into the lumen of the guts. Finally, duodena, caeca and colons were cut into small squares and separately incubated in 0.85% physiological sodium chloride solution at 37 °C for 24 h. By carefully scraping the mucosa the histotropic larvae became visible using a dissecting microscope. Seven mice, in which not a single stage of *T. muris* was found, were excluded from the study.

Female/male ratio in the course of infection

On day 35 *p.i.,* faecal examinations were performed for each of the 60 infected mice individually to confirm patency of the infection. Only animals found positive for eggs in their faeces were included in the study. Weekly, starting from day 35 until day 152 *p.i.,* three mice were euthanised and dissected. Female and male whipworms in caecum and colon were counted. Two independent experiments with 60 mice in each experiment were performed.

In vitro embryonation of T. muris eggs

The embryonation of eggs was analysed and compared under several different conditions. Freshly isolated and purified eggs were suspended in (i) 0.5% formaldehyde in physiological sodium chloride solution, (ii) physiological sodium chloride solution or (iii) tap water and transferred into 40 ml stender dishes (see [37]) to compare the rate and speed of development. The progress of embryonation was assessed weekly by microscopic analysis of three 10 µl aliquots. Eggs were counted and categorised as (i) unembryonated, (ii) partially embryonated, (iii) fully embryonated or (iv) degraded. The latter category was chosen according to the following criteria: a) vesicular appearance of unsegmented eggs or b) deformed larval structures within the eggs.

Furthermore, incubation temperatures of 4 °C, 19 °C, 27 °C and 37 °C as well as the influence of the presence of antibiotic (*i.e.* 10 µg/ml sisomycin plus 1 µg/ml clotrimazole), relative humidity (75%, 85% and 95%) and light conditions (light versus no light) were evaluated in tap water using the same method.

Finally, the influence of storage at 4 °C after full embryonation of eggs was compared to storage at 27 °C to determine the best storage condition. After embryonation at 27 °C, eggs were stored at 27 °C or at 4 °C for 70 days. For each incubation temperature, 5 mice were infected. On day 45 *p.i.,* mice were euthanised and worm counts were determined.

In vivo efficacy against T. muris in mice

In 24 consecutive experimental blocks, 655 mice were randomised into 132 groups, each consisting of five animals. One group of five mice was used for each dosage and for each administration route tested. In each block, five infected mice served as untreated control and received the vehicle only.

In vivo efficacy against mature adult stages of T. muris

On day 42 *p.i.,* a faecal examination was performed for each mouse to confirm patency of the infection. Only animals positive for *T. muris* eggs in their faeces were included in the study. Based on the individual body weight on day 45 *p.i.,* exact dosages were calculated. In case of multiple dose regimens, three doses of the respective drug were administered orally, intraperitoneally or subcutaneously (nuchal fold) on days 46-48 *p.i.* Dosages used are summarised in Tab. 5-1. For single doses, 50, 75, 100, 150, 200, 250, 300 or 500 mg/kg PF1022A were administered on day 48 *p.i.* On day 49 *p.i.,* mice were euthanised. Subsequently, necropsy was performed and worms in colons and caeca were counted.

Tab. 5-1. Single and multiple drug dosages evaluated *in vivo* against mature adults of *T. muris*, classified by route of administration

Dosage (mg/kg)	0.5	1.0	2.5	5.0	7.5	10	15	20	25	50	75	100	150	200	250	300	400	500
PF1022A																		
3x oral		X	X	X	X	X			X	X		X						
1x oral										X	X	X	X	X	X	X		X
3x subcutaneous										X		X	X	X	X	X	X	X
3x intraperitoneal						X			X	X	X	X	X	X	X			
Emodepside																		
3x oral	X	X	X	X	X	X				X		X						
3x subcutaneous		X				X	X	X	X	X	X	X						
3x intraperitoneal		X		X		X	X	X	X	X		X						
Amidantel																		
3x oral																		X
3x subcutaneous																		
3x intraperitoneal																		
dAMD																		
3x oral		X		X		X			X	X		X						
3x subcutaneous												X						X
3x intraperitoneal				X		X			X	X		X						
Tribendimidine																		
3x oral		X		X		X			X	X		X						
3x subcutaneous												X						X
3x intraperitoneal				X		X			X	X		X						

'X' indicated that the respective drug was evaluated in the given dose using the indicated route of administration.

In vivo efficacy against larval and immature adult stages of T. muris

Based on the parasitological characterisation (duration of prepatent period and time-dependent localisation of larvae during period of prepatency), the *in vivo* efficacy of PF1022A was also investigated against larval and immature adult stages of *T. muris*. According to the time course of localisation of developmental stages in the present study and the analysis on the moulting pattern in *T. muris* [36], the following time points for drug administration were chosen:

Individual body weight was determined on the day of infection for L1, on day 11 *p.i.* for L2 and on day 25 *p.i.* for further developed stages. PF1022A dosages of 10, 100, 250, and 500 mg/kg or 1.0, 10, 50 and 100 mg/kg were administered on day 3 *p.i.* or on days 1-3 *p.i.* to target L1. For the evaluation of efficacy against the histotropic L2, treatments were carried out with PF1022A dosages of 10, 100, 250, and 500 mg/kg or 1.0, 10, 50 and 100 mg/kg on day 14 *p.i.* or on days 12-14 *p.i.,* respectively. Since the following moults of males and females are less synchronous [36], treatments were directed against further developed immature stages in general. Treatments with 1.0, 10, 50 and 100 mg/kg PF1022A were performed on three consecutive days (26-28 *p.i.*) and treatments with 10, 100, 250, and 500 mg/kg PF1022A only on day 28 *p.i.* Independently of the targeted stage euthanasia of mice and worm counts were performed on day 49 *p.i.*

Calculation of dose-response curves and statistical analysis

For parasitological characterisation of the *T. muris* isolate used in C57BL/10 mice, descriptive statistics were performed using GraphPad Prism 5.03. Differences in worm counts between different weeks of infection and in sex ratio were determined by a One Way ANOVA followed by Dunnet's post hoc test using the first week of the patent period as the control against which all other time points were tested.

For all drugs and routes of administration tested against patent *T. muris* infections, the reduction of the worm burden expressed in percent of the corresponding control groups of five mice was plotted against the $\log_{10}$ of the drug dosages. Efficacies were set to zero if mean of the worm counts was higher than the mean of the corresponding control group. Furthermore, the corresponding SEM values of the affected groups start from 0 (all figures showing dose-response curves). Four-parameter-logistic curves were fitted using GraphPad Prism 5.03 [38]. The top was constrained to values between 0% and 100%. The no-drug controls were set to 10^{-4} mg/kg to allow $\log_{10}$ transformation of dosages. Calculated ED_{50} and ED_{95} values were compared using the extra sum of squares F test. If multiple tests were

performed, *P*-values were corrected using the Bonferroni-Holmes procedure.

The absolute number of recovered mature adult worms after treatment against larval and immature adult stages was compared to the number of worms isolated from the negative controls by using the non-parametric Kruskal-Wallis test with Dunn's *post hoc* test for identification of significant differences between individual groups.

5.5 Results

Time course of T. muris development in C57BL/10 mice

Periods of prepatency and patency of the infection

In each of the three independent experiments determining presence of eggs in faeces in weekly intervals, first eggs were found on day 35 *p.i.* Therefore, prepatent period lasted for at least four but not longer than five weeks. None of the mice that became patent stopped shedding eggs before week 14 *p.i*. However, starting from week 15 *p.i*., samples were much more heterogeneous. Mice in experiment 1 remained patent at least until week 16 *p.i*., whereas animals in experiment 2 stopped shedding eggs in week 15 *p.i*. In experiment 3, faecal examination was negative in week 16 *p.i.,* but was positive in week 17 *p.i.,* again. Patency of the infection ended in week 18 *p.i.*

Detailed analysis on egg shedding

Nine out of ten mice were found positive for eggs in their faeces on day 35 *p.i*. Therefore, only a single mouse was excluded from the study. Furthermore, one individual mouse died on day 67 *p.i*. and was only included in the statistics until day 63 *p.i.* Variation in egg shedding in the course of patency of the infection is summarised in Fig. 5-1. Strong variation in individual egg counts was observed, indicated by the relatively large standard deviations (Fig. 5-1). In general, the mean epg increased during the first three weeks of patency of the infection starting with a mean epg of 2919 ± 1182 (range 800-5600) at day 35 *p.i.* The peak in egg output was observed in week 8 *p.i.* (epg = 12200 ± 3813, range 4200-19800). Starting from week 9 *p.i.*, a gradual reduction of the average egg count was observed. In week 14 *p.i.* the first mouse was found to be negative for eggs in its faeces. In another two mice patency of the infection ceased in week 15 *p.i.* For the remaining five mice the mean (± SD) of epg faeces was calculated to be 1120 ± 1110.5 (range 200-3600) on week 15 *p.i.*, being the final week of this analysis.

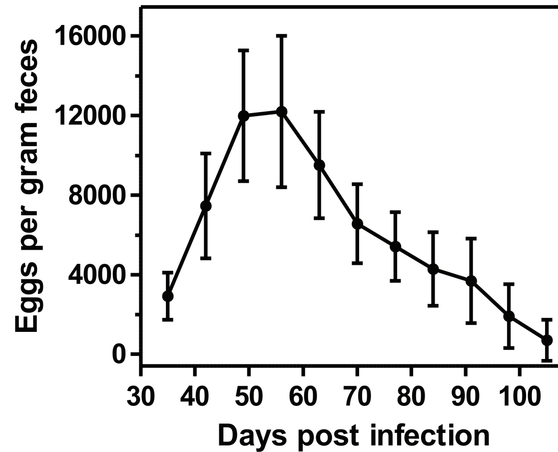

Fig. 5-1. Analysis on egg shedding in the course of patency of the infection

The graph shows the arithmetic mean values with standard deviations of the absolute numbers of epg faeces between days 35 and 105 *p.i.* with a group size of nine animals. Due to the death of one mouse, group size was reduced to eight starting from day 70 *p.i.*

Localisation of developmental stages throughout infection

The analysis of the time course of the migration of *T. muris* stages during the period of prepatency revealed distinct phases of localisation. Fig. 5-2 summarises the trend in absolute numbers of recovered stages in the course of the prepatent period. Supplementary Tab. 5-3 shows the individual counts divided by duodenum, caecum, colon and luminal debris.

On days 1-4 *p.i.* a small number (2.8 ± 1.9, mean ± SD) of embryonated eggs was recovered from the intestinal debris of duodenum, caecum and colon. After day 5 *p.i.* no embryonated eggs were found in the gastrointestinal tract. Free larvae were identified in the debris of guts also for a very limited period during the first days after infection. Whereas 9.0 ± 6.2 free larvae were recovered between day 1 and day 5 *p.i.* only one sample on day 8 was found positive for two free larvae. However, starting from day 27 an increasing number of immature and mature adult worms in the debris was counted (see Supplementary Tab. 5-3). Histotropic larvae were recovered almost throughout the whole evaluation period. However, during the period of prepatency two relative maxima in histotropic larval counts were observed. A high number of histotropic larvae was detected on days 1 and 2 *p.i.* (45.8 ± 4.5), while only a small number was recovered between days 3 and 12 *p.i.* (10.3 ± 4.1). Starting from day 13 the number steadily increased until day 19 *p.i.,* where 50.3 ± 17.4 larvae were counted (see Supplementary Tab. 5-3). From day 20 *p.i.* on, the number of histotropic larvae decreased again and finally, starting from day 24 *p.i.,* the majority of the guts was found to be negative. Further developed stages were not found before day 21 *p.i.* The number of these stages then

increased until day 24 and remained stable (42.6 ± 13.9) until the end of the evaluation period (see Fig. 5-2). As expected, neither histotropic larvae nor any further developed stages were found in the duodenum (Supplementary Tab. 5-3). On days 30 and 31, the intestinal debris became positive for unembryonated eggs, indicating the start of patency of the infection (Supplementary Tab. 5-3).

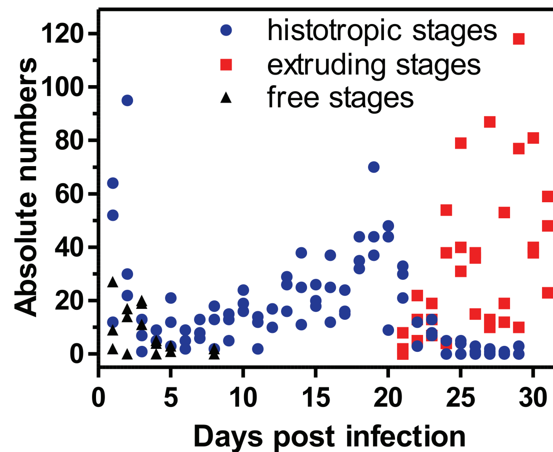

Fig. 5-2. Analysis on the occurrence of specific stages of *T. muris* in the course of the period of prepatency

Presented is the occurrence of L1 in the luminal content of the guts (free stages), of histotropic first, L2 and L3 (histotropic stages) and of L3 and L4 as well as immature and mature adults attached to the epithelium while extruding their posterior parts into the lumen of the guts (extruding stages) between days 1 and 31 *p.i.* Based on dissection of three mice per time point, the graph shows three data points for each stage and time point. If the count was found to be zero for a specific stage in each of the three independent counts, data points are not shown.

Worm counts and sex ratio

On day 35 *p.i.,* 107/120 mice harboured a patent infection (infection rate of 89.17%). 13 uninfected mice and four mice which had died in the course of the experiment were exclude from the analysis.

The absolute worm counts per infected host are summarised in Fig. 5-3A. Mean worm counts were not significantly different from those on day 35 *p.i.* up to day 70 *p.i.* (One Way ANOVA followed by Dunnet's post hoc test, $P>0.05$) although a tendency to lower and steadily decreasing mean worm counts was observed already at earlier time-points. Thereafter, mean worm counts were significantly lower than on day 35 ($P<0.01$) and a continuous decrease in recovered worms was observed (Fig. 5-3A). On day 112 *p.i.* only five worms per mouse where recovered on average and finally on days 145 and 152 *p.i.* only two whipworms were

found in one of the necropsied mice. In addition to the absolute worm counts, Fig. 5-3B shows the relative sex distribution of the worms during the same evaluation period. The male/female ratio was progressively skewed towards male worms. The ratio was 1:1.27 five weeks *p.i,* 1:0.97 nine weeks *p.i.*, 1:0.56 14 weeks *p.i.,* and 1:0.12 17 weeks *p.i.* Starting in week 9, the male/female ratio was significantly higher than on day 35 *p.i.* (One Way ANOVA followed by Dunnet's post hoc test, *P*<0.001). From week 18 on, 100% of the recovered worms were males (see Fig. 5-3B).

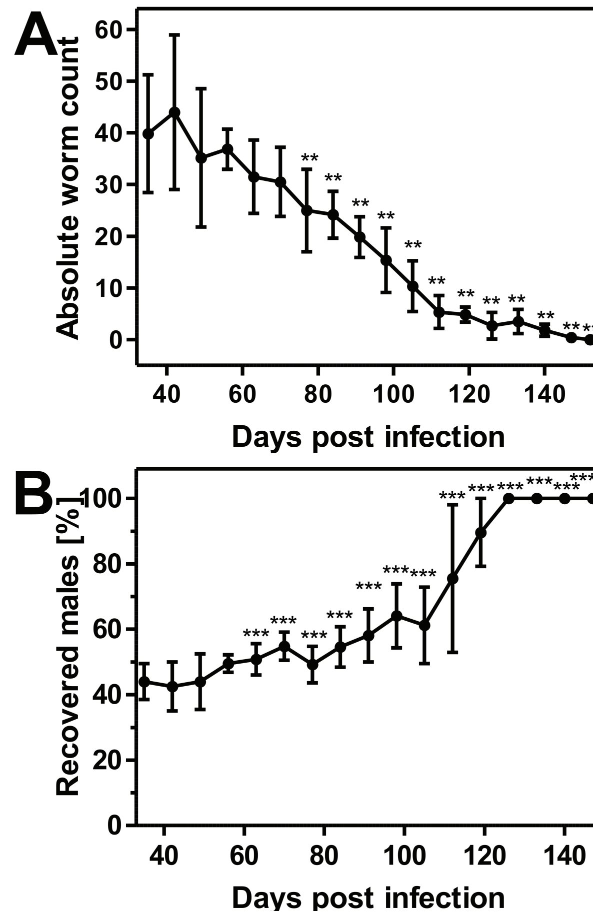

Fig. 5-3. Analysis on the occurrence of *T. muris* in the course of patency of the infection

A) Absolute worm counts in the course of patency of the infection. The graph shows the arithmetic mean values and SD of the absolute number of recovered worms during time with a group size of six animals per time point. Mean worm counts were compared to day 35 *p.i.* using One Way ANOVA followed by Dunnet's post hoc test. **, *P*<0.01 vs. day 35. B) Sex ratio of *T. muris* in the course of patency of the infection. Graph shows the arithmetic means with standard deviations of the recovered male worms expressed as percentage of total recovered worms with a group size of six animals per time point. ***, *P*<0.001 vs. day 35.

Optimised conditions for in vitro embryonation of T. muris eggs

The influence of different media on the rate and speed of embryonation were compared. No significant difference was observed between (i) 0.5% formaldehyde in physiological sodium chloride solution, (ii) physiological sodium chloride solution and (iii) tap water (data not shown). Therefore, tap water was used as medium for the following analyses. The incubation temperature (4 °C, 19 °C, 27 °C or 37 °C) had an enormous impact on both speed and embryonation rate (Supplementary Tab. 5-4). Speed of embryonation steadily increased with temperature. However, at 37 °C the absolute number of degenerated eggs was also increased. Additives such as sisomycin plus clotrimazole or lighting conditions did not influence embryonation and were therefore neglected. However, relative humidity (75%, 85% and 95%) strongly affected the loss of medium by evaporation and therefore 95% humidity was chosen for routine purposes.

Finally, the influence on storage temperature on egg infectivity after full embryonation was tested. Mice infected with eggs stored at 27 °C or at 4 °C for at least 70 days were necropsied on day 45 *p.i.* The infection levels between both groups were not found to differ significantly, as illustrated by worm counts ranging between 28 and 45 or 12 and 59 (P=0.69 using the Mann Whitney U test).

In vivo efficacy of cyclooctadepsipeptides and aminophenylamidines against T. muris

The average number of worms recovered from caecum and colon from untreated control mice on day 49 was 33.77 ± 15.59. Worm counts after treatment against developmental stages were also determined on day 49 *p.i.,* while four mice died before evaluation and were, therefore, not included in the statistics. The highest worm count was 80, whereas no worms were recovered in two cases.

In vivo efficacy of aminophenylamidines against mature adult stages of T. muris

Three oral doses of 500 mg/kg of the aminophenylamidine amidantel led to no significant reduction of the worm burden. Since three high consecutive doses of amidantel did not reduce worm counts in comparison to the no-drug control, this derivative was not further evaluated in the present study. In contrast to amidantel, both oral and intraperitoneal treatments with either tribendimidine or dAMD resulted in dose-dependent reductions of the *T. muris* burden. Dose-response curves for both drugs and both routes of administration are given in Fig. 5-4. Furthermore, ED_{50} and ED_{95} values with 95% confidence intervals as well as P values from comparisons between the derivatives and R^2 values are summarised in Tab. 5-2. The ED_{95} of

tribendimidine was found to be approximately eight times lower than the ED_{95} of dAMD following three oral consecutive doses, whereas the ED_{95} of tribendimidine was approximately four times higher than the ED_{95} of dAMD after three intraperitoneal administrations (Tab. 5-2). However, three subcutaneous doses with 100 mg/kg or 500 mg/kg of either tribendimidine or dAMD had no effect on worm counts in comparison to the vehicle treated group (data not shown).

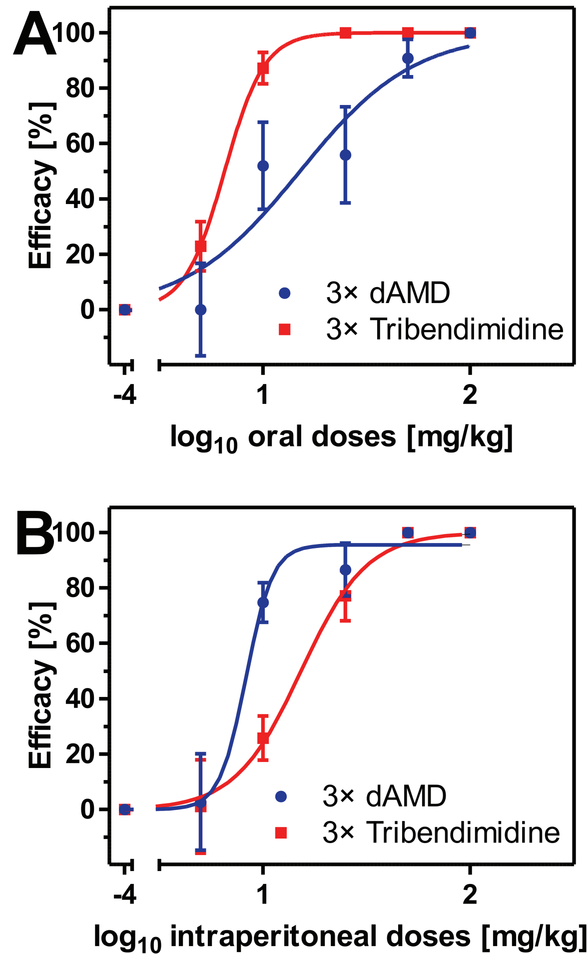

Fig. 5-4. *In vivo* dose-response curves for aminophenylamidines against mature adult *T. muris* in mice

Dose-response curves show the arithmetic mean values and standard errors of the mean for dAMD (blue) and tribendimidine (red) after A) oral and B) intraperitoneal treatment with a group size of five animals per drug and dose. Efficacy was calculated as relative number of recovered worms compared to the no-drug control in percentage. Dosages were log_{10} transformed and logistic regressions were calculated with top values constrained between 0% and 100%. Efficacies were set to zero if mean of the worm counts was higher than the mean of the corresponding control group. Furthermore, the corresponding SEM values of the affected groups start from zero. The no-drug controls were set to 10^{-4} mg/kg to allow log_{10} transformation of dosages.

In vivo efficacy of cyclooctadepsipeptides against mature adult stages of T. muris

Three oral, intraperitoneal or subcutaneous doses of either emodepside or PF1022A on days 46-48 *p.i.,* resulted in dose-dependent reductions of the *T. muris* burden. Tab. 5-2 summarises ED_{50} and ED_{95} values as well as comparisons between them by administration route. By comparing the three routes of administration, oral treatments diminished the worm burden at significantly lower doses than intraperitoneal or subcutaneous administrations (Fig. 5-5A, B, C and Tab. 5-2). For emodepside, the ED_{50} values for intraperitoneal and subcutaneous treatments were approximately twofold and fivefold higher than for oral treatment (Tab. 5-2). The differences for PF1022A were even more pronounced. The ED_{50} values for intraperitoneal and subcutaneous treatments were approximately ten and 43-times higher in comparison to the ED_{50} values for the oral treatments.

By comparing the ED_{50} values of the two cyclooctadepsipeptides, the results were very diverse depending on the respective route of administration. However, the calculated ED_{50} value for PF1022A after three intraperitoneal doses was approximately nine times higher than the ED_{50} value of emodepside. A comparison of the two drugs after three subcutaneous doses resulted in an approximately 15-fold higher ED_{50} value for PF1022A. Surprisingly, the ED_{50} value of emodepside using three oral administrations was only twofold lower than that of PF1022A. Since the costs of PF1022A are much lower than those of emodepside and the difference between both drugs was only small for oral administration, a single oral dose against mature adult stages of *T. muris* was only evaluated for PF1022A.

A single oral administration of PF1022A on day 48 *p.i.* also resulted in dose-dependent reduction of the whipworm burden. A dose-response curve was calculated (Fig. 5-5A) and ED_{50} and ED_{95} values with 95% CI as well as R^2 values are presented in Tab. 5-2. The ED_{50} value for PF1022A using a single oral dose was approximately 36-fold higher in comparison to the three oral administrations.

Tab. 5-2. Comparison of the *in vivo* efficacies of monotherapies against mature adults *T. muris* in mice

Drug	Admin.	ED_{50} with 95% CI (in mg/kg)	*P* value[a]	ED_{95} with 95% CI (in mg/kg)	*P* value[b]	R^2
dAMD	3× oral	15.1 (9.9-22.9)	< 0.0001 (vs. 3× tribendimidine oral)	97.3 (28.3-334.2)	0.0007 (vs. 3× tribendimidine oral)	0.8039
	3× i.p.	8.3 (7.3-9.5)	< 0.0001 (vs. 3× tribendimidine i.p.)	12.8 (10.6-15.4)	< 0.0001 (vs. 3× tribendimidine i.p.)	0.9349
Tribendimidine	3× oral	6.5 (6.0-7.2)	< 0.0001 (vs. 3× dAMD oral)	12.6 (9.9-15.9)	0.0007 (vs. 3× dAMD oral)	0.9447
	3× i.p.	15.3 (13.2-17.7)	< 0.0001 (vs. 3× dAMD i.p.)	44.8 (30.9-65.0)	< 0.0001 (vs. 3× dAMD i.p.)	0.9279
Emodepside	3× oral	2.7 (1.9-3.9)	0.0009 (vs. 3× PF1022A oral)	24.5 (8.7-68.8)	0.3684 (vs. 3× PF1022A oral)	0.8368
	3× i.p.	6.1 (4.8-7.7)	< 0.0001 (vs. 3× PF1022A i.p.)	40.0 (18.9-84.5)	< 0.0001 (vs. 3× PF1022A i.p.)	0.9274
	3× s.c.	15.2 (13.0-17.7)	< 0.0001 (vs. 3× PF1022A s.c.)	40.7 (24.5-67.4)	< 0.0001 (vs. 3× PF1022A s.c.)	0.8481
PF1022A	3× oral	5.2 (4.0-6.8)	0.0009 (vs. 3× emodepside oral)	36.5 (14.9-89.8)	0.3684 (vs. 3× emodepside oral)	0.8681
	3× i.p.	55.7 (44.4-70.0)	< 0.0001 (vs. 3× emodepside i.p.)	208.5 (99.2-438.2)	< 0.0001 (vs. 3× emodepside i.p.)	0.8657
	3× s.c.	225.7 (180.2-282.6)	< 0.0001 (vs. 3× emodepside s.c.)	515.0 (254.8-1041)	< 0.0001 (vs. 3× emodepside s.c.)	0.7432
	1× oral	186.6 (111.0-313.5)	< 0.0001 (vs. 3× PF1022A oral)	686.7 (168.5-2798)	< 0.0001 (vs. 3× PF1022A oral)	0.6086

Presented are the ED_{50} and ED_{95} values with 95% confidence intervals (CI) and coefficients of determination (R^2) as well as *P* values, for determination of significant differences. [a] Significant difference in ED_{50} to drug in brackets. [b] Significant difference in ED_{95} to drug in brackets

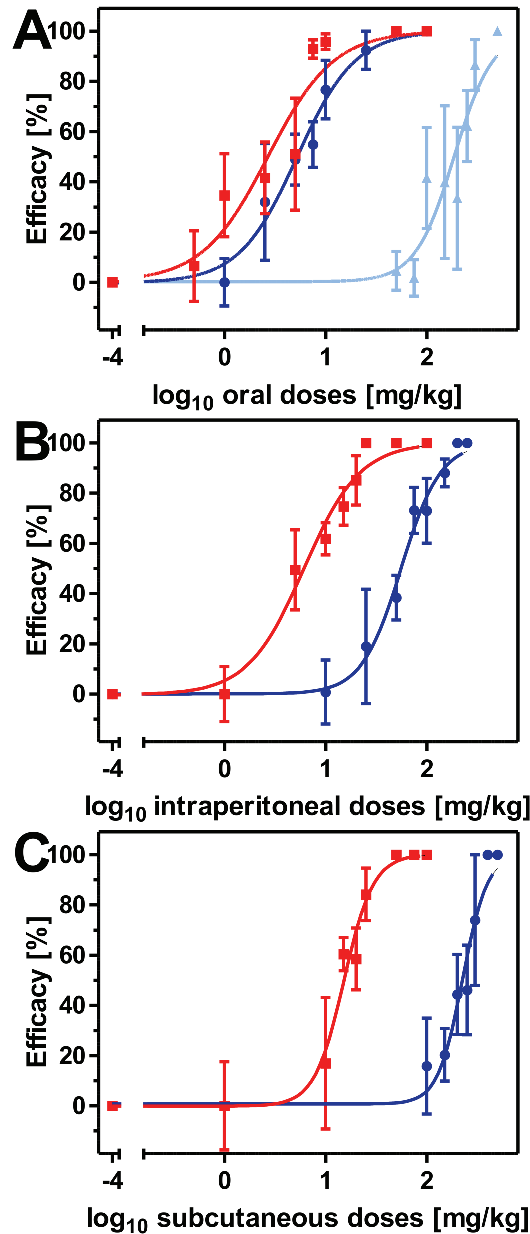

Fig. 5-5. *In vivo* dose-response curves of emodepside and PF1022A against mature adults of *T. muris*

Dose-response curves show the arithmetic mean values with SEM with a group size of five animals per drug and dose. Efficacy was calculated as relative number of recovered worms compared to the no-drug control in percentage. Dosages were $\log_{10}$ transformed and logistic regressions were calculated with top values constrained between 0% and 100%. Triangles indicate a single dose of PF1022A (light blue), circles three doses of PF1022A (dark blue) and squares three doses of emodepside (red) after A) oral, B) intraperitoneal and C) subcutaneous administration. Efficacies were set to zero if mean of the worm counts was higher than the mean of the corresponding control group. Furthermore, the corresponding SEM values of the affected groups start from zero. The no-drug controls were set to 10^{-4} mg/kg to allow $\log_{10}$ transformation of dosages.

In vivo efficacy of PF1022A against developmental stages of T. muris

Both single and multiple PF1022A doses on day 28 and days 26-28 resulted in dose-dependent reductions in the number of recovered worms (Fig. 5-6A, B and Supplementary Tab. 5-5). While a single administration of 10 mg/kg did not result in any apparent effects, 100 mg/kg or higher dosages already eliminated the worm burden completely. Three oral doses of PF1022A against developmental stages on days 26-28 also resulted in nearly complete or complete cure rates starting from 10 mg/kg. Therefore, an approximately 10-fold lower dosage of PF1022A was sufficient to cure the infection with further developed larval stages and immature adult worms with three doses in comparison to a single dose.

The efficacy of single and multiple PF1022A doses on day 14 and days 12-14 respectively, targeting the histotropic L2, also resulted in dose-dependent significant reductions of the worm burden (Fig. 5-6C, D and Supplementary Tab. 5-5). In particular, three dosages of 100 mg/kg PF1022A or a single administration of 250 mg/kg PF1022A were required for complete elimination of whipworms.

In contrast to the efficacy against L2, the effects of PF1022A against L1 were not sufficient in the single dose regimen. While three doses of 100 mg/kg PF1022A on days 1-3 *p.i.* were sufficient to completely cure the mice, a single dose on day 3 *p.i.*, using even 500 mg/kg, was not able to significantly reduce worm burdens (Fig. 5-6E, F and Supplementary Tab. 5-5).

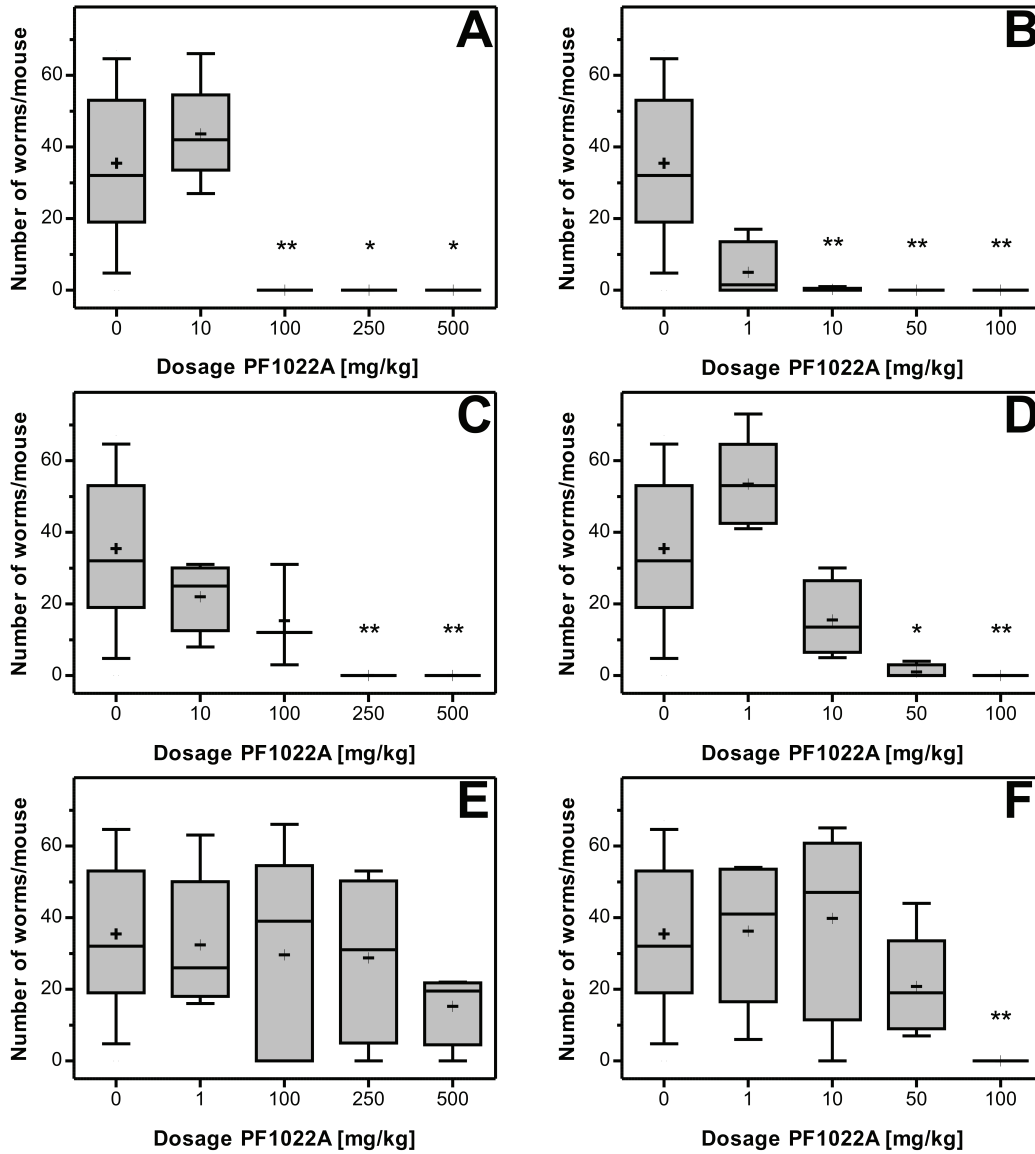

Fig. 5-6. *In vivo* efficacy of PF1022A against larval and immature stages of *T. muris* in mice

Box plots show the median numbers and quartiles of recovered adult *T. muris* after treatment against L3, L4 and immature adults (A, B), histotropic L2 (C, D) and histotropic L1 (E, F) using both, single (A, C, E) and triple (B, D, F) dose regimens with wiskers representing minimal and maximal values. Group sizes were 5 mice per drug and dose. + arithmetic mean; * $P<0.01$ vs. control; ** $P<0.001$ vs. control.

5.6 Discussion

The majority of human gastrointestinal nematode infections are caused by *A. lumbricoides*, *A. duodenale*, *N. americanus, S. stercoralis* and *T. trichiura* [8]. Whereas available drugs are usually highly effective against *A. lumbricoides* in a single dose regimen, at least multiple dosages of those drugs are required to cure hookworm, threadworm and particularly whipworm infections [9,39].

In addition to the enormous impact on human medicine, the genus *Trichuris,* like *T. suis,* is also considered to be a dose-limiting nematode for most current anthelmintics in a variety of hosts of veterinary importance [13]. However, treatment options are often limited. For example, a large number of drugs (diethylcarbamazine, ivermectin, piperazine, pyrantel) registered to treat nematode infections in dogs are lacking sufficient efficacy against *T. vulpis* [12]. Among the new anthelmintics that entered the market in the recent past, especially the cyclooctadepsipeptides [21-23] and partially the aminophenylamidines [20,24,27] are active against *Trichuris* spp., whereas paraherquamide has only poor efficacy [40] and monepantel lacks efficacy [41]. For derquantel, only data describing a high efficacy of the combination with abamectin against *T. ovis* have been published [42]. However, if these effects are attributed to derquantel, abamectin or only the combination of both needs to be clarified.

Persistent underdosing of *Trichuris* spp. in both humans (during the reinforced mass drug administration campaigns against lymphatic filariosis and soil-transmitted nematodes) and animals of veterinary importance may favour selection of highly resistant genotypes [9] as already described for *T. trichiura* [11]. Therefore, the urgent need for new drugs for the treatment against *Trichuris* spp., preferably in a single dose regimen, is obvious for both human and veterinary medicine.

Due to the long prepatent period of *Trichuris* spp. and the lack of efficacy of most drugs against the histotropic phase of larval forms, multiple blocks with one to three doses each are usually necessary to completely eliminate the infections [12]. Larvae of several gastrointestinal nematode species penetrate into the pits and glands of the mucosa (*e.g. Haemonchus* spp., *Ostertagia* spp., *Teladorsagia* spp.) or even penetrate and feed on individual cells (*e.g. Trichuris* spp., *Trichinella* spp.) to survive the lethargy associated with moulting without losing their place in the gut [43]. These histotropic larvae are often difficult to eliminate and require higher or repeated doses when compared with luminal or mucosal stages.

In order to evaluate the effects of drugs against the histotropic stages of *T. muris*, a detailed knowledge of the time course of development within the host is required. Since data in the literature are often quite old and differ in many observations, especially regarding the number and the time course of moults (for review see [15]), the isolate used in the present study was subjected to an in-depth parasitological analysis. Furthermore, the course of infection strongly depends on the respective mouse strain [17] and *T. muris* isolate [18], making a detailed characterisation even more crucial.

The parasitological data obtained here were in agreement with findings of Panesar and Croll [16]. They reported, that on day 20 *p.i.,* all larvae were found embedded in the surface epithelium with their posterior ends extruding into the lumen of the gut. In the present study, this observation was made from day 21 onwards. In contrast to Panesar and Croll, we still found a small but significant number of histotropic stages until day 29 *p.i.* However, the period in which histotropic stages were exclusively present was almost the same. Interestingly, observations by Pike [37] were also in line with data shown here. They have shown, that the female/male ratio steadily develops towards more male worms and that male *T. muris* survive longer than females, which is in marked contrast to other parasitic nematode species, where females survive longer than males [37].

In the present study, no *in vivo* efficacy of the aminophenylamidine amidantel was found against patent *T. muris* infections in mice. Three oral doses of 500 mg/kg amidantel did not reduce the worm burden in comparison to the no-drug control. The efficacy of amidantel against *T. muris* was investigated previously and was also found to be only moderate [24]. Therefore, amidantel was not further evaluated in the presented study. In contrast, three consecutive oral doses with either tribendimidine or dAMD resulted in ED_{50} values of 6.5 mg/kg and 15.1 mg/kg, respectively. Complete elimination of the worm burden was achieved by three oral doses using either 25 mg/kg tribendimidine or 100 mg/kg dAMD. Oral doses of 1× 400 mg [44] or 3× 400 mg [26] tribendimidine have been shown to result in cure rates of 76.8% and 33.3%, respectively, against *T. trichiura* in humans. Intraperitoneal injections of the drugs, which to our knowledge were evaluated for the first time, resulted in reversed potency with ED_{50} values of 15.3 mg/kg for tribendimidine and 8.3 mg/kg for dAMD and complete elimination at dosages above 50 mg/kg in both cases. This is somewhat surprising since tribendimidine is known to rapidly disintegrate in aqueous environments releasing two molecules of dAMD [45]. Differences in release of the highly hydrophobic drugs from the used formulation (dispersion containing Cremophor EL/deionised water) are the most likely explanation for the observed phenomenon. The larger tribendimidine molecule

can be suspected to diffuse more slowly into the aqueous environment. It can be assumed that release of drugs from the dispersion occurs more rapidly in the digestive track under mechanical mixing in the presence of bile salts than in the peritoneum and that passive diffusion is of minor importance in the gut. The absence of efficacy of tribendimidine and dAMD using subcutaneous administrations might also be due to the very basic formulation of the drugs. However, neither intraperitoneal nor subcutaneous administrations, using such a basic formulation, were able to significantly improve the efficacy of tribendimidine or dAMD against *T. muris* in mice.

In contrast to the aminophenylamidines, the cyclooctadepsipeptide, emodepside, has previously been shown to be completely effective against *T. vulpis* [21] and also *T. muris* [22,23]. A single dosage of 7.16 mg/kg emodepside in the Profender® spot on formulation for cats was sufficient to clear patent *T. muris* infections of mice within 48 h [22] and even treatments of mice against immature stages using 6.0 mg/kg emodespide of the same formulation on day 3, day 20 or day 35 *p.i.*, resulted in significantly reduced worm counts (>95% efficacy) [23]. Next to the oral tablet formulation of Profender® for dogs with 1 mg/kg emodepside [21], also a single dose of 0.45 mg/kg emodepside of the oral Procox® suspension was sufficient to completely eliminate immature and mature *T. vulpis* from dogs [46]. However, almost all investigations on the efficacy and safety of emodepside were conducted on nematodes of veterinary importance and only few *in vitro* data on important nematodes of humans are available [47], and PF1022A has not been evaluated against *Trichuris* spp. at all. However, while no clinical signs of intolerability were found, a high degree of efficacy against a large number of helminths in a variety of hosts including *H. bakeri* in mice [48], *S. ratti* and *N. brasiliensis* in rats, *A. caninum* in dogs, cyathostomes in horses, *T. colubriformis* and *H. contortus* in sheep and *D. viviparus* in cattle using fairly low dosages of 1-10 mg/kg PF1022A were reported [49].

There were also differences in efficacy comparing emodepside and PF1022A in the present study, but the magnitude of these differences was dependent on the route of administration. However, emodepside always performed significantly better than PF1022A using the ED_{50} value as criterion. The difference between both drugs was particularly small for the oral administration, which also performed better than the intraperitoneal and the subcutaneous route. The ED_{50} and ED_{95} values for PF1022A were only 1.9 and 1.5 fold higher than those for emodepside, respectively.

For its suitability in mass-drug-treatment programs, drugs need a high safety and production costs should be as low as possible. Due to the fact that the class of aminophenylamidines is

still considered to be potentially hazardous [50] and PF1022A does have much cheaper production costs than emodepside (due to omission of synthetic derivatisation) [47], single dose experiments and treatments targeting developmental stages were only performed with PF1022A. In addition, no data regarding the effects of PF1022A on any stages of *Trichuris* spp. have been published previously. To examine whether PF1022A has the potential to replace the more expensive emodepside in therapy of *Trichuris* spp., it is important to determine the suitability of PF1022A as a broad-spectrum anthelmintic.

At least in the triple dose regimen, PF1022A was able to completely eliminate all developmental stages of *T. muris*. However, the required dosages inversely correlated with the time span after infection, *i.e.* the earlier stages had to be treated with higher dosages. Single drug administration needed 2.5 to 10-fold higher dosages to achieve complete resolution of the infections, and against L1 larvae no significant effect on worm burdens could be obtained using only a single dose. The most likely reason for this observation is the localisation of the larvae. Larvae develop deep in the epithelium of the basal parts of the crypts of Lieberkühn until day 5 *p.i.,* while they were found closer to the surface of the epithelium between days 5 and 10 *p.i.* On day 15 *p.i.,* a large proportion of histotropic larvae was already found in the epithelial surface, where a higher drug concentration might be present [16].

One might think that the relatively high dosages of PF1022A required to completely eliminate developmental *T. muris* stages, especially in single dose regimens, could prevent its further development as trichuricidal drug. However, potential improvement of efficacy through optimised galenic formulations should be taken into account. The potential of cyclooctadepsipeptides for efficient treatment against *Trichuris* spp. has been shown using single oral administration of Profender® tablets (Bayer Animal Health GmbH, Leverkusen, Germany). A dose rate of 1 mg/kg emodepside resulted in almost complete elimination of immature and mature stages of *T. vulpis* in dogs (>99%) [20], suggesting that optimised formulations can dramatically improve drug performance in this drug class. The formulation in Profender® tablets is optimised to eliminate all relevant parasitic nematodes of dogs and optimisation can be considered to improve drug efficacy. Emodepside is also the only nematocidal ingredient of Profender® spot-on for cats and Procox® suspension for puppies. Although using a different route of administration (dermal) Mehlhorn *et al*. [22] have shown that a single dosage of 7.16 mg/kg emodepside in the Profender® spot on formulation for cats was sufficient to clear *T. muris* infections of mice within 48 h. In sharp contrast to that, three consecutive oral doses of 75 mg/kg emodepside using the Cremophor EL/water dispersion

were required to achieve a complete elimination of patent *T. muris* infections in the present study. The more than 10-fold increase in efficacy between three doses using Cremophor/water and a single dose using the optimised Profender® formulation emphasises that every drug formulation has to be optimised for each drug and host species and that dramatic decreases in required drug dosages are possible when using an optimised formulation. In addition, optimization of formulations also decreases the risk of intoxications and the costs of treatment, in particular, if drugs can be targeted specifically towards the location of the parasite, *e.g.,* the gut, avoiding high drug concentrations in tissues, *e.g.,* the brain, which may be important for side effects.

In conclusion, *in vivo* treatments with relatively high doses of PF1022A resulted in complete elimination of *T. muris*, including mature adult and immature adult worms as well as histotropic and further developed larval stages in a single-dose regimen. Since only non-optimised formulations were evaluated in this study, considerably lower dosages might be achievable, using formulations optimised for particular host species. Despite the fact that detailed safety and pharmacokinetic studies are still completely missing for humans, distinct effects of PF1022A against the usually dose-limiting genus *Trichuris* in the mouse model suggest that cyclooctadepsipeptides are useful candidates for development as agents against human STHs and nematode infections of livestock animals.

5.7 References

1. Awasthi S, Bundy DA, Savioli L, 2003, Helminthic infections. BMJ 327, 431-433.
2. Lustigman S, Prichard RK, Gazzinelli A, Grant WN, Boatin BA, McCarthy JS, Basáñez MG, 2012, A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 6, e1582.
3. Knopp S, Steinmann P, Keiser J, Utzinger J, 2012, Nematode infections: soil-transmitted helminths and trichinella. Infect Dis Clin North Am 26, 341-358.
4. Hotez PJ, Fenwick A, Savioli L, Molyneux DH, 2009, Rescuing the bottom billion through control of neglected tropical diseases. Lancet 373, 1570-1575.
5. Pullan RL, Brooker SJ, 2012, The global limits and population at risk of soil-transmitted helminth infections in 2010. Parasit Vectors 5, 81.
6. Stephenson LS, Holland CV, Cooper ES, 2000, The public health significance of *Trichuris trichiura*. Parasitology 121, 73-95.
7. World Health Organization, 2011, WHO model list of essential medicines: 17th list, March 2011. Geneva: World Health Organization.
8. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ, 2006, Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367, 1521-1532.
9. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, Yang GJ, Sripa B, Lustigman S, 2012, A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6: e1549.
10. Keiser J, Utzinger J, 2010, The drugs we have and the drugs we need against major helminth infections. Adv Parasitol 73, 197-230.
11. Diawara A, Drake LJ, Suswillo RR, Kihara J, Bundy DA, Scott ME, Halpenny C, Stothard JR, Prichard RK, 2009, Assays to detect beta-tubulin codon 200 polymorphism in *Trichuris trichiura* and *Ascaris lumbricoides*. PLoS Negl Trop Dis 3: e397.
12. Traversa D, 2011, Are we paying too much attention to cardio-pulmonary nematodes and neglecting old-fashioned worms like *Trichuris vulpis*? Parasit Vectors 4, 32.
13. Arends J, Vercruysse J, 2002, The use of macrocyclic lactones to control parasites of pigs, In: Vercruysse J, Rew RS (Eds.), Macrocyclic lactones in antiparasitic therapy. CABI Publishing,Wallingford.

14. Keeling JE, 1961, Experimental trichuriasis. II. Anthelmintic screening against *Trichuris muris* in the albino mouse. J Parasitol 47, 647-651.
15. Anderson RC, 2000, Nematode parasites of vertebrates: their development and transmission. CABI Publishing Wallingford.
16. Panesar TS, Croll NA, 1980, The location of parasites within their hosts: site selection by *Trichuris muris* in the laboratory mouse. Int J Parasitol 10, 261-273.
17. Wakelin D, 1967, Acquired immunity to *Trichuris muris* in the albino laboratory mouse. Parasitology 57, 515-524.
18. Johnston CE, Bradley JE, Behnke JM, Matthews KR, Else KJ, 2005, Isolates of *Trichuris muris* elicit different adaptive immune responses in their murine host. Parasite Immunol 27, 69-78.
19. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, Welz C, von Samson-Himmelstjerna G, 2012, Anthelmintic cyclcooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28, 385-394.
20. Xiao SH, Utzinger J, Tanner M, Keiser J, Xue J, 2013, Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. Acta Trop 126, 115-126.
21. Schimmel A, Altreuther G, Schroeder I, Charles S, Cruthers L, Kok DJ, Kraemer F, Krieger KJ, 2009, Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature adult *Trichuris vulpis* infections in dogs. Parasitol Res 105, 17-22.
22. Mehlhorn H, Schmahl G, Frese M, Mevissen I, Harder A, Krieger KJ, 2005, Effects of a combinations of emodepside and praziquantel on parasites of reptiles and rodents. Parasitol Res 97, 65-69.
23. Schmahl G, Mehlhorn H, Harder A, Klimpel S, Krieger KJ, 2007, Efficacy of combination of emodepside plus praziquantel against larval and adult stages of nematodes (*Trichuris muris, Angiostrongylus cantonensis*) in rodents. Parasitol Res 101, 77-84.
24. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, Thomas H, 1979, Amidantel, a potent anthelminthic from a new chemical class. Arzneimittelforschung 29, 31-32.

25. Steinmann P, Zhou XN, Du ZW, Jiang JY, Xiao SH, Wu ZX, Zhou H, Utzinger J, 2008, Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. PLoS Negl Trop Dis 2, e322.
26. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W, 2005, Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94, 1-14.
27. Xiao SH, Wu ZX, Zhang JH, Wang SQ, Wang SH, Qiu DC, Wang C, 2007, Clinical observation on 899 children infected with intestinal nematodes and treated with tribendimidine enteric coated tablets. Chin J Parasit Dis 25, 372-375.
28. Hu Y, Xiao SH, Aroian RV, 2009, The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3, e499.
29. Miltsch SM, Krucken J, Demeler J, Ramunke S, Harder A, von Samson-Himmelstjerna G, 2013, Interactions of anthelmintic drugs in *Caenorhabditis elegans* neuro-muscular ion channel mutants. Parasitol Int 62, 591-598.
30. Welz C, Kruger N, Schniederjans M, Miltsch SM, Krucken J, Guest M, Holden-Dye L, Harder A, von Samson-Himmelstjerna G, 2011, SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in *Caenorhabditis elegans* slo-1 loss of function mutants. PLoS Pathog 7, e1001330.
31. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, Harder A, Holden-Dye L, Hopper NA, 2007, The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. Int J Parasitol 37, 1577-1588.
32. Saeger B, Schmitt-Wrede HP, Dehnhardt M, Benten WP, Krucken J, Harder A, von Samson-Himmelstjerna G, Wiegand H, Wunderlich F, 2001, Latrophilin-like receptor from the parasitic nematode *Haemonchus contortus* as target for the anthelmintic depsipeptide PF1022A. FASEB J 15, 1332-1334.
33. Chen W, Terada M, Cheng JT, 1996, Characterization of subtypes of gamma-aminobutyric acid receptors in an *Ascaris* muscle preparation by binding assay and binding of PF1022A, a new anthelmintic, on the receptors. Parasitol Res 82, 97-101.

34. Miltsch SM, Krucken J, Demeler J, Janssen IJ, Kruger N, Harder A, von Samson-Himmelstjerna G, 2012, Decreased emodepside sensitivity in unc-49 gamma-aminobutyric acid (GABA)-receptor-deficient *Caenorhabditis elegans*. Int J Parasitol 42, 761-770.
35. Stoll NR, 1923, Investigations on the control of hookworm disease. XV. An effective method of counting hookworm eggs in feces. Am J Epidemiol 3, 59-70.
36. Panesar TS, 1989, The moulting pattern in *Trichuris muris* (Nematoda: Trichuroidea). Can J Zool 67, 2340-2343.
37. Pike EH, 1969, Egg output of *Trichuris muris* (Schrank, 1788). J Parasitol 55, 1046-1049.
38. Motulsky H, Christopoulos A, 2004, Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting. Oxford University Press, New York.
39. Suputtamongkol Y, Premasathian N, Bhumimuang K, Waywa D, Nilganuwong S, Karuphong E, Anekthananon T, Wanachiwanawin D, Silpasakorn S, 2011, Efficacy and safety of single and double doses of ivermectin versus 7-day high dose albendazole for chronic strongyloidiasis. PLoS Negl Trop Dis 5, e1044.
40. Shoop WL, Eary CH, Michael BF, Haines HW, Seward RL, 1991, Anthelmintic activity of paraherquamide in dogs. Vet Parasitol 40, 339-341.
41. Tritten L, Silbereisen A, Keiser J, 2011, *In vitro* and *in vivo* efficacy of Monepantel (AAD 1566) against laboratory models of human intestinal nematode infections. PLoS Negl Trop Dis 5, e1457.
42. Little PR, Hodge A, Maeder SJ, Wirtherle NC, Nicholas DR, Cox GG, Conder GA, 2011, Efficacy of a combined oral formulation of derquantel-abamectin against the adult and larval stages of nematodes in sheep, including anthelmintic-resistant strains. Vet Parasitol 181, 180-193.
43. Sutherland I, Scott I, 2010, Gastrointestinal nematodes of sheep and cattle - biology and control. Blackwell Publishing, Oxford.
44. Wu ZX, Fang YY, Liu YS, 2006, Effect of a novel drug – enteric coated tribendimidine in the treatment of intestinal nematode infections. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 24, 23–26.
45. Yuan G, Xu J, Qu T, Wang B, Zhang R, Wei C, Guo R, 2010, Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs 10, 83-90.

46. Petry G, Altreuther G, Wolken S, Swart P, Kok DJ, 2013, Efficacy of emodepside plus toltrazuril oral suspension for dogs (Procox®, Bayer) against *Trichuris vulpis* in naturally infected dogs. Parasitol Res 112, 133-138.
47. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, Don R, Keiser J, 2011, Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5, e1138.
48. Nwosu U, Vargas M, Harder A, Keiser J, 2011, Efficacy of the cyclooctadepsipeptide PF1022A against *Heligmosomoides bakeri in vitro* and *in vivo*. Parasitology 138, 1193-1201.
49. von Samson-Himmelstjerna G, Harder A, Schnieder T, Kalbe J, Mencke N, 2000, *In vivo* activities of the new anthelmintic depsipeptide PF 1022A. Parasitol Res 86, 194-199.
50. Epe C, Kaminsky R, 2013, New advancement in anthelmintic drugs in veterinary medicine. Trends Parasitol 29, 129-134.

5.8 Supporting information

Supplementary Tab. 5-3. Localisation of *T. muris* stages throughout prepatency

	Eggs				Free larvae/adults				Histotropic larvae				Extruding larvae			
Day p.i.	Duodenum	Colon	Caecum	Debris	Duodenum	Colon	Caecum	Debris	Duodenum	Colon	Caecum	Debris	Duodenum	Colon	Caecum	Debris
1	0, 0, 0;	0, 0, 0;	0, 0, 0;	12, 3, 0;*	0, 0, 0;	0, 0, 0;	0, 0, 0;	27, 9, 1;	0, 0, 0;	8, 5, 1;	55, 47, 10;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
2	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 3, 2;*	0, 0, 0;	0, 0, 0;	0, 0, 0;	17, 14, 0;	0, 0, 0;	7, 0, 0;	88, 50, 22;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
3	0, 0, 0;	0, 0, 0;	0, 0, 0;	4, 3, 1;*	0, 0, 0;	0, 0, 0;	0, 0, 0;	20, 19, 11;	0, 0, 0;	1, 0, 0;	13, 7, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
4	0, 0, 0;	0, 0, 0;	0, 0, 0;	1, 0, 0;*	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 4, 0;	0, 0, 0;	0, 0, 0;	9, 5, 5;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
5	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	3, 3, 1;	0, 0, 0;	0, 0, 0;	21, 12, 8;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
6	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	1, 0, 0;	9, 4, 2;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
7	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	2, 1, 0;	11, 7, 6;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
8	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	2, 0, 0;	0, 0, 0;	0, 0, 0;	18, 13, 2;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
9	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 0, 0;	15, 12, 5;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
10	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 4, 0;	19, 16, 15;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
11	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	14, 12, 2;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
12	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	17, 10, 10;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
13	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	29, 26, 16;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
14	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	1, 0, 0;	58, 24, 11;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
15	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	26, 20, 18;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
16	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	8, 2, 0;	29, 25, 10;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
17	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	2, 0, 0;	24, 15, 14;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
18	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	4, 4, 1;	43, 31, 28;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
19	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	11, 5, 0;	59, 39, 37;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
20	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	3, 1, 0;	47, 41, 9;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;
21	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	53, 30, 21;	0, 0, 0;	0, 0, 0;	0, 0, 0;	8, 2, 0;	0, 0, 0;
22	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	12, 3, 3;	0, 0, 0;	0, 0, 0;	5, 2, 0;	17, 15, 5;	0, 0, 0;
23	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	13, 8, 7;	0, 0, 0;	0, 0, 0;	1, 1, 0;	19, 12, 6;	0, 0, 0;
24	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 0, 0;	0, 0, 0;	0, 0, 0;	11, 0, 0;	54, 27, 4;	0, 0, 0;
25	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 4, 0;	0, 0, 0;	0, 0, 0;	8, 0, 0;	79, 40, 23;	0, 0, 0;
26	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	2, 0, 0;	1, 1, 0;	0, 0, 0;	0, 0, 0;	9, 9, 1;	35, 29, 6;	0, 0, 0;
27	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	9, 0, 0;	0, 0, 0;	0, 0, 0;	2, 1, 0;	0, 0, 0;	0, 0, 0;	23, 3, 2;	62, 30, 8;	0, 0, 0;
28	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	23, 0, 0;	0, 0, 0;	1, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	17, 11, 0;	36, 19, 1;	0, 0, 0;
29	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	3, 2, 0;	0, 0, 0;	0, 0, 0;	3, 0, 0;	0, 0, 0;	0, 0, 0;	22, 20, 5;	95, 55, 5;	0, 0, 0;
30	0, 0, 0;	0, 0, 0;	0, 0, 0;	82, 0, 0;**	0, 0, 0;	0, 0, 0;	0, 0, 0;	6, 0, 0	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	15, 10, 0;	71, 38, 27;	0, 0, 0;
31	0, 0, 0;	0, 0, 0;	0, 0, 0;	127, 0, 0;**	0, 0, 0;	0, 0, 0;	0, 0, 0;	5, 1, 1;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	0, 0, 0;	7, 5, 0;	54, 31, 23;	0, 0, 0;

Table shows localisation of *T. muris* stages within their murine host in the course of prepatent period. Stages are subdivided into (i) eggs, (ii) larvae found in the intestinal contents (free larvae), (iii) larvae completely penetrated into the epithelium of duodenum, colon or caecum (histotropic larvae) and (iv) larvae extruding their posterior ends into the lumen of the guts (extruding larvae). Absolute numbers of stage counts per region are given for each mouse individually.*, fully embryonated eggs; **, unembryonated eggs.

Supplementary Tab. 5-4. Temperature dependency of *T. muris* larval development in eggs

	Unsegmented (in %)				Partially segmented (in %)				Fully developed (in %)				Degenerated (in %)			
DPP	**4°C**	**19°C**	**27°C**	**37°C**	**4°C**	**19°C**	**27°C**	**37°C**	**4°C**	**19°C**	**27°C**	**37°C**	**4°C**	**19°C**	**27°C**	**37°C**
0	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
7	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
14	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0
	100	100	100	96.0	0	0	0	4.0	0	0	0	0	0	0	0	0
	100	100	100	89.5	0	0	0	10.5	0	0	0	0	0	0	0	0
	100	100	94.7	75.0	0	0	5.3	25.0	0	0	0	0	0	0	0	0
21	100	100	100	0	0	0	0	100	0	0	0	0	0	0	0	0
	100	100	95.0	10.5	0	0	5.0	89.5	0	0	0	0	0	0	0	0
	100	100	88.9	11.7	0	0	11.1	88.3	0	0	0	0	0	0	0	0
	100	100	86.9	15.8	0	0	13.1	84.2	0	0	0	0	0	0	0	0
	100	100	76.2	22.8	0	0	23.8	77.2	0	0	0	0	0	0	0	0
	100	86.4	68.4	33.3	0	13.7	31.6	66.7	0	0	0	0	0	0	0	0
28	100	100	73.7	0	0	0	26.3	100	0	0	0	0	0	0	0	0
	100	100	68.4	0	0	0	31.6	100	0	0	0	0	0	0	0	0
	100	100	61.9	0	0	0	38.1	100	0	0	0	0	0	0	0	0
	100	69.6	48.0	0	0	30.4	52.0	61.9	0	0	0	0	0	0	0	38.9
	100	61.1	40.0	0	0	38.9	60.0	57.1	0	0	0	0	0	0	0	42.9
	100	55.2	36.8	0	0	44.8	64.8	42.9	0	0	0	9.5	0	0	0	47.6
35	100	81.1	0	0	0	18.9	100	5.0	0	0	0	20.0	0	0	0	75.0
	100	76.5	0	0	0	23.5	100	3.7	0	0	0	25.9	0	0	0	70.4
	100	73.3	0	0	0	26.7	95.7	0	0	0	0	44.0	0	0	4.3	66.0
	100	26.9	7.4	0	0	73.1	92.6	0	0	0	0	82.1	0	0	0	17.9
	100	26.7	5.3	0	0	73.3	94.7	0	0	0	0	71.4	0	0	0	28.6
	100	5.9	4.4	0	0	94.1	95.6	0	0	0	0	87.5	0	0	0	12.5
42	100	78.9	0	0	0	21.1	73.3	0	0	0	26.7	73.3	0	0	0	26.7
	100	78.6	0	0	0	21.4	72.7	0	0	0	27.3	76.2	0	0	0	23.8
	100	80.0	0	0	0	20.0	70.0	0	0	0	30.0	72.7	0	0	0	27.3
	100	0	0	0	0	100	52.9	0	0	0	47.1	35.7	0	0	0	64.3
	100	0	0	0	0	100	41.2	0	0	0	58.8	35.3	0	0	0	64.7
	100	0	0	0	0	100	35.7	0	0	0	64.3	12.5	0	0	0	87.5
49	100	54.2	0		0	45.8	42.9		0	0	57.1		0	0	0	
	100	41.4	0		0	58.6	36.4		0	0	63.6		0	0	0	
	100	38.9	0		0	61.1	35.0		0	0	65.0		0	0	0	
	100	0	0		0	100	14.3		0	0	85.7		0	0	0	
	100	0	0		0	100	11.8		0	0	88.2		0	0	0	
	100	0	0		0	100	10.0		0	0	90.0		0	0	0	
56	100	0	0		0	100	0		0	0	100		0	0	0	
	100	0	0		0	100	0		0	0	100		0	0	0	
	100	0	0		0	100	0		0	0	100		0	0	0	
	100	42.1	0		0	57.9	0		0	0	100		0	0	0	
	100	40.9	0		0	59.1	0		0	0	96.6		0	0	4.4	
	100	33.3	0		0	66.7	0		0	0	94.7		0	0	5.3	
63	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	10.5			0	89.5			0	0			0	0		
70	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	0			0	100			0	0			0	0		
	100	4.5			0	95.5			0	0			0	0		
	100	5.9			0	94.1			0	0			0	0		

DPP: Days post purification

Supplementary Tab. 5-5. Descriptive statistics for each treatment group (classified by individual drugs, dose regimen and route of administration)

dAMD, three oral doses

	Control	1.0 mg/kg	5.0 mg/kg	10 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	46.80	56.00	23.60	11.20	2.33	0
SD	13.66	12.03	16.93	15.95	9.83	3.83	0
N	89	5	5	5	5	5	5

dAMD, three intraperitoneal doses

	Control	5.0 mg/kg	10 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	36.20	9.40	3.40	0	0
SD	13.66	14.45	5.98	5.41	0	0
N	89	5	5	5	5	5

Tribendimidine, three oral doses

	Control	1.0 mg/kg	5.0 mg/kg	10 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	59.20	35.00	5.80	0	0	0
SD	13.66	22.00	9.03	5.72	0	0	0
N	89	5	5	5	5	5	5

Tribendimidine, three intraperitoneal doses

	Control	5.0 mg/kg	10 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	36.80	27.60	8.80	0	0
SD	13.66	14.03	6.62	7.85	0	0
N	89	5	5	5	5	5

PF1022A, three oral doses

	Control	1.0 mg/kg	2.5 mg/kg	5.0 mg/kg	7.5 mg/kg	10 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	36.60	26.80	13.60	17.80	6.20	3.00	0	0
SD	13.66	5.59	20.46	6.03	7.98	6.94	6.71	0	0
N	89	5	5	5	5	5	5	5	5

PF1022A, three intraperitoneal doses

	Control	10 mg/kg	25 mg/kg	50 mg/kg	75 mg/kg	100 mg/kg	150 mg/kg	200 mg/kg	250 mg/kg
Mean	32.97	48.00	39.20	29.80	13.00	5.20	5.80	0	0
SD	13.66	13.75	24.61	9.63	9.92	5.54	6.02	0	0
N	89	5	5	5	5	5	5	5	5

PF1022A, three subcutaneous doses

	Control	50 mg/kg	100 mg/kg	150 mg/kg	200 mg/kg	250 mg/kg	300 mg/kg	400 mg/kg	500 mg/kg
Mean	32.97	32.60	16.20	21.60	15.80	15.30	7.40	0	0
SD	13.66	7.50	8.23	7.27	10.13	11.32	16.55	0	0
N	89	5	5	5	5	5	5	5	5

PF1022A, one oral dose

	Control	50 mg/kg	75 mg/kg	100 mg/kg	150 mg/kg	200 mg/kg	250 mg/kg	300 mg/kg	500 mg/kg
Mean	32.97	33.20	33.40	11.00	16.00	12.80	12.40	2.60	0
SD	13.66	5.97	5.50	8.46	18,10	12.15	10.36	4.34	0
N	89	5	5	5	5	5	5	5	5

Emodepside, three oral doses

	Control	0.5 mg/kg	1.0 mg/kg	2.5 mg/kg	5.0 mg/kg	7.5 mg/kg	10 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	28.80	12.29	18.00	9.20	2o20	0.80	0	0
SD	13.66	9.68	6.94	9.82	9.36	2,49	1.30	0	0
N	89	5	5	5	5	5	5	5	5

Emodepside, three intraperitoneal doses

	Control	1.0 mg/kg	5.0 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Mean	32.97	37.00	17.60	13.00	8.80	5.20	0	0	0
SD	13.66	8.34	12.40	4.85	5.85	7.66	0	0	0
N	89	5	5	5	5	5	5	5	5

Emodepside, three subcutaneous doses

	Control	1.0 mg/kg	10 mg/kg	15 mg/kg	20 mg/kg	25 mg/kg	50 mg/kg	75 mg/kg	100 mg/kg
Mean	32.97	37.00	17.60	13.00	8.80	5.20	0	0	0
SD	13.66	8.34	12.40	4.85	5,85	7.66	0	0	0
N	89	5	5	5	5	5	5	5	5

Chapter 6

Characterisation of the calcium-gated and voltage-dependent potassium channel SLO-1 of nematodes and its interaction with emodepside

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6 Characterisation of the calcium-gated and voltage-dependent potassium channel SLO-1 of nematodes and its interaction with emodepside

6.1 Abstract

The cyclooctadepsipeptide emodepside and its parental compound PF1022A are very broad spectrum nematicidal drugs which are able to eliminate nematodes resistant to other anthelmintics. The mode of action of cyclooctadepsi-peptides is only partially understood, but involves the latrophilin LAT-1 receptor and the voltage- and calcium-activated potassium channel SLO-1. Genetic evidence suggests that emodepside exerts its anthelmintic activity predominantly through SLO-1. In deed *slo-1* deficient *C. elegans* strains are completely emodepside resistant. However, direct effects of emodepside on this channel have not been reported and SLO-1 channels have only been characterised for *C. elegans* and related Strongylida. Molecular and bioinformatic analyses identified full-length SLO-1 cDNAs of *A. suum*, *P. equorum*, *T. canis*, *D. immitis*, *B. malayi*, *O. gutturosa* and *S. ratti*. Two paralogs were identified in the trichocephalids *T. muris*, *T. suis* and *T. spiralis*. Several splice variants encoding truncated channels were identified in *Trichuris* spp. SLO-1 channels of trichocephalids form a monophyletic group, showing that duplication occurred after the divergence of Enoplea and Chromadorea. To explore function of a representative protein, *C. elegans* SLO-1 was expressed in *X. laevis* oocytes and studied in electrophysiological (voltage-clamp) experiments. Incubation of oocytes with 1-10 µM emodepside caused significantly increased currents over a wide range of holding potentials in the absence of calcium signalling suggesting that emodepside directly opens SLO-1. Emodepside wash out did not reverse the effect and the SLO-1 inhibitor verruculogen was only effective when applied before but not after emodepside. The identification of several splice variants and paralogs in some parasitic nematodes suggests that there are substantial differences in channel properties among species. Most importantly, this study showed for the first time that emodepside directly opens SLO-1 channels, significantly improving the understanding of the mode of action of this drug class.

6.2 Introduction

In Ecdysozoa, large conductance potassium channels (also BK or Maxi-K channels) are encoded by *slo-1* genes named after the "slowpoke" phenotype observed in *slo-1* deficient *D. melanogaster* [1]. Due to their large conductance in the open state, typically exceeding 200 pS, SLO-1 channels are of major importance for repolarisation of excitable cells such as neurons, muscle cells and exocrine as well as endocrine cells. These channels are tetramers and, in almost every animal species, all subunits are encoded by a single gene – with teleost fish as the only known exception, with multiple *slo-1* paralogs resulting from whole genome duplications [2]. The opening of SLO-1 channels is controlled by the membrane potential and intracellular free calcium-concentrations $[Ca^{2+}]_i$. Depolarisation of the membrane as well as very high transient local $[Ca^{2+}]_i$ are required to open SLO-1 channels [3]. Indeed, at the subcellular level, SLO-1 colocalises with voltage-gated calcium channels in calcium nanodomains, and mislocalisation interferes with its function in *C. elegans* [4]. Most other ion channels produce subtypes with altered physiological properties through a combination of different subunits as recently reviewed for acetylcholine and glutamate receptors [5,6]. In contrast, several different *slo-1* splice variants have been described *e.g.* for *C. elegans*, *D. melanogaster*, mice and humans and differential splicing is known to affect channel properties [7-10]. In addition, the use of different tissue-specific promoters has been described for *D. melanogaster* [11,12]. Indeed, the number of different splice variants has dramatically increased during evolution, which is demonstrated by the fact that the human *slo-1* ortholog *kcnma1* encodes 13 alternative exons [13]. In fact, the number of splice variants in Mammalia and Diptera is so high that systematic analysis of the effects of the channel variants generated by alternative splicing on voltage- and calcium sensitivity has only been performed for a few of them. The complexity of SLO-1 channel heterogeneity is further increased by the ability to form heterotetramers [14] containing different splice variants and the fact that channel responses to depolarisation and calcium are modulated by posttranslational modifications such as palmitoylation and phosphorylation at several distinct sites [15-17].

The *C. elegans slo-1* gene is expressed in the pharyngeal neuronal network, in motoneurons of the dorsal and ventral nerve cord and in body wall muscles, but not in pharyngeal muscles [18]. Splice variants in *C. elegans* are more clearly arranged, with four different splice sites giving rise to 12 well characterised splice variants [19] plus three additional variants annotated in WormBase. Expression levels of these variants in whole *C. elegans* as well as

voltage and calcium sensitivity profiles of different homomeric tetramers have been reported recently [7,19]. Whether differential splicing is influenced by the cell- or tissue-type (as shown for *D. melanogaster*) and whether the heteromerisation of subunits encoded by different splice variants further influences voltage and calcium sensitivity, are still open questions.

Nematode SLO-1 channels have received particular attention in recent years due to their central involvement in the mode of action of the broad-spectrum nematicidal drug emodepside, a cyclooctadepsipeptide [20]. *C. elegans* strains with *slo-1* loss-of-function mutations are completely resistant to emodepside [21]. Emodepside sensitivity can be rescued by re-introducing SLO-1 expression cassettes obtained from either *C. elegans* or the parasitic nematodes *A. caninum* or *C. oncophora,* but only partially by the human ortholog [21-23]. These results can be explained by either a direct and specific interaction of emodepside with nematode SLO-1 channels or a necessary function of SLO-1 in a signal transduction pathway downstream of an emodepside target. Ectopic expression of *slo-1* in the pharynx muscle in a *slo-1* deficient genetic background conferred emodepside sensitivity to pharyngeal pumping [23] suggesting that this channel is either the direct target of the drug or that an unidentified target is present in pharyngeal muscle cells that is activated downstream of SLO-1. Although SLO-1 channels are widely considered to be the most likely receptors for emodepside in nematodes [18,20,24,25], direct interaction of the channel with emodepside or its activation by this drug have not been reported. This is in contrast to another putative emodepside target, the G protein-coupled receptor LAT-1, an ortholog of mammalian latrophilin receptors. Binding of the drug to its target and activation of LAT-1 by PF1022A and emodepside have been demonstrated [26]. However, *lat-1* loss-of-function mutations in *C. elegans* cause only partial emodepside resistance; the effects of emodepside on the pharynx but not on the body muscle were impaired.

Knowing the mode of action of new drugs has important advantages for the prediction of efficacy in new target species [27] and can exclude potential receptor-dependent deleterious side effects in host species [28]. Structure-activity relationships are also facilitated if the receptor is known. The cyclooctadepsipeptides have a very broad anthelmintic spectrum against parasitic nematodes [20] representing all major clades [29], including strongylids (clade V), *Strongyloides* (clade IV), Ascaridoidea and Filarioidea (clade III) as well as the trichocephalids *Trichuris* and *Trichinella* (clade I) [20]. This broad spectrum nematicidal activity is in marked contrast to other anthelmintics that have been developed in the last decade. Efficacy of tribendimidine against *S. stercoralis* and *T. trichiura* is only moderate to

low [30], although repeated dosing was effective in a *T. muris* mouse model [31]. Monepantel appears to have good efficacy against parasites from clade V, especially in small ruminants [32], although activity against human hookworms appeared to be limited [33]. Poor or no efficacy against parasites from other clades was observed [33]. For *S. ratti* this is presumably due to the fact that no member of the DEG-3/DES-2 subfamily of AChRs encoded in its genome [27]. Members of this subfamily are implicated in the monepantel mode of action in clade V nematodes [28,34,35]. These examples clearly show that tremendous differences exist among nematodes regarding susceptibility to anthelmintics, based at least in part on the presence of anthelmintic targets encoded in their genomes. Despite their relatively uniform body shape, nematodes are genetically and physiologically extremely diverse [36,37].

slo-1 genes and gene products have thus far only been investigated in clade V of the phylum Nematoda [29]. Since parasitic nematodes of vertebrates are found in four of five clades [29] and SLO-1 is apparently a validated drug target, the present study aimed to analyse the diversity of SLO-1 channels within the phylum Nematoda and to determine effects of emodepside on SLO-1 channel opening characteristics.

6.3 Material and methods

Parasites

All animal experiments were approved by the local administrations in charge and were in accordance with local laws regarding animal welfare (Animal Welfare Act in the United States and the Tierschutzgesetze in Germany and in Switzerland as well as in accordance with and the European Union directive 2010/63/EU). Adult *T. muris* were obtained during controlled drug trials performed for other studies at Bayer HealthCare AG, Global Drug Discovery Animal Health in Monheim, Germany (approved by LANUV, North Rhine-Westphalia, Germany) under no. 200/V14) [31] and at the Swiss Tropical and Public Health Institute in Basel, Switzerland (approved by the local veterinary agency based on Swiss cantonal and national regulations under permission no. 2070) [38]. *P. equorum* were collected during a slaughter trial approved by the Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) under the reference no. 33.9-42502-05-07A499. *A. suum* were obtained from a German slaughterhouse. *D. immitis* macrofilariae were collected during routine necropsy of a naturally infected, moribund, euthanised dog in Athens (Georgia, USA). Macrofilariae of *O. gutturosa* were collected from slaughtered cattle at an abattoir in Banjul, The Gambia. *B. malayi* microfilariae were provided by the NIAID/NIH Filariasis Research Reagent Resource centre (FR3). *T. canis* RNA was obtained from a previously published study [39].

RNA isolation and cDNA synthesis

Nematodes were homogenized on ice in Trizol (Invitrogen) or TriFast (Peqlab) reagent using a TissueRuptor and transparent disposable probes (Qiagen). RNA was isolated according to the manufacturer's instructions, except that the volume of Trizol was increased to 5 ml per 100 mg wet tissue weight. Volumes of all subsequently used reagents were adjusted accordingly. RNAs were precipitated twice in the presence of glycogen (Thermo Fischer Scientific), dissolved in water and stored at -80 °C until use. For cDNA synthesis, 1 µg total RNA was mixed with 100 pM of either random hexamer primers (for PCR with degenerate primers) or oligo dT primers (for full-length PCRs; both primers from Thermo Fischer Scientific), incubated at 65 °C for 5 min and then chilled on ice. Reverse transcription was performed in 20 µl using 200 U Maxima Reverse Transcriptase (Thermo Fischer Scientific), 1 mM dNTPs and 20 U RiboLock® RNase inhibitor (Thermo Fischer Scientific) by incubation at 42 °C for 30 min and 60 °C for 30 min. Finally, the enzyme was inactivated at 70 °C for 5

min. Alternatively, 200 U RevertAidM-MuLV reverse transcriptase were used with 20 U RiboLock RNase inhibitor, 0.5 mM dNTPs and 25 µM oligo-dT primers.

Amplification of full-length cDNAs

Partial genomic data from *T. muris* and *T. canis* and a deduced full-length sequence of *S. ratti* (for phylogenetic analysis only) were provided by the Parasite Genomics group at the Wellcome Trust Sanger Institute (www.sanger.ac.uk/research/projects/parasitegenomics/). Based on comparisons among available slo-1 cDNA sequences of *H. contortus*, *C. oncophora* and *C. elegans,* degenerate primers were designed and used to amplify and sequence small slo-1 fragments of *P. equorum* and *A. suum*. A 126 bp long cDNA sequence of *T. canis* slo-1 published on nematode.net [40] was identified by BLAST. Specific primers for nested 5'- and 3'-RACE PCR were designed using these sequence data. PCRs contained 0.3 mM dNTPs, 0.4 µM of the gene-specific and the universal primer, 1 µl cDNA and 0.5 µl Advantage® 2 Polymerase Mix (Clontech) in 25 µl 1×Advantage buffer. Full-length sequences were amplified using two gene-specific primers and the same PCR protocol. PCR fragments were gel purified, cloned into pCR4 TOPO (Invitrogen) and sent to GATC Biotech for sequencing. Primers for all full-length amplifications are provided in Supplementary Tab. 6-2.

For other nematodes, primers derived from partial slo-1 sequences identified in the *T. muris*, *D. immitis*, *Onchocerca volvulus* and *B. malayi* genome projects were used. Using these sequences, primers for 5'- and 3'- RACE PCR were designed and RACE PCRs were carried out with the 5'/3' RACE Kit (second generation) as detailed previously [39]. PCRs were performed in 25 µl 1×Phusion II buffer containing 1 U Phusion II DNA polymerase (Thermo Fischer), 0.3 mM dNTPs, 0.3 µM of each primer, 1×Q solution (Qiagen) and 1 µl cDNA synthesised with RevertAidM-MuLV reverse transcriptase. Thermocycling was conducted in a Biorad C1000 or S1000 cycler with initial denaturation at 98 °C for 1 min followed by 35 cycles with denaturation at 98 °C for 10 s, annealing at a primer pair specific temperature for 30 s and elongation at 72 °C for 20 s to 2 min. For full-length PCRs, primers flanking the open reading frame were chosen. Full-length primers for *B. malayi*, *D. immitis* and *O. gutturosa* were obtained from genome data [41]. Further details about primer sequences and PCR conditions are available in Supplementary Tab. 6-2. PCR products were gel-purified and cloned into pCR4 TOPO blunt vector and sequenced by GATC Biotech.

Identification of protein motifs in SLO-1 channels

Molecular weight and putative isoelectric points were calculated with Clone Manger 9 (Scientific and Educational Software). Localisation of transmembrane regions was predicted using TMPred software [42]. Conserved domains and Prosite motifs were identified using CD-BLAST [43,44] and InterProScan [45,46]. Prediction of phosphorylation sites was performed using NetPhosK 1.0 [47].

Phylogenetic analysis

SLO-1 sequences from the present study were aligned with homologs available in GenBank or Wormbase, and homologs from *Meloidogyne incognita* [48], *S. ratti* (obtained from the Sanger genome project). Deduced protein sequences were aligned using ClustalX2 [49]. Alignments were analysed with Prottest 3.0.1 [50] to identify the most appropriate amino acid substitution model. PhyML 3.0.1 [51,52] was then used to determine tree topology and branch support as described [53]. In brief, the JTT model for amino acid substitution [54] with 16 Γ distributed substitution rate categories was used. PhyML was set to estimate Γ shape parameter and proportion of invariable sites while amino acid frequencies were set to be based on the substitution model. Nearest neighbour interchange (NNI) and subtree pruning and regraftment (SPR) moves were allowed to optimise the tree topology. Both, Bayesian transformation and Shimodaira-Hasegawa-like modifications of the approximate likelihood test were used to calculate branch support. Tree optimisation started with one neighbour joining and five random trees and the tree with the highest likelihood was finally chosen and visualised in MEGA5 [55].

For analysis of the conserved alternative exon, ProtTest 3.0 identified the same model as for the full-length sequence and PhyML was executed using identical parameters as for the full-length sequence.

Experimental detection of differential splice products

The primers flanking the putative splice site (Supplementary Tab. 6-2) were used in a two-step RT-PCR as described above. PCR products were analysed on 2.0% agarose gels and with the DNA 1000 kit on the Bioanalyzer 2100 (Agilent). Gel-purified DNA fragments were cloned and sequenced. Quantification of fragments was performed using the Bioanalyzer Expert software.

Trichuris suis transcriptome data

For RNA-seq experiments, total RNAs were extracted from larvae at day 10 *p.i.* (approximately 50000 L1 and L2 from five experimentally infected pigs), day 18 *p.i.* (15000 L3 from four pigs) and day 28 *p.i.* (3000 L4 from two pigs), from whole adult male (n=10), adult female (n=10) or from multiple stichosomes (mixed sex; n=10), posterior portions of adult females (n=10) and of adult males (n=10) using the TriPure reagent (Roche). Yield and quality were verified using the 2100 Bioanalyzer (Agilent). For library production, purification of polyadenylated (polyA$^+$) RNA from 10 µg total RNA from each sample was carried out using Sera-mag oligo(dT) beads (Thermo Fisher Scientific). Then RNAs were fragmented to a size of 300-500 bp, reverse-transcribed using random hexamer primers, end-repaired and adapter-ligated according to the manufacturer's protocol (Illumina). Ligation products of approximately 400 bp were eluted from agarose gels, PCR-amplified (15 cycles) as recommended and purified on MinElute columns (Qiagen). Finally, libraries were subjected to paired-end RNA-seq using HiSeq 2000 (Illumina) and assessed for quality and adapter sequences. After sequencing, raw reads were trimmed of Illumina adapters, filtered for length (≥ 40 nucleotides) and low-quality data (reads containing four or more consecutive bases showing a PHRED quality below 20).

Transcripts for each stage and/or body portion as well as the Illumina RNA-seq data from a mixed-sex adult *T. suis* transcriptome generated previously [56] were reconstructed and quantified by Jex *et al.* (unpublished data) using the Tuxedo suite [57] and a draft assembly of the *T. suis* genome (Jex *et al.*, unpublished). Differential transcription was assessed using NOISeq [58] with 20% of the evaluated reads for each library used in 5 iterations to simulate technical replicates (note: all RNA-seq libraries were constructed from multiple parasites, *i.e.,* 10 adults or 50,000 larvae retrieved from multiple host individuals to compensate for inter-individual biological variation).

Preparation of slo-1 cRNAs

Full-length cDNAs encoding *C. elegans* SLO-1a and *T. muris* SLO-1.1a in pCR4TOPO (Invitrogen) were used. Plasmid DNA was linearised in 100 µl 1×buffer containing 8 µg plasmid DNA and 100 U XbaI (for *C. elegans* SLO-1a) or 100 U BcuI (for *T. muris* SLO-1.1a) at 37 °C for 2 h. Linearised plasmid DNA was purified via the GeneJet PCR Purification kit (Thermo Fisher Scientific) and eluted with 50 µl UltraPure DNase/RNase-free distilled water. Linearisation of the plasmid DNA was monitored by agarose gel electrophoresis. DNA concentration was determined using the DNA 12000 kit and the 2100

Bioanalyzer (Agilent Technologies). For cRNA synthesis, the mMessage mMachine T7 transcription kit (Ambion) was used. To determine the integrity and concentration cRNA, RNA 6000 Nano kit and Bioanalyzer were used.

Microinjection of X. laevis oocytes

Defolliculated *X. laevis* oocytes were obtained from EcoCyte Bioscience (Castrop-Rauxel, Germany). After delivery, single oocytes were transferred into individual cavities of a 48 well plate containing Barth's solution (88 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 15 mM Tris HCl, 0.5 mM $CaCl_2$, pH 7.4) and incubated for at least 2 h at 19 °C before use. 75 nl of either 200 ng/µL *C. elegans* SLO-1a cRNA or *T. muris* SLO-1.1a were microinjected into each oocyte using the Roboinject (Multi Channel Systems MCS GmbH). Oocytes injected with 75 nl water served as negative control. Position of impalement was set to 350 µm, whereas position of injection was set to 400 µm. Oocytes were incubated at 19 °C for 3-4 days post microinjection. Barth's solution was replaced daily.

Voltage clamp experiments with X. laevis oocytes

Experiments were carried out using the Roboocyte (Multi Channel Systems MCS GmbH). In the Roboocyte, oocytes were perfused with normal frog Ringer solution (NFR: 90 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) for 1 min before electrodes were inserted and membrane potential was clamped to -70 mV. Experiments were carried out as summarised in Fig. 6-1. In general, IVCs were recorded after another perfusion for 1 min. Currents were recorded during clamping the membrane potential between -120 and +60 mV for 500 ms using voltage steps of 20 mV (Fig. 6-1B). Between individual steps, the membrane potential was clamped to -70 mV for 3 s. After initial recordings of IVCs, a perfusion with NFR was performed for 2 min before repeating the recording to ensure that responses were stable over time (Fig. 6-1A(I)). Oocytes showing currents exceeding 250 nA at +60 mV were excluded from further experiments. To determine the effects of drugs, oocytes were first incubated with the vehicle (0.1% DMSO, 0.003% Pluronic F-68 (Sigma Aldrich) for 2 min. Vehicle, emodepside and blockers were always added manually into the well. IVCs were recorded and oocytes were again perfused for 1 min before incubation with emodepside and another recording (Fig. 6-1a(II)). To test whether potential emodepside effects were reversible, oocytes were perfused for 5 to 10 min with NFR after an IVC recording in the presence of emodepside (Fig. 6-1A (III), (IV)). Effects of the blocker verruculogen (1 µM) were determined both before and after addition of emodepside (1 µM) to

the oocytes (Fig. 6-1A (V), (VI)). Currents were recorded using Roboocyte ClampAmp software 2.2.0.15.

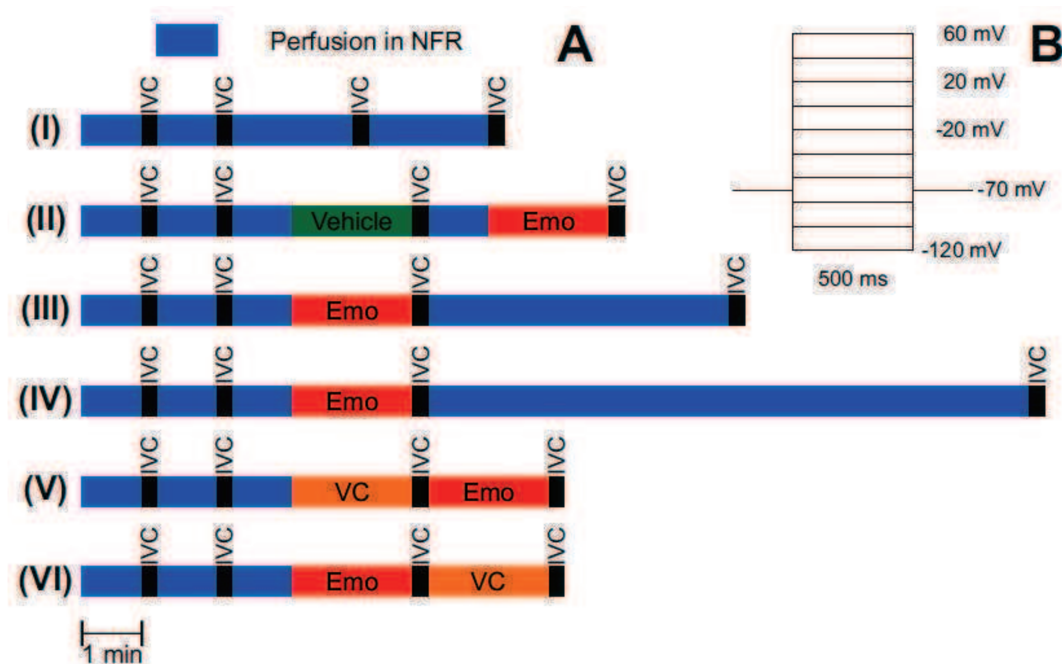


Fig. 6-1. Description of the electrophysiological recordings

Individual oocytes were initially tested in the absence of drugs repeatedly to ensure that oocyte responses were stable over time (Fig. 6-1A (i)). Current-voltage curves (IVCs) were recorded after clamping the membrane potential to -70 mV. Then, holding potentials were clamped from -120 mV to +60 mV in 20 mV steps with 3 s at -70 mV between individual voltage steps as shown in Fig. 1B. The vehicle contained 0.1% DMSO and 0.003% Pluronic F-68. Drugs and vehicle were added manually in the absence of perfusion. Emo, emodepside; VC, verruculogen; NFR, normal frog ringer.

Statistical analysis of electrophysiological data

Mean currents during each voltage step were calculated for individual oocytes in Roboocyte ClampAmp. To obtain IVCs, mean currents ± SEM for all replicate oocytes were plotted against the voltage used to clamp the membrane potential in GraphPad Prism 6.00. Mean currents between different groups of oocytes were compared separately for every holding potential applied using the multiple t-test function in GraphPad Prism. *P* values were corrected for multiple comparisons using the Holm-Sidak method.

6.4 Results

Comparison of slo-1 cDNAs and encoded proteins

Supplementary Tab. 6-3 summarises the physico-chemical properties of predicted nematode SLO-1 subunits compared to homologous proteins from other nematodes and some orthologs from other species used as outgroup in further phylogenetic analysis. Only a single full-length cDNA sequence was identified from clade III nematodes (*B. malayi*, *O. gutturosa*, *P. equorum*, *A. suum* and *T. canis*), although alternative exons were identified in partial PCR products (data not shown). These sequences were not included in the initial analysis of differential splicing since no information regarding possible combinations of different exons is currently available for them. For *B. malayi*, six different isoforms (*Bma*SLO-1c-h) are annotated in WormBase and isoform *Bma*SLO-1f was identical to the one identified in the present study (Fig. 6-2). For *D. immitis* two variants were identified differing only in a short region encoding amino acids 678-692 in *Dim*SLO-1a, which is missing in *Dim*SLO-1b (Fig. 6-2).

In the clade I nematode *T. muris*, two partial genomic sequences encoding SLO-1 homologs were identified in contigs NODE_15952_length_5825_cov_10.724463 and NODE_133417_length_23144_cov_11.427541. Full-length cDNAs of both paralogs were amplified and cloned. Comparison with *T. spiralis* sequences in GenBank revealed that this clade I nematode also encodes two distinct SLO-1 homologs in its genome. The *T. spiralis* SLO-1.1 protein sequence can be found as two partial entries, which cover the whole length under the GenBank accession numbers XP_003370273.1 and XP_003370274. The *T. spiralis* SLO-1.2 database entries XP_003370270.1, XP_003370271.1 and XP_003370272.1 correspond to the *T. muris* SLO-1.2. In both trichocephalids, the two paralogs are juxtaposed in the genome in a tail-to-tail orientation.

Two splice variants encoding full-length *T. muris* SLO-1.1 channels were identified. In addition, two splice variants encoding severely truncated proteins were cloned; truncation is caused by retention of a partial or entire intron in the mature RNA, leading to premature stop codons (*Tmu*SLO1.1c and *Tmu*SLO-1.1d in Fig. 6-2, respectively).

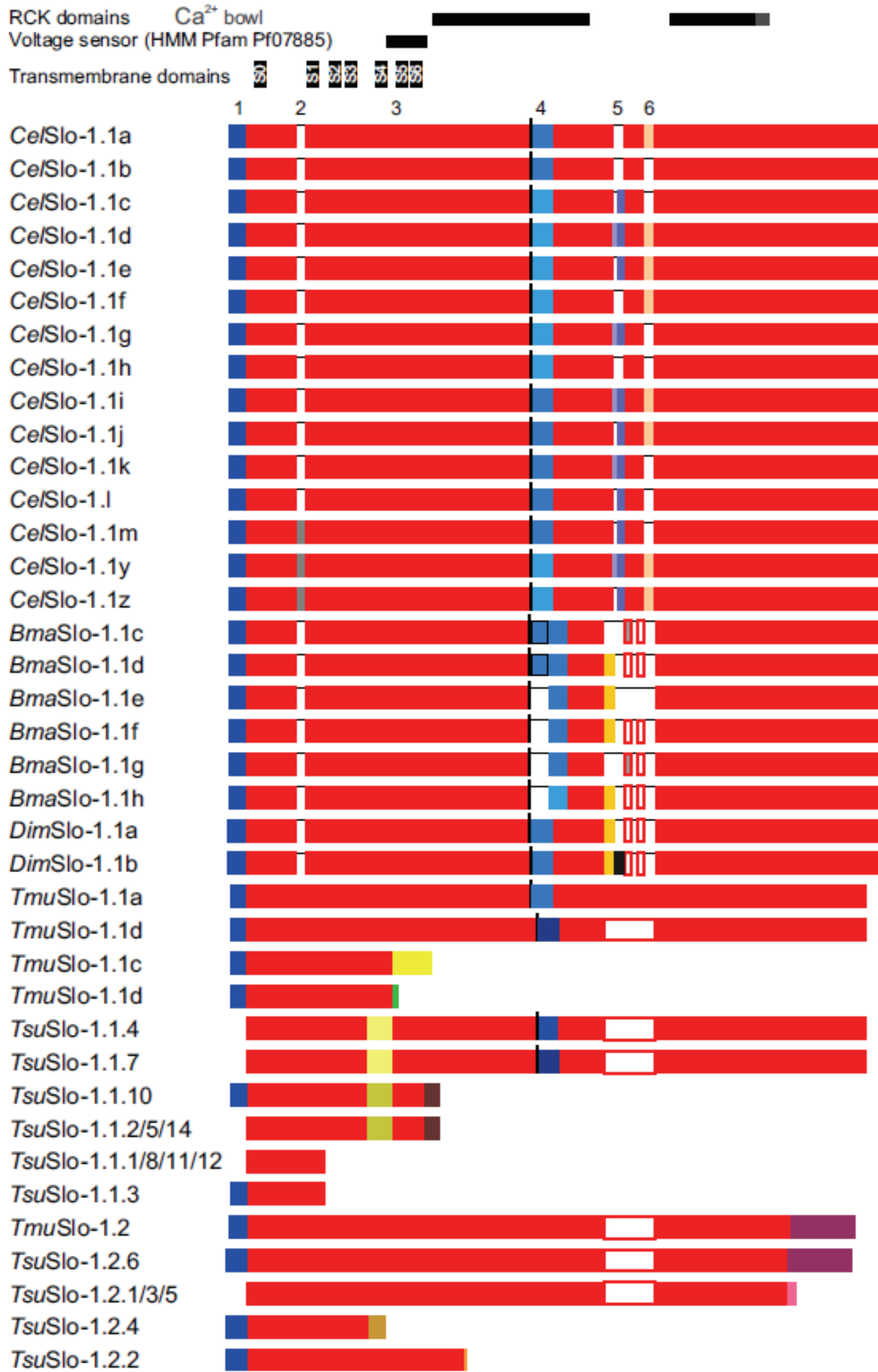

RCK domains
Ca2+ bowl
Voltage sensor (HMM Pfam Pf07885)
Transmembrane domains
1
2
3
4
5
6
CelSlo-1.1a
CelSlo-1.1b
CelSlo-1.1c
CelSlo-1.1d
CelSlo-1.1e
CelSlo-1.1f
CelSlo-1.1g
CelSlo-1.1h
CelSlo-1.1i
CelSlo-1.1j
CelSlo-1.1k
CelSlo-1.l
CelSlo-1.1m
CelSlo-1.1y
CelSlo-1.1z
BmaSlo-1.1c
BmaSlo-1.1d
BmaSlo-1.1e
BmaSlo-1.1f
BmaSlo-1.1g
BmaSlo-1.1h
DimSlo-1.1a
DimSlo-1.1b
TmuSlo-1.1a
TmuSlo-1.1d
TmuSlo-1.1c
TmuSlo-1.1d
TsuSlo-1.1.4
TsuSlo-1.1.7
TsuSlo-1.1.10
TsuSlo-1.1.2/5/14
TsuSlo-1.1.1/8/11/12
TsuSlo-1.1.3
TmuSlo-1.2
TsuSlo-1.2.6
TsuSlo-1.2.1/3/5
TsuSlo-1.2.4
TsuSlo-1.2.2

Fig. 6-2. Comparison of SLO-1 splice variants in nematodes

Schematic representations of different splice variants annotated in WormBase for *C. elegans* (*Cel*SLO-1a-m, y, z) and *B. malayi* (*Bma*SLO-1c-h) in comparison with variants identified in full-length cDNAs cloned from *D. immitis* (*Dim*SLO-1a, b), *T. muris* (*Tmu*SLO-1.1a-d and *Tmu*SLO-1.2). In addition, splice variants predicted from transcriptome data for *T. suis* were included. For the latter splice variants are denominated with numbers (e.g. *Tsu*SLO-1.1.2/5/14 refers to the splice variants 2, 5 and 14 of *T. suis* SLO-1.1 which all encode the same protein but differ in alternative exons downstream of the stop codon. Related exons are shown in similar colors. *Bma*SLO-1c and *Bma*SLO-1d include a partial duplication of the alternative exon in region 4, which is marked by a black box around the partial duplicated region. Regions in which no alternative splicing was detected are indicated by red boxes. In *Trichuris* spp. SLO-1 sequences, regions corresponding to the alternatively spliced regions 5 and 6 in clade III and V nematodes are shown as an empty red box. Thin black lines indicate splice variants in which the corresponding region is missing. The location of transmembrane helices (S0-S6) and functional domains is shown at the top of the scheme. The various regions were alternative splicing was detected are enumerated (1-6). Two highly conserved phosphorylation sites for PKC, located immediately before alternative splice region 4, are depicted as thin vertical lines.

Using primers flanking these introns, a semi-quantitative estimate of the frequency of the splice variants was possible in two independent *T. muris* isolates. PCR products were separated on agarose gels (Fig. 6-3A) and the Bioanalyzer (Fig. 6-3B). The latter was used to quantify the amount of DNA in the peaks (Tab. 6-1). In contrast to the initial impression from agarose gels, where the large PCR products encoding the truncated versions produced the brightest bands, the Bioanalyzer clearly shows that the smallest fragment encoding full-length channels represents the majority of amplicons in terms of molecules (77% and 84%).

Tab. 6-1. Relative abundance of cDNA amplicons encoding full-length or truncated *T. muris* SLO-1 channels

T. muris isolate:	Monheim		Edinburgh Zoo	
-RT control	no amplicon		no amplicon	
Genomic PCR	353 bp	100% ± 0.0%	353 bp	100% ± 0.0%
RT-PCR	67 bp	77.6 ± 0.6%	67 bp	83.8% ± 0.4%
	180 bp	7.8% ± 0.4%	180 bp	4.5% ± 1.7%
	353 bp	12.7% ± 0.6%	353 bp	11.6 % ± 2.0%

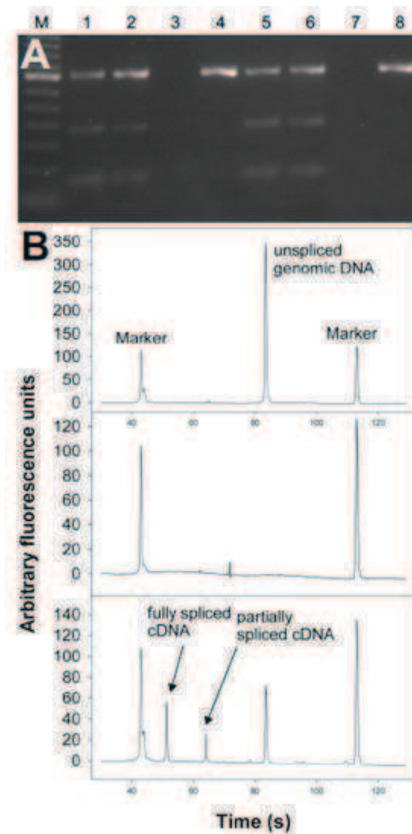


Fig. 6-3. Estimation of splice variant abundances encoded truncated or full-length versions of *T. muris* SLO-1.1.

A) Primers flanking the introns retained in the cDNAs encoding the truncated proteins (*Tmu*SLO-1.1c and *Tmu*SLO-1.1d) were used in RT-PCR (lanes 1, 2, 5, and 6) and genomic PCR (lanes 4 and 8). Template was either derived from the Monheim (lanes 1-4) or the Edinburgh Zoo (lanes 5-8) isolate. Lanes 3 and 7 show no reverse transcription controls. M, 100 bp ladder. B) Representative samples separated on the Bioanalyzer. The upper panel shows a genomic PCR product, the middle panel the control without reverse transcription and the bottom panel the RT-PCR.

To reveal if these truncated channel versions are present in related species, unpublished data from a *T. suis* genome/transcriptome project were analysed for the presence of different slo-1 splice products. Truncated versions due to retained introns were observed in both *Tsu*SLO-1.1 and *Tsu*SLO-1.2 in the same region of the channel (*i.e.,* the transmembrane helix S4 and the voltage sensor comprised of S5 and S6), but the introns involved in these events were not at exactly the same position. Remarkably, alternative exons were found immediately before the retained introns, with one splice variant only present among cDNAs encoding full-length channels (*Tsu*SLO-1.1.4/7) and the alternative variant only in the truncated versions (*Tsu*SLO-1.1.2/5/10/14). In addition, splice variants encoding even shorter channels than those found in *T. muris* were identified (*Tsu*SLO-1.1.1/3/8/12).

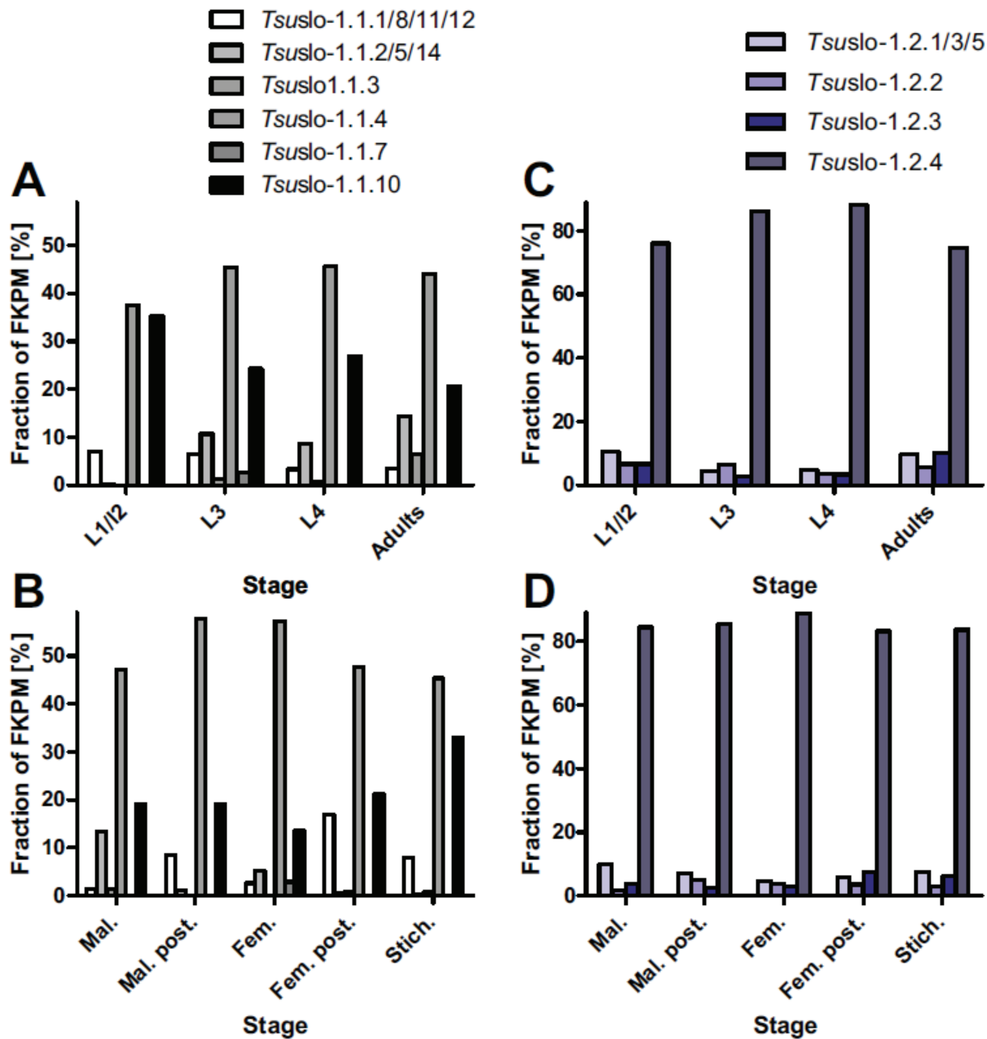


Fig. 6-4. Frequencies of *T. suis* slo-1 splice variants comparing developmental stages, different sexes and tissues

Frequencies of splice variants in terms of fragments per kilobase of exons per million fragments mapped (FKPM). Libraries were compared according to developmental stage (A and C) as well as different sexes or tissues (B and D). Splice variants were grouped according to the encoded protein, *i.e.* frequencies of splice variants encoding the same protein and differing in sequence downstream of the stop codon were added together. A) and B) show expression patterns of *Tsu*slo-1.1 whereas C) and D) indicate patterns for *Tsu*slo-1.2 splice variants. Mal., males, Mal. post., posterior part of males; Fem. Females; Fem. post., posterior part of females; Stich., stichosome.

Quantitative analysis of transcriptome data revealed for *Tsu*slo.1.1 that the most abundantly detected splice variant corresponds to the full-length splice variant *Tsu*slo-1.1.4 in terms of fragments per kilobase of exons per million fragments mapped (FKPM), whereas the other full-length variant *Tsu*slo1.1.7 was found at much lower abundance (Fig. 6-4). The second most abundantly detected splice variant (*Tsu*slo1.1.10) encodes a severely truncated protein of similar length as the truncated *T. muris* variants detected by RT-PCR (Fig. 6-2). Remarkably, there are very obvious differences in frequency of splice variants, although these differences could not be evaluated statistically since only a single library was sequenced per stage or tissue. For example, the frequency of *Tsu*slo-1.1.10 ranged between 20% in adults and 35% in L1, with L3 and L4 showing intermediate frequencies (Fig. 6-4A). Additional stage specific splicing is exemplified by the channels encoded by *Tsu*slo-1.1.2/5/14 and *Tsu*slo-1.1.3: While the former encodes a channel very similar to *Tsu*slo-1.1.10, but differing in its NH_2 terminus, the latter variant is extremely truncated (Fig. 6-2). Notably, the abundance of *Tsu*slo-1.1.10 is relatively high among slo-1.1 transcripts during larval development, peaking in the L1/L2 stage (Fig. 6-4A). In contrast, transcription of this highly truncated slo-1.1 splice variant was relatively low (statistically significant at a false discovery rate of 0.1) in most adult libraries, with the major exception being the stichosome, in which it displayed its highest abundance. Other minor slo-1.1 isoforms, including minor slo-1.1 variants, showed evidence of differential transcription during development and in adulthood among genders/tissues. However, because single library replicates were used for each tissue and stage of *T. suis*, statistical assessment of the differential transcription of these minor isoforms is not advisable. We consider these differences both preliminary and qualitative at this stage. In contrast to *Tsu*slo-1.1, little variation in transcription of *Tsu*slo-1.2 channel variants was observed among developmental stages or adult tissues/genders, with *Tsu*slo-1.2.6 (the full length transcript) being the major isoform (Fig. 6-4C and D).

Comparison of splice variants in parasitic nematodes with the 15 splice variants described for *C. elegans* reveals five main regions where differential splicing occurs (Fig. 6-2). The first site is an alternative exon (*i.e.,* present or not), the absence of which results in the use of a downstream ATG start codon only in *T. suis* SLO-1.1 and 1.2. Insufficient data are available to determine whether this splicing also occurs in *T. muris*. The first alternative exon identified in *C. elegans* (region 2 in Fig. 6-2; also a 'present or not' type) was not found in any of the parasitic nematode slo-1 cDNAs. Splicing region 3 refers to a larger region where differential splicing occurs in all four *Trichuris* genes in different positions. Many but not all of the encoded proteins are severely truncated. The fourth splicing site (alternative exons) has been

conserved throughout the evolution of SLO-1 channels in nematodes and two versions are present in *T. muris* and *T. suis* SLO-1.1. In all seven sequences cloned from ascarids and filariae, the corresponding region shows higher similarity to *C. elegans* SLO-1a. However, alternative sequences were identified in the genomes of *A. suum*, *O. volvulus* and *B. malayi* (*Bma*SLO-1h in Fig. 6-2 and Supplementary Tab. 6-3). Alternative splicing occurs in exactly the same position in arthropods and vertebrates (data not shown). This alternative exon immediately follows two highly conserved phosphorylation sites. Remarkably, two of the predicted *B. malayi* splice variants contain a partial duplication (approximately two thirds of the whole exon (*Bma*SLO-1c/d). Splicing site four in *C. elegans* (position 5 in Fig. 6-2; ‘present or not’ type with two different 5' splice sites) has no equivalent in any of the *Trichuris* sequences, but there is alternative splicing in the same area in *B. malayi* and *D. immitis*. The sixth splice region is found in *C. elegans* but is apparently not present in clade III and clade I parasitic nematodes.

Phylogenetic analysis of SLO-1 channels

A maximum-likelihood approach was used to calculate a phylogenetic tree from the SLO-1 amino acid sequences (Fig. 6-5A). As outgroup, three sequences from vertebrates, one from a mollusc and five from arthropods were included in the analysis. SLO-1 channels of nematodes and arthropods formed a monophyletic group, in accordance with their phylogenetic position within the Ecdysozoa. In general, most of the tree topology is consistent with current views on nematode evolution. However, the position of the SLO-1 proteins from the clade IV nematodes *M. incognita* and *S. ratti* is basal to a group containing all clade III and clade V sequences, but the support values for this position are rather low in both versions of the approximate likelihood test (Fig. 6-5A). The additional paralog SLO-1.2 present in clade I parasitic nematodes is placed as a sister operational taxonomic unit (OTU) to the SLO-1.1 paralog in this clade. This suggests that duplication of the *slo-1* gene occurred after the trichocephalids diverged from the other groups and is not an ancestral feature of nematode genomes.

To prove that differential splice variants were conserved throughout nematode evolution at the *C. elegans* alternative splice site four, only the amino acid sequences of this exon were aligned and subjected to phylogenetic analysis. For this purpose, alternative exons identified in the genomes of *A. suum* and *O. volvulus* were included. The partial duplication of this exon in *Bma*SLO-1c/d was also included as a separate OTU (*Bma*SLO-1c (part. dupl.) in Fig. 6-2). Results are depicted in Fig. 6-5B. Due to the small size of the sequence, statistical support for

individual nodes is lower than for full-length sequences. However, there is clearly a nematode-specific group with high similarity to the sequence in *C. elegans* SLO-1a, although the exon sequences in the trichocephalid species strongly diverge from those in clade III and V nematodes. The second group contains exon sequences from both nematodes and arthropods, including both alternative exons encoded in the *D. melanogaster* genome. Diversity within this group is much higher and deep divergence patterns could not be resolved. Therefore, the group consists of three major lineages, *i.e.* (i) *Trichuris* SLO-1.1b-like and arthropod sequences which might therefore be considered to be "ancestral", (ii) the exon encoded in *slo-1.2* genes, (iii) the sequences derived from clade III and V nematodes.

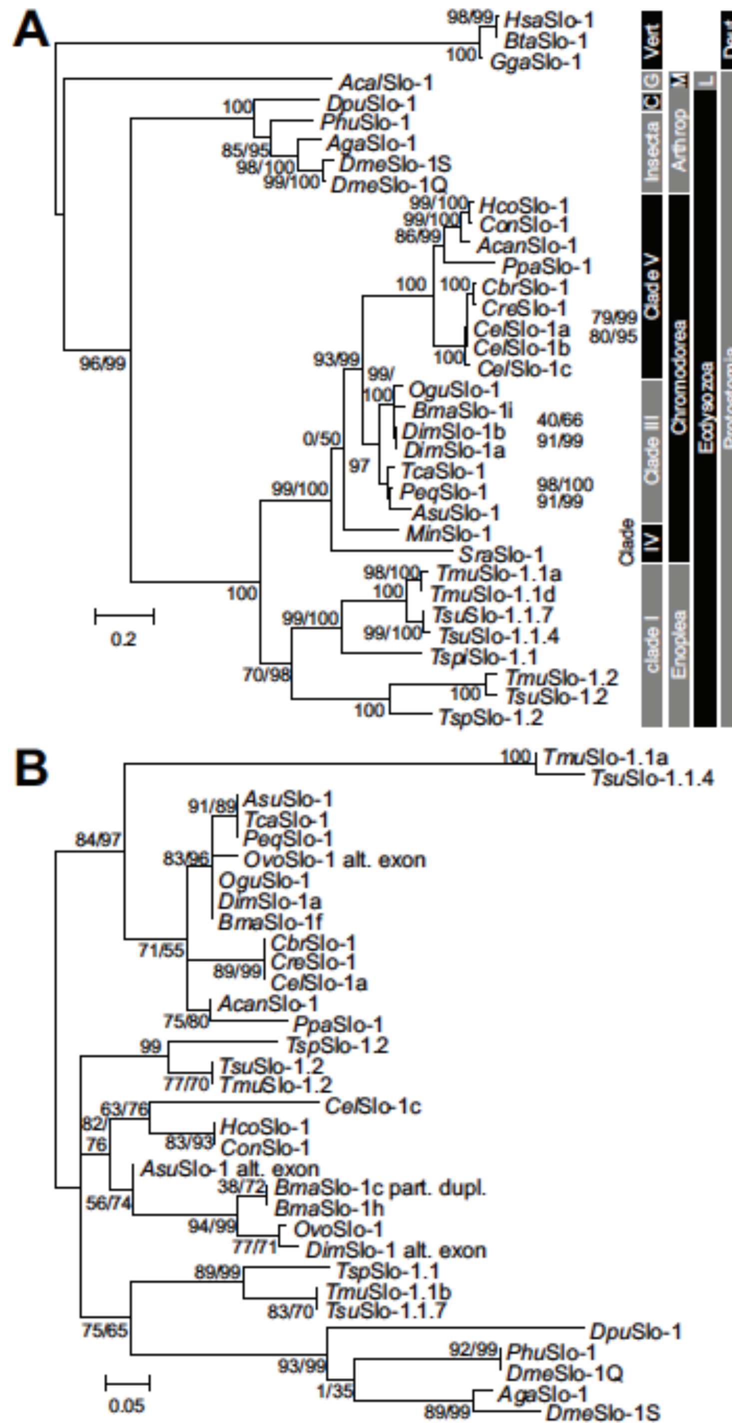


Fig. 6-5. Phylogenetic analysis of nematode SLO-1 channels

A) Phylogram obtained by maximum likelihood analysis from full-length SLO-1 channels. SLO-1 protein sequences from the nematode species *C. elegans* (*Cel*), *C. brigssae* (*Cbr*), *C. remanei* (*Cre*), *P. pacificus* (*Pca*), *H. contortus* (*Hco*), *C. oncophora* (*Con*), *A. caninum* (*Acan*), *O. gutturosa* (*Ogu*), *B. malayi* (*Bma*), *D. immitis* (*Dim*), *T. canis* (*Tca*), *P. equorum* (*Peq*), *A. suum* (*Asu*), *M. incognita* (*Min*), *S. ratti* (*Sra*), *T. muris* (*Tmu*) and *T. suis* (*Tsu*) were aligned together with orthologs from *D. melanogaster* (*Dme*), *Anopheles gambiae* (*Aga*), *Pediculus humanus corporis* (*Phu*), *Daphnia pulex*, *Aplysia callifornica* (*Acal*), *Gallus gallus* (*Gal*), *Bos taurus* (*Bta*) and *H. sapiens* (*Hsa*), which were used as outgroup, using ClustalX2.

For *B. malayi* only the experimentally identified splice variant SLO-1f and for *C. elegans* only the variants SLO-1a-c were included. The JTT model of amino acid substitutions was used and PhyML was set to optimise the number of invariable sites while amino acid frequencies were based on the model. The number of Γ distributed substitution rate categories was set to 16 and PhyML optimised the Γ shape parameter. Support for individual nodes was calculated using the Shimodaira-Hasegawa modification and a baysian transformation of the approximate likelihood ratio test and results are shown close to the nodes before and after the slash, respectively. For those cases were support values were not shown next to the node they are shown on the right and refer to the most terminal node on the same vertical position. The scale bar represents 0.2 substitutions per site. C, Crustacea; G, Gastropoda; Vert, Vertebrata; Arthropod, Arthropoda; M, Mollusca; L, Lophotrophora; Deut, Deuerostomia. B) Phylogenetic tree calculated on an alignment of the conserved alternative exons from all Ecdysozoa included in the tree in A). In addition, four alternative exons identified in *Bma*SLO-1h and in the genome sequences of *O. volvulus* (*Ovo*SLO-1 and *Ovo*SLO-1 alt. exon) and *A. suum* (*Asu*SLO-1 alt. exon) were included. Parameters were identical to those used to calculate the tree from full-length sequences.

Voltage clamp experiments

Voltage-dependent currents were measured in water-injected oocytes (Fig. 6-6A). An inward-directed current was detected at very low negative holding potentials (-120 mV to -80 mV) whereas an approximately linear increase of outward currents was detected between -60 mV and +60 mV (Fig. 6-6A and 6-7A). In water- as well as in *Cel*SLO-1a- and *Tmu*SLO-1.1a-injected oocytes no significant differences were observed between repeatedly recorded IVCs, indicating that the oocytes were in good physiological condition (data not shown). Currents detected in water-injected oocytes were not significantly influenced by the vehicle (0.1% DMSO/ 0.003% Pluronic F-68) or by 10 µM emodepside (Fig. 6-6A). In the absence of emodepside, basal IVCs between water and CelSLO-1a injected oocytes were very similar. However, there was a small but significant higher current at +20 mV in *Cel*SLO-1a injected oocytes, which corresponds to the voltage of approximately 0 mV to +20 mV needed to open *Cel*SLO-1a at a low calcium concentration of 10 µM [59]. This difference was also observed in the presence of the vehicle and there was no significant difference between basal IVCs and IVCs in the presence of the vehicle (Fig. 6-7B). Interestingly, currents did not increase further when holding potion above +20 mV were applied (Fig. 6-6B) and no differences to water injected oocytes were observed at +40 mV and +60 mV (Fig. 6-7B). No increase in currents at +20 mV was detected in oocytes injected with *Tmu*SLO-1a cRNA (Supplementary Fig. 6-8). In fact, currents measured in *Tmu*SLO-1a-injected oocytes were significantly lower than in water-injected oocytes at 0 mV and +20 mV holding potential. At high holding potentials (+40 mV - +60 mV), these IVCs deviated from linearity, suggesting a nonphysiological response of the oocytes. In an attempt to increase the difference for *Cel*SLO-1a or *Tmu*SLO-1a, oocytes were preincubated in 2 µg/ml of the calcium ionophore A23187 (Sigma Aldrich)

in calcium-free Barth's medium for 15 min followed by three washes with normal Barth's medium. However, this pre-treatment did not increase the response but caused severe leak currents (data not shown).

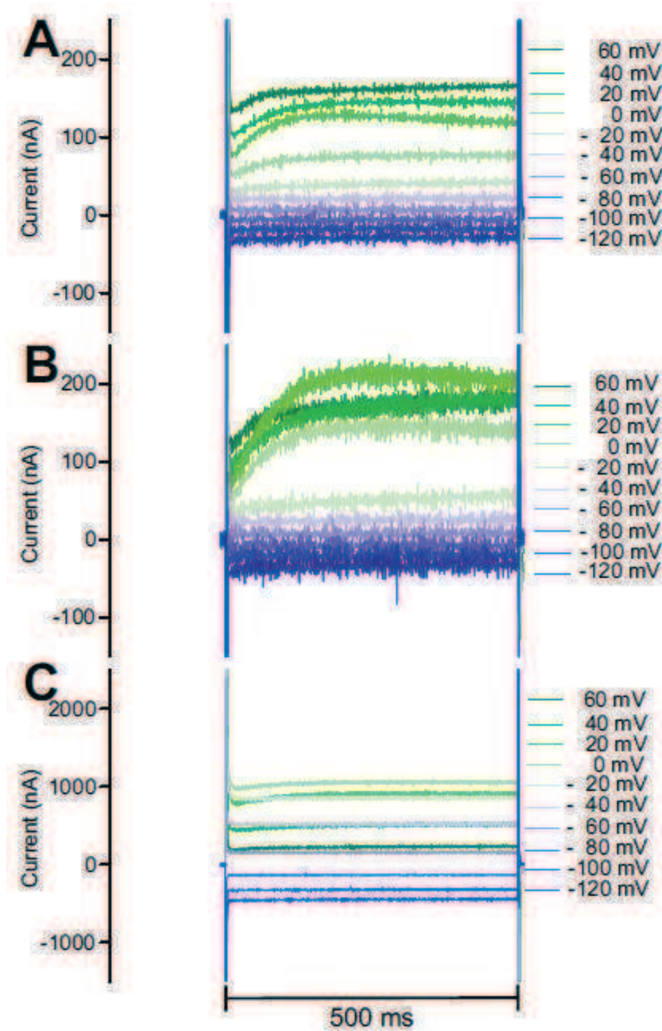


Fig. 6-6. Currents observed in voltage-clamp experiments

Representative currents determined in oocytes injected with A) water, B) *Cel*slo-1a cRNA in the absence of emodepside and C) *Cel*slo-1a cRNA in the presence of 10 µM emodepside.

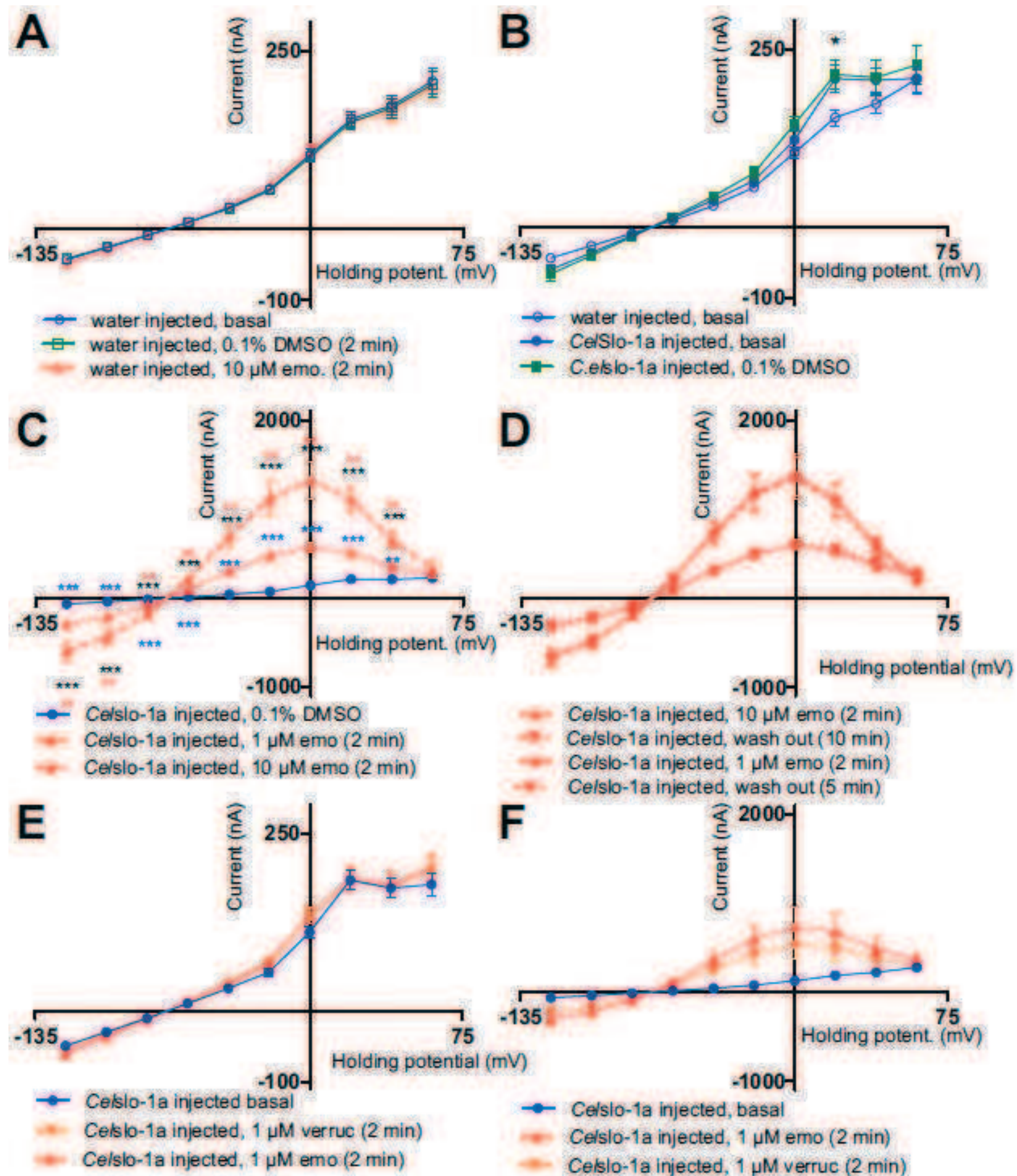


Fig. 6-7. Determination of emodepside effects on current voltage curves in *X. laevis* oocytes injected with *Cel*SLO-1a cRNA

A) IVCs (means ± SEM) were recorded in water injected oocytes without addition of drugs (basal), after addition of the vehicle (0.1% DMSO, 0.003% Pluronic F-68) and after addition of 10 µM emodepside (emo) (n=10). B) Currents obtained from oocytes injected with water (n=10) or *Cel*slo-1a cRNA in the absence of any drug (basal, n=8) or in the presence of vehicle (0.1% DMSO, 0.003% Pluronic F-68) (n=8). The asterisk highlights a significant difference of both curves obtained from *Cel*slo-1a cRNA injected groups of oocytes to the water injected oocytes. C) Currents were recorded in *Cel*slo-1a injected oocytes in the presence of vehicle (0.1% DMSO, 0.003% Pluronic F-68), or emodepside (1 µM or 10 µM

emo) (n=10). For 10 µM emodepside the upper asterisks symbolise the comparison to 1 µM emodepside and the lower the comparison to the vehicle control. The asterisks at the 1 µM emodepside curve indicate significant differences to the control. D) Oocytes injected with *Cel*slo-1a were incubated with 1 µM (n=6) or 10 µM (n=7) emodepside (emo) before recording the IVC curves. Then oocytes were perfused with normal frog ringer for 5 or 10 min, respectively, before a second IVC was recorded from the same oocytes. E) Oocytes were preincubated in the absence of drugs (basal) or with 1 µM verruculogen (verruc) before IVCs were recorded. Then, oocytes were perfused for 2 min before 1 µM emodepside (emo) was added (n=8). F) Oocytes injected with water (n=10) or with *Cel*slo-1 cRNA (n=6) were incubated with 1 µM emodepside (emo) before IVCs were recorded. After perfusion with normal frog ringer for 2 min, verruculogen (verruc) was added for 2 min followed by recording of IVCs.*, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$.

In the absence of a stimulus increasing $[Ca^{2+}]_i$, addition of emodepside to *Cel*slo-1a-injected oocytes resulted in dramatically increased currents (Fig. 6-6C) with significantly stronger effects at 10 µM than 1 µM emodepside (Fig. 6-7C). Differences were significant at all holding potentials except for +60 mV at both emodepside concentrations. IVCs obtained in the presence of emodepside are characterised by two very remarkable features. Firstly, currents increased even at holding potentials far below the expected opening potential of SLO-1 channels, suggesting that channel opening can occur independently of membrane potential. Secondly, currents peaked at 0 mV and significantly decreased at higher holding potentials, resulting in bell shaped IVCs (Fig. 6-7C). At the highest holding potential used, differences between vehicle controls and emodepside-treated oocytes were no longer significant.

Wash out of 1 µM emodepside for 5 min and wash out of 10 µM emodepside for 10 min had no effect on IVCs. Even perfusion for 25 min did not reduce the emodepside effects (n=3, data not shown). Effects of the SLO-1 specific blocker verruculogen revealed that the order of addition strongly influenced the outcome. Preincubation of oocytes with verruculogen alone had no effects on IVCs; even the slight increase in currents at +20 mV observed in *Cel*slo-1a-injected oocytes was not blocked (compare Fig. 6-7B and 6-7E). However, addition of verruculogen before emodepside completely prevented emodepside effects on IVCs (Fig. 6-7E). In contrast, when verruculogen was added to oocytes in the presence of emodepside, no decrease in emodepside-induced currents was detected (Fig. 6-7F).

6.5 Discussion

The mode of action of cyclooctadepsipeptides has long been a matter of debate, with ionotropic $GABA_A$ receptors [39,60], the latrophilin receptor LAT-1 [26,61] and the SLO-1 channel [21-23] consecutively deemed to be the most likely receptors. Although SLO-1 is generally considered to be most important target based experiments using forward and reverse genetics, there has been no direct evidence for emodepside activation of the SLO-1 channel. Responses of excitable cells to emodepside develop slowly [23,62], even more so effects on LAT-1 in transfected mammalian cells [26]. Direct gating of ionotropic receptors is usually characterised by much faster kinetics, which meant that indirect effects of emodepside on SLO-1 could never be completely excluded [62]. Using an oocyte expression system eliminating all nematode-related confounding factors, the present study for the first time demonstrates direct effects of emodepside on SLO-1 channels. In the presence of emodepside, highly increased currents were observed without depolarisation up to a threshold of 0 mV and in the absence of elevated $[Ca^{2+}]_i$ levels. These novel findings confirm that SLO-1 is a direct target of emodepside.

The cyclooctadepsipeptides, exemplified by emodepside, represent an important new class of anthelmintics characterised by their resistance-breaking properties [63]. Cyclooctadepsipeptides have a very broad nematicidal activity in a wide range of host species [64]. Here it has clearly been demonstrated that SLO-1 is encoded in the genomes of parasitic nematodes in all major phylogenetic groups (clade I and clades III-V). This is in remarkable contrast to the target of another new class of anthelmintics, the amino acetonitrile derivatives such as monepantel, for which no receptor has been found in *S. ratti* [27] and *D. immitis* [65]. Despite these findings, microfilaricidal efficacy of monepantel was detected (albeit at high concentrations) [65], whereas it was inactive against *S. ratti* [33]. The other new anthelmintic, derquantel, was found to be only a mid-spectrum anthelmintic in the dose range tested (0.5 – 8 mg/kg bodyweight), showing full efficacy (>95%) against L4 and adults of *Trichostrongylus* spp. and *Nematodirus* spp. as well as against adults of *H. contortus*. However, efficacy was suboptimal against L4 and adults of *T. circumcincta*, L4 of *H. contortus*, and some large intestinal nematodes [66].

All these drug classes have been considered to be candidates for development of new medicines to treat human soil transmitted helminthoses and filarioses [67-69]. Although activity of cyclooctadepsipeptides against *B. malayi* and *B. pahangi* appears to be limited to microfilaria, *in vitro* and in animal models [70-72], the spectrum of emodepside exceeds that

of the other current drug candidates as illustrated by the efficacy against the hard to treat nematodes of the genus *Trichuris*. No drug is available which reliably cures human *T. trichiura* infections using a single dose, but emodepside has been shown to have excellent activity against *T. vulpis* [73,74] and *T. muris* [75,76].

The *X. laevis* expression system has been widely used to characterise the function of nematode ion channels and in particular receptors for neurotransmitters [77-79]. It has several advantages including robustness, simultaneous expression of proteins (*e.g.*, auxiliary proteins or multiple subunits) [78], and large cells which allow easy application of the two electrode voltage clamp technique [80]. However, there is also a drawback of using oocytes when working with calcium responsive channels such as SLO-1. The cell is so large that it is not possible to buffer the cytoplasm to a defined calcium concentration. Inside out patch clamp recording would be required to perform this type of experiment [59]. Therefore, activation of the SLO-1 channel by simultaneously increasing $[Ca^{2+}]_i$ and depolarisation of the cells was not possible in this configuration. Attempts to increase the $[Ca^{2+}]_i$ by preincubation of oocytes with the calcium ionophore A23187 were not successful and resulted in cells looking very unhealthy and exhibiting large leak currents (not shown).

Activity of the *Cel*SLO-1a channel was only observed in the presence of emodepside, and currents mediated by *Tmu*SLO-1.1a could not be observed at all. In the absence of any other agonist of nematode SLO-1 channels and without the ability to prove functional expression of *Tmu*SLO-1.1a by determining currents evoked by increased $[Ca^{2+}]_i$ and depolarisation, the failure to detect emodepside effects cannot be interpreted as emodepside unresponsiveness. In fact, currents in *Tmu*SLO-1.1a-injected oocytes were lower than those observed in water-injected oocytes, suggesting that some nonphysiological changes have occurred in the cells. This is supported by the fact that the IVCs in the absence of emodepside significantly deviated from linearity at high voltages, unlike currents observed in water-injected oocytes. Since *T. muris* is fully susceptible to emodepside [31,75,76], it is unlikely that SLO-1 from this parasite should not respond to this drug. However, due to the presence of two *slo-1* paralogs in the *T. muris* genome, it cannot be ruled out that only *Tmu*SLO-1.2 is emodepside responsive. Another possibility is that emodepside acts on *T. muris* only via the LAT-1 pathway. Optimised expression systems will be needed to distinguish between these possibilities in future experiments. For example, codon optimisation might improve expression levels. Moreover, vectors that are particularly adapted for protein expression in *X. laevis* oocytes are available [81]. Finally, other expression systems, such as insect or mammalian cells, should be considered for *T. muris* SLO-1.

In the absence of emodepside, no large currents were observed for *Cel*SLO-1a, which is expected since $[Ca^{2+}]_i$ was not increased. However, there was a slight but significant and highly reproducible increase in current at +20 mV holding potential, which corresponds to the opening potential reported for *C. elegans* SLO-1 at low calcium concentrations [59]. This small current may represent a minor activation of a few channels even in the absence of calcium signalling. However, it remains unclear why this was not observed at more positive holding potentials. Moreover, increased currents at +20 mV were also observed in the presence of the SLO-1 channel blocker verruculogen regardless if addition of verruculogen was followed by exposure to emodepside. Preincubation with verruculogen completely abolished the effects of emodepside and again only the small additional current at +20 mV was detected. In contrast, verruculogen had no significant effect when emodepside was applied first. This suggests that both drugs bind very tightly to *Cel*SLO-1a and cannot displace each other once they have bound. High-affinity binding of emodepside to its target is also suggested by the fact that prolonged perfusion of the oocytes after removal of emodepside did not reverse channel opening. Very similar observations have been reported for effects of emodepside on *A. suum* muscle flaps [62]. Together with the slow onset of emodepside effects [62], the fact that the drug effects were not reversible might suggest that emodepside does not bind to the extracellular domain of SLO-1, but instead binds to an intracellular domain or to the transmembrane helices. The latter possibility is in agreement with its highly lipophilic nature. However, since emodepside needs even longer times to activate the G protein coupled receptor LAT-1 in a mammalian cell expression system, one might speculate that physical/chemical properties of the drug predominantly contribute to the slow onset of effects on two different target molecules, since the emodepside precursor PF1022A was shown to bind to the extracellular NH_2 terminal part of LAT-1 [26]. Since verruculogen cannot inhibit currents through SLO-1 when applied after emodepside, it is also possible that emodepside induces larger conformational changes in the channel compared to many other drugs and that these changes require prolonged time.

Emodepside opened the *Cel*Slo-1a channel at virtually all holding potentials, since currents were much higher than in the controls except at +60 mV. Unexpectedly however, the response did not increase linearly with holding potential, but peaked at 0 mV, which is unusually low. The decreasing current at higher potentials and the lack of emodepside effects at +60 mV might be explained due to inhibition of Slo-1 channels by high intracellular concentrations of calcium or magnesium as reported for mammalian BK channels (see [82] and references therein). Although $[Ca^{2+}]_i$ can be expected to be fairly low since there was no

increased current in the absence of *C. elegans* SLO-1, the concentration of magnesium in *X. laevis* oocytes has been reported to be approximately 0.7 mM [83]. Whether this is also the case in the present study could not be resolved using the current voltage-clamp set up, but requires patch-clamp studies in which the concentration of these cations can be tightly controlled. Indeed, decreased currents at high holding potentials in response to emodepside have not been observed for expression of *C. elegans* SLO-1 in HEK293 cells [84].

Despite their very similar morphology, genetic diversity of nematodes is known to be huge [85]. Although SLO-1 is a highly conserved channel in metazoans, it was not clear, whether emodepside exerts its nematicidal effects through the same mechanisms in all nematode clades. The present study now offers the tools to compare the physiology of SLO-1 channels from different parasitic nematode lineages in the future. However, the failure to detect currents in oocytes injected with *T. muris* slo-1.1 cRNA suggests that optimised or alternative expression systems are required for this purpose. Patch clamp experiments should be the next step to characterise channel properties on a single channel level and tightly control $[Ca^{2+}]_i$ and determine its effect on channel responses. The various *slo-1* splice variants identified in parasitic nematodes here show that evolution has occurred at highly conserved alternative splice sites (splice region 4 with alternative exons present in nematodes, arthropods and vertebrates at the same position) as along with evolution of nematode or clade-specific splice variants. Functional analysis can address the effects of alternative splicing on the probability of posttranslational modifications, channel electrochemical and pharmacological properties and possible heteromerisation between subunits encoded by different splice variants or even paralogs. For instance, the alternative exon in splice region 4 is located immediately behind two highly conserved phosphorylation sites, and it is likely that sequence variation in the neighbourhood of these sites also affects the phosphorylation pattern and frequency.

That *B. malayi* and *B. pahangi* are rather unresponsive to emodepside *in vivo* and *in vitro* [70-72], cannot be easily explained by the primary structure of *Bma*SLO-1, which is virtually identical to that of other filariae. Whether this difference in susceptibility is due to target site related differences (*e.g.,* different splice variants or combination of subunits) or due to other (not SLO-1 related) mechanisms protecting *Brugia* from emodepside effects could be answered by comparing electrophysiological properties of different splice variants between filarial species.

Moreover, the high number of splice variants encoding severely truncated SLO-1 proteins in both *Trichuris* species should be further analysed. On the one hand, truncated versions of SLO-1 with stop codons between the S4 membrane helix and the end of the first RCK domain

(regulator of conduction of potassium) have been shown to be highly resistant to emodepside [21]. On the other hand, a truncated version containing only the NH_2 terminal part of a mouse SLO-1 ortholog from the start codon to the S6 transmembrane region forms a functional channel [86]. Therefore, a functional role of some of the truncated versions cannot be excluded, especially in light of the relatively high number of variants and the fact that expression levels also appear to be high in both *Trichuris* species. Truncated versions of the AChR subunits UNC-63 and ACR-8 have been implicated in resistance to levamisole [87] and truncated SLO-1 subunits might have dominant negative effects if they are able to heteromerise with full-length subunits but prevent the formation of a fully functional channel. That truncated channel are expressed in variants of both, *Tsu*SLO.1.1 and *Tmu*SLO-1.2, and are apparently more frequent at least in some developmental stages and worm tissues than others suggests that these variants might have a physiological function, perhaps in modulating the activity of full-length gene products. More detailed experiments allowing statistical analyses of stage- and tissue-specific differences regarding splice variants as well as more detailed spatial analysis expression sites of individual splice variants will be required. To determine the function of tissue-specific splice variants, recombinant expression systems allowing combined expression of full-length and truncated channel variants should be used. The fact that temporal as well as spatial variation of splicing is *de facto* limited to *Tsu*SLO-1.1 suggests that this splice variant is under less stringent evolutionary pressure than *Tsu*SLO-1.2. A more detailed spatio-temporal picture of the expression pattern of the splice variants would help define their physiological roles.

In conclusion, these data show that the SLO-1 channel of *C. elegans* is a direct target of emodepside and that the channel is present in every important group of parasitic nematodes of vertebrates. Sequence diversity of SLO-1 channels among these groups and within species involves several alternative splice variants and gene duplications. Interactions of subunit isoforms and effects on channel physiology and drug susceptibility are important aspects for more research on nematode neurobiology and parasitology.

6.6 References

1. Elkins T, Ganetzky B, Wu CF, 1986, A *Drosophila* mutation that eliminates a calcium-dependent potassium current. Proc Natl Acad Sci U S A 83, 8415-8419.
2. Rohmann KN, Deitcher DL, Bass AH, 2009, Calcium-activated potassium (BK) channels are encoded by duplicate slo1 genes in teleost fishes. Mol Biol Evol 26, 1509-1521.
3. Tao Q, Kelly ME, 1996, Calcium-activated potassium current in cultured rabbit retinal pigment epithelial cells. Curr Eye Res 15, 237-246.
4. Kim H, Pierce-Shimomura JT, Oh HJ, Johnson BE, Goodman MB, McIntire SL, 2009, The dystrophin complex controls bk channel localization and muscle activity in *Caenorhabditis elegans*. PLoS Genet 5, e1000780.
5. Wolstenholme AJ, 2012, Glutamate-gated chloride channels. J Biol Chem 287, 40232-40238.
6. Arteaga JL, Orensanz LM, Martinez MP, Barahona MV, Martinez-Saenz A, Fernandes VS, Bustamante S, Carballido J, Benedito S, Garcia-Sacristan A, Prieto D, Hernandez M, 2012, Endothelin ET(B) receptors are involved in the relaxation to the pig urinary bladder neck. Neurourol Urodyn. 31, 688-694.
7. Glauser DA, Johnson BE, Aldrich RW, Goodman MB, 2011, Intragenic alternative splicing coordination is essential for *Caenorhabditis elegans slo-1* gene function. Proc Natl Acad Sci108, 20790-20795.
8. Schreiber M, Yuan A, Salkoff L, 1999, Transplantable sites confer calcium sensitivity to BK channels. Nat Neurosci 2, 416-421.
9. Brenner R, Yu JY, Srinivasan K, Brewer L, Larimer JL, Wilbur JL, Atkinson NS, 2000, Complementation of physiological and behavioral defects by a slowpoke Ca^{2+} -activated K^{+} channel transgene. J Neurochem 75, 1310-1319.
10. Tseng-Crank J, Foster CD, Krause JD, Mertz R, Godinot N, DiChiara TJ, Reinhart PH (1994) Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^{+} channel isoforms from human brain. Neuron 13, 1315-1330.
11. Brenner R, Atkinson NS, 1997, Calcium-activated potassium channel gene expression in the midgut of *Drosophila*. Comp Biochem Physiol B Biochem Mol Biol 118, 411-420.

12. Bohm RA, Wang B, Brenner R, Atkinson NS, 2000, Transcriptional control of Ca^{2+}-activated K^+ channel expression: identification of a second, evolutionarily conserved, neuronal promoter. J Exp Biol 203, 693-704.
13. Beisel KW, Rocha-Sanchez SM, Ziegenbein SJ, Morris KA, Kai C, Kawai J, Carninci P, Hayashizaki Y, Davis RL, 2007, Diversity of Ca^{2+}-activated K^+ channel transcripts in inner ear hair cells. Gene 386, 11-23.
14. Chiu YH, Alvarez-Baron C, Kim EY, Dryer SE, 2010, Dominant-negative regulation of cell surface expression by a pentapeptide motif at the extreme COOH terminus of an Slo1 calcium-activated potassium channel splice variant. Mol Pharmacol 77, 497-507.
15. Dai S, Hall DD, Hell JW, 2009, Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev 89, 411-452.
16. Tian L, Jeffries O, McClafferty H, Molyvdas A, Rowe IC, Saleem F, Chen L, Greaves J, Chamberlain LH, Knaus HG, Ruth P, Shipston MJ, 2008, Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels. Proc Natl Acad Sci 105, 21006-21011.
17. Liu Q, Chen B, Ge Q, Wang ZW, 2007, Presynaptic Ca^{2+}/calmodulin-dependent protein kinase II modulates neurotransmitter release by activating BK channels at *Caenorhabditis elegans* neuromuscular junction. J Neurosci 27, 10404-10413.
18. Holden-Dye L, O'Connor V, Hopper NA, Walker RJ, Harder A, Bull K, Guest M, 2007, SLO, SLO, quick, quick, slow: calcium-activated potassium channels as regulators of *Caenorhabditis elegans* behaviour and targets for anthelmintics. Invert Neurosci 7, 199-208.
19. Johnson BE, Glauser DA, Dan-Glauser ES, Halling DB, Aldrich RW, Goodman MB, 2011, Alternatively spliced domains interact to regulate BK potassium channel gating. Proc Natl Acad Sci 108, 20784-20789.
20. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, Welz C, von Samson-Himmelstjerna G, 2012, Anthelmintic cyclooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28, 385-394.
21. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, Harder A, Holden-Dye L, Hopper NA, 2007, The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclooctadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. Int J Parasitol 37, 1577-1588.

22. Welz C, Krüger N, Schniederjans M, Miltsch SM, Krücken J, Guest M, Holden-Dye L, Harder A, von Samson-Himmelstjerna G, 2011, SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in *Caenorhabditis elegans* slo-1 loss of function mutants. PLoS Pathog 7, e1001330.

23. Crisford A, Murray C, O'Connor V, Edwards RJ, Krüger N, Welz C, von Samson-Himmelstjerna G, Harder A, Walker RJ, Holden-Dye L, 2011, Selective toxicity of the anthelmintic emodepside revealed by heterologous expression of human KCNMA1 in *Caenorhabditis elegans*. Mol Pharmacol 79, 1031-1043.

24. Holden-Dye L, Crisford A, Welz C, von Samson-Himmelstjerna G, Walker RJ, O'Connor V, 2012, Worms take to the slo lane: a perspective on the mode of action of emodepside. Invert Neurosci 12, 29-36.

25. Martin RJ, Buxton SK, Neveu C, Charvet CL, Robertson AP, 2011, Emodepside and SL0-1 potassium channels: A review. Exp Parasitol 132, 40-46.

26. Saeger B, Schmitt-Wrede HP, Dehnhardt M, Benten WP, Krücken J, Harder A, von Samson-Himmelstjerna G, Wiegand H, Wunderlich F, 2001, Latrophilin-like receptor from the parasitic nematode *Haemonchus contortus* as target for the anthelmintic depsipeptide PF1022A. FASEB J 15, 1332-1334.

27. Rufener L, Keiser J, Kaminsky R, Maser P, Nilsson D, 2010, Phylogenomics of ligand-gated ion channels predicts monepantel effect. PLoS Pathog 6, e1001091.

28. Kaminsky R, Ducray P, Jung M, Clover R, Rufener L, Bouvier J, Weber SS, Wenger A, Wieland-Berghausen S, Goebel T, Gauvry N, Pautrat F, Skripsky T, Froelich O, Komoin-Oka C, Westlund B, Sluder A, Maser P, 2008, A new class of anthelmintics effective against drug-resistant nematodes. Nature 452, 176-180.

29. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas WK, 1998, A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71-75.

30. Xiao SH, Utzinger J, Tanner M, Keiser J, Xue J, 2013, Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. Acta Trop 126, 115-126.

31. Kulke D, Krücken J, Harder A, von Samson-Himmelstjerna G, 2014, Efficacy of cyclooctadepsipeptides and aminophenylamidines against larval, immature and mature adult stages of a parasitologically characterized trichurosis model in mice. PLoS Negl Trop Dis 8, e2698.

32. Sager H, Hosking B, Bapst B, Stein P, Vanhoff K, Kaminsky R, 2009, Efficacy of the amino-acetonitrile derivative, monepantel, against experimental and natural adult stage gastro-intestinal nematode infections in sheep. Vet Parasitol 159, 49-54.
33. Tritten L, Silbereisen A, Keiser J, 2011, In vitro and in vivo efficacy of Monepantel (AAD 1566) against laboratory models of human intestinal nematode infections. PLoS Negl Trop Dis 5, e1457.
34. Rufener L, Baur R, Kaminsky R, Mäser P, Sigel E, 2010, Monepantel allosterically activates DEG-3/DES-2 channels of the gastrointestinal nematode *Haemonchus contortus*. Mol Pharmacol 78, 895-902.
35. Rufener L, Bedoni N, Baur R, Rey S, Glauser DA, Bouvier J, Beech R, Sigel E, Puoti A, 2013, *acr-23* Encodes a monepantel-sensitive channel in *Caenorhabditis elegans*. PLoS Pathog 9, e1003524.
36. Kumar S, Koutsovoulos G, Kaur G, Blaxter M, 2012, Toward 959 nematode genomes. Worm 1, 42-50.
37. De Ley P, 2006, A quick tour of nematode diversity and the backbone of nematode phylogeny. WormBook, 1-8.
38. Keiser J, Tritten L, Adelfio R, Vargas M, 2012, Effect of combinations of marketed human anthelmintic drugs against *Trichuris muris in vitro* and *in vivo*. Parasit Vectors 5, 292.
39. Miltsch SM, Krücken J, Demeler J, Janssen IJ, Krüger N, Harder A, von Samson-Himmelstjerna G, 2012, Decreased emodepside sensitivity in unc-49 gamma-aminobutyric acid (GABA)-receptor-deficient Caenorhabditis elegans. Int J Parasitol 42, 761-770.
40. Martin J, Abubucker S, Heizer E, Taylor CM, Mitreva M, 2012, Nematode.net update 2011: addition of data sets and tools featuring next-generation sequencing data. Nucleic Acids Res 40, 720-728.
41. Ghedin E, Wang S, Spiro D, Caler E, Zhao Q, Crabtree J, Allen JE, Delcher AL, Guiliano DB, Miranda-Saavedra D, Angiuoli SV, Creasy T, Amedeo P, Haas B, El-Sayed NM, Wortman JR, Feldblyum T, Tallon L, Schatz M, Shumway M, Koo H, Salzberg SL, Schobel S, Pertea M, Pop M, White O, Barton GJ, Carlow CK, Crawford MJ, Daub J, Dimmic MW, Estes CF, Foster JM, Ganatra M, Gregory WF, Johnson NM, Jin J, Komuniecki R, Korf I, Kumar S, Laney S, Li BW, Li W, Lindblom TH, Lustigman S, Ma D, Maina CV, Martin DM, McCarter JP, McReynolds L, Mitreva M, Nutman TB, Parkinson J, Peregrin-Alvarez JM, Poole C, Ren Q, Saunders L, Sluder

AE, Smith K, Stanke M, Unnasch TR, Ware J, Wei AD, Weil G, Williams DJ, Zhang Y, Williams SA, Fraser-Liggett C, Slatko B, Blaxter ML, Scott AL, 2007, Draft genome of the filarial nematode parasite *Brugia malayi.* Science 317, 1756-1760.

42. Hoffmann K, Stoffel W, 1993, TMbase - A database of membrane spanning proteins segments. Biol Chem Hoppe-Seyler 374, 166.
43. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH, 2011, CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39, 225-229.
44. Marchler-Bauer A, Bryant SH, 2004, CD-Search: protein domain annotations on the fly. Nucleic Acids Res 32, 327-331.
45. Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R, 2010, A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res 38, 695-699.
46. Zdobnov EM, Apweiler R, 2001, InterProScan - an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17, 847-848.
47. Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S, Brunak S, 2004, Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4, 1633-1649.
48. Abad P, Gouzy J, Aury JM, Castagnone-Sereno P, Danchin EG, Deleury E, Perfus-Barbeoch L, Anthouard V, Artiguenave F, Blok VC, Caillaud MC, Coutinho PM, Dasilva C, De Luca F, Deau F, Esquibet M, Flutre T, Goldstone JV, Hamamouch N, Hewezi T, Jaillon O, Jubin C, Leonetti P, Magliano M, Maier TR, Markov GV, McVeigh P, Pesole G, Poulain J, Robinson-Rechavi M, Sallet E, Segurens B, Steinbach D, Tytgat T, Ugarte E, van Ghelder C, Veronico P, Baum TJ, Blaxter M, Bleve-Zacheo T, Davis EL, Ewbank JJ, Favery B, Grenier E, Henrissat B, Jones JT, Laudet V, Maule AG, Quesneville H, Rosso MN, Schiex T, Smant G, Weissenbach J, Wincker P, 2008, Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. Nat Biotechnol 26, 909-915.
49. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG, 2007, Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

50. Darriba D, Taboada GL, Doallo R, Posada D, 2011, ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27, 1164-1165.

51. Anisimova M, Gil M, Dufayard JF, Dessimoz C, Gascuel O, 2011, Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. Syst Biol 60, 685-699.

52. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O, 2010, New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59, 307-321.

53. Janssen IJ, Krücken J, Demeler J, Basiaga M, Kornas S, von Samson-Himmelstjerna G, 2013, Genetic variants and increased expression of *Parascaris equorum* P-glycoprotein-11 in populations with decreased ivermectin susceptibility. PLoS One 8, e61635.

54. Jones DT, Taylor WR, Thornton JM, 1992, The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8, 275-282.

55. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, 2011, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28, 2731-2739.

56. Cantacessi C, Young ND, Nejsum P, Jex AR, Campbell BE, Hall RS, Thamsborg SM, Scheerlinck JP, Gasser RB, 2011, The transcriptome of *Trichuris suis* - first molecular insights into a parasite with curative properties for key immune diseases of humans. PLoS One 6, e23590.

57. Schulz MH, Zerbino DR, Vingron M, Birney E, 2012, Oases: robust de novo RNA-seq assembly across the dynamic range of expression levels. Bioinformatics 28, 1086-1092.

58. Zerbino DR, 2010, Using the Velvet de novo assembler for short-read sequencing technologies. Curr Protoc Bioinformatics Chapter 11: Unit 11-15.

59. Chen B, Ge Q, Xia XM, Liu P, Wang SJ, Zhan H, Eipper BA, Wang ZW, 2010, A novel auxiliary subunit critical to BK channel function in *Caenorhabditis elegans*. J Neurosci 30, 16651-16661.

60. Chen W, Terada M, Cheng JT, 1996, Characterization of subtypes of gamma-aminobutyric acid receptors in an *Ascaris* muscle preparation by binding assay and binding of PF1022A, a new anthelmintic, on the receptors. Parasitol Res 82, 97-101.

61. Willson J, Amliwala K, Davis A, Cook A, Cuttle MF, Kriek N, Hopper NA, O'Connor V, Harder A, Walker RJ, Holden-Dye L, 2004, Latrotoxin receptor signaling engages the UNC-13-dependent vesicle-priming pathway in *C. elegans*. Curr Biol 14, 1374-1379.
62. Buxton SK, Neveu C, Charvet CL, Robertson AP, Martin RJ, 2011, On the mode of action of emodepside: slow effects on membrane potential and voltage-activated currents in *Ascaris suum*. Br J Pharmacol 164, 453-470.
63. von Samson-Himmelstjerna G, Harder A, Sangster NC, Coles GC, 2005, Efficacy of two cyclooctadepsipeptides, PF1022A and emodepside, against anthelmintic-resistant nematodes in sheep and cattle. Parasitology 130, 343-347.
64. Harder A, von Samson-Himmelstjerna G, 2002, Cyclooctadepsipeptides - a new class of anthelmintically active compounds. Parasitol Res 88, 481-488.
65. Godel C, 2012, Drug targets of the heartworm, "*Dirofilaria immitis*". PhD thesis, University of Basel, Basel, Switzerland.
66. Little PR, Hodges A, Watson TG, Seed JA, Maeder SJ, 2010, Field efficacy and safety of an oral formulation of the novel combination anthelmintic, derquantel-abamectin, in sheep in New Zealand. N Z Vet J 58, 121-129.
67. Keiser J, Utzinger J, 2010, The drugs we have and the drugs we need against major helminth infections. Adv Parasitol 73, 197-230.
68. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, Don R, Keiser J, 2011, Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5, e1138.
69. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, Yang GJ, Sripa B, Lustigman S, 2012, A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6, e1549.
70. Townson S, Freeman A, Harris A, Harder A, 2005, Acitivty of the cyclooctadepsipeptide emodepside against *Onchocerca gutturosa*, *Onchocerca lienalis* and *Brugia pahangi*. Am J Trop Med Hyg 73, 93.
71. Zahner H, Taubert A, Harder A, von Samson-Himmelstjerna G, 2001, Effects of Bay 44-4400, a new cyclodepsipeptide, on developing stages of filariae (*Acanthochelonema viteae*, *Brugia malayi*, *Litomosoides sigmodontis*) in the rodent *Mastomys coucha*. Acta Trop 80, 19-28.

72. Zahner H, Taubert A, Harder A, von Samson-Himmelstjerna G, 2001, Filaricidal efficacy of the anthelmintically active cyclodepsipeptides. Int J Parasitol 31, 1515-1522.

73. Petry G, Altreuther G, Wolken S, Swart P, Kok DJ, 2013, Efficacy of emodepside plus toltrazuril oral suspension for dogs (Procox®, Bayer) against *Trichuris vulpis* in naturally infected dogs. Parasitol Res 112, 133-138.

74. Schimmel A, Altreuther G, Schroeder I, Charles S, Cruthers L, Kok DJ, Kraemer F, Krieger KJ, 2009, Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature adult *Trichuris vulpis* infections in dogs. Parasitol Res 105, 17-22.

75. Schmahl G, Mehlhorn H, Harder A, Klimpel S, Krieger K, 2007, Efficacy of a combination of emodepside plus praziquantel against larval and adult stages of nematodes (*Trichuris muris*, *Angiostrongylus cantonensis*) in rodents. Parasitol Res 101, 77-84.

76. Mehlhorn H, Schmahl G, Frese M, Mevissen I, Harder A, Krieger K, 2005, Effects of a combinations of emodepside and praziquantel on parasites of reptiles and rodents. Parasitol Res 97, 65-69.

77. Brown DD, Siddiqui SZ, Kaji MD, Forrester SG, 2012, Pharmacological characterization of the *Haemonchus contortus* GABA-gated chloride channel, *Hco*-UNC-49: modulation by macrocyclic lactone anthelmintics and a receptor for piperazine. Vet Parasitol 185, 201-209.

78. Buxton SK, Charvet CL, Neveu C, Cabaret J, Cortet J, Peineau N, Abongwa M, Courtot E, Robertson AP, Martin RJ, 2014, Investigation of acetylcholine receptor diversity in a nematode parasite leads to characterization of tribendimidine- and derquantel-sensitive nAChRs. PLoS Pathog 10, e1003870.

79. Forrester SG, Prichard RK, Dent JA, Beech RN, 2003, *Haemonchus contortus*: *Hc*GluCla expressed in *Xenopus* oocytes forms a glutamate-gated ion channel that is activated by ibotenate and the antiparasitic drug ivermectin. Mol Biochem Parasitol 129, 115-121.

80. Bianchi L, Driscoll M, 2006, Heterologous expression of *C. elegans* ion channels in *Xenopus* oocytes. WormBook, 1-16.

81. Dufour V, Beech RN, Wever C, Dent JA, Geary TG, 2013, Molecular cloning and characterization of novel glutamate-gated chloride channel subunits from *Schistosoma mansoni*. PLoS Pathog 9, e1003586.

82. Geng Y, Wang X, Magleby KL, 2013, Lack of negative slope in I-V plots for BK channels at positive potentials in the absence of intracellular blockers. J Gen Physiol 141: 493-497.
83. Günzel D, Kucharski LM, Kehres DG, Romero MF, Maguire ME, 2006, The MgtC virulence factor of *Salmonella enterica serovar Typhimurium* activates Na^+, K^+-ATPase. J Bacteriol 188: 5586-5594.
84. Crisford, A, 2011, An investigation into mode of action and selective toxicity of the novel antiparasitic emodepside. PhD thesis, University of Southampton, Southampton, England.
85. Meldal BH, Debenham NJ, De Ley P, De Ley IT, Vanfleteren JR, Vierstraete AR, Bert W, Borgonie G, Moens T, Tyler PA, Austen MC, Blaxter ML, Rogers AD, Lambshead PJ, 2007, An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. Mol Phylogenet Evol 42, 622-636.
86. Piskorowski R, Aldrich RW, 2002, Calcium activation of BK(Ca) potassium channels lacking the calcium bowl and RCK domains. Nature 420, 499-502.
87. Martin RJ, Robertson AP, Buxton SK, Beech RN, Charvet CL, Neveu C, 2012, Levamisole receptors: a second awakening. Trends Parasitol 28, 289-296.

6.7 Supporting information

Supplementary Tab. 6-2. Details for SLO-1 channel protein sequences using for phylogenetic analysis and identification of splice variants

Species	Systematic position	Protein	Amino acid length	Molecular weight (kDa)	pI	Accession no.[a]
Caenorhabditis elegans	Chromadorea Rhabditida Rhabditoidea clade V	*Cel*SLO-1a	1140	129.5	5.75	NP_001024259
Caenorhabditis elegans	Chromadorea Rhabditida Rhabditoidea clade V	*Cel*SLO-1b	1118	127.0	5.67	NP_001024260
Caenorhabditis elegans	Chromadorea Rhabditida Rhabditoidea clade V	*Cel*SLO-1c	1131	128.6	5.58	NP_001024261
Caenorhabditis briggsae	Chromadorea Rhabditida Rhabditoidea clade V	*Cbr*SLO-1	1142	129.7	5.69	XP_2638493
Caenorhabditis remanei	Chromadorea Rhabditida Rhabditoidea clade V	*Cre*SLO-1	1167	132.6	5.77	XP_003094347
Haemonchus contortus	Chromadorea Rhabditida Rhabditoidea clade V	*Hco*SLO-1	1105	125.2	5.77	ABS45068
Cooperia oncophora	Chromadorea Rhabditida Rhabditoidea clade V	*Con*SLO-1	1111	125.9	5.80	ABS45069
Ancylostoma caninum	Chromadorea Rhabditida Rhabditoidea clade V	*Aca*SLO-1	1116	126.7	5.44	ACC68842

Pristionchus pacificus	Chromadorea Rhabditida Rhabditoidea clade V	*Ppa*SLO-1	1188	134.1	5.56	PPA29145[b]
Strongyloides ratti	Chromodorea Rhabditida Panagrolaimoidea Clade IV	*Sra*SLO-1	1170	131.4	5.51	Sr321_0X0013600.t1[c]
Meloidogyne incognita	Chromodorea Tylenchida CladeIV	*Min*SLO-1	1120	126.4	5.39	Minc04076a[d]
Dirofilaria immitis	Chromadorea Spirurida clade III	*Dim*SLO-1a	1119	126.7	5.27	AFH88396
Dirofilaria immitis	Chromadorea Spirurida clade III	*Dim*SLO-1b	1104	125.0	5.44	AFX93730
Onchocerca gutturosa	Chromadorea Spirurida clade III	*Ogu*SLO-1	1119	126.8	5.27	ADY18306
Brugia malayi	Chromadorea Spirurida clade III	*Bma*SLO-1c	1115	126.3	5.19	BM6719c[b]
Brugia malayi	Chromadorea Spirurida clade III	*Bma*SLO-1d	1132	128.2	5.39	BM6719d[b]
Brugia malayi	Chromadorea Spirurida clade III	*Bma*SLO-1e	1079	122.3	5.30	BM6719e[b]
Brugia malayi	Chromadorea Spirurida clade III	*Bma*SLO-1f	1104	125.1	5.40	BM6719f[b] KJ531222
Brugia malayi	Chromadorea Spirurida clade III	*Bma*SLO-1g	1087	123.2	5.19	BM6719g[b]
Brugia malayi	Chromadorea Spirurida clade III	*Bma*SLO-1h	1104	125.0	5.44	BM6719h[b]
Ascaris suum	Chromadorea Ascaridoidea clade III	*Asu*SLO-1	1117	126.4	5.04	ACC68842

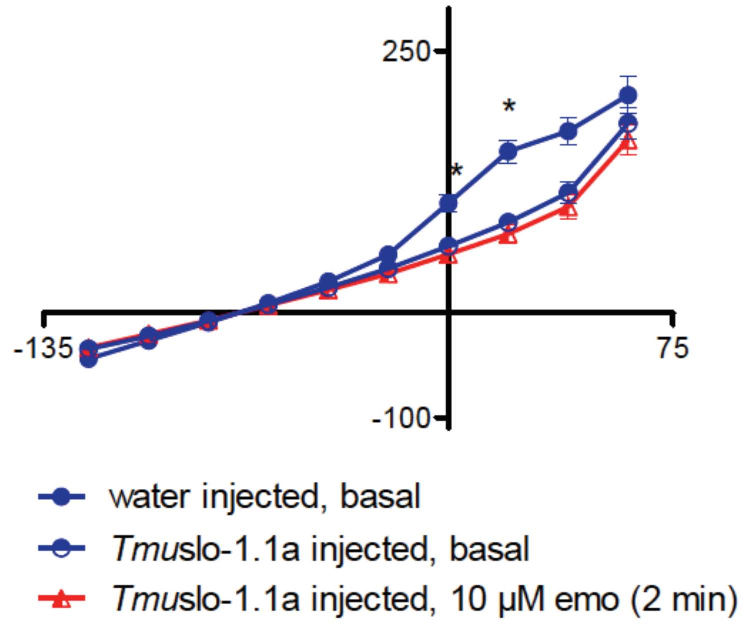
Parascaris equorum	Chromadorea Ascaridoidea clade III	*Peq*SLO-1	1108	125.2	5.51	ACC68843
Toxocara canis	Chromadorea Ascaridoidea clade III	*Tca*SLO-1	1123	126.9	5.38	ACJ64718
Trichuris muris	Enoplea Trichocephalida clade I	*Tmu*SLO-1.1a	1151	130.1	5.70	AEB96250
Trichuris muris	Enoplea Trichocephalida clade I	*Tmu*SLO-1.1b	1151	130.2	5.67	KJ531218
Trichuris muris	Enoplea Trichocephalida clade I	*Tmu*SLO-1.1c	346	41.5	6.21	KJ531219
Trichuris muris	Enoplea Trichocephalida clade I	*Tmu*SLO-1.1d	303	34.7	6.48	KJ531220
Trichuris muris	Enoplea Trichocephalida clade I	*Tmu*SLO-1.2	1123	126.9	5.59	KJ531221
Trichinella spiralis	Enoplea Trichocephalida clade I	*Tsp*SLO-1.1	1134	128.5	5.57	XP_003370273 XP_003370274[e]
Trichinella spiralis	Enoplea Trichocephalida clade I	*Tsp*SLO-1.2	1129	128.0	5.2	XP_003370270 XP_003370271 XP_003370272[e]
Daphnia pulex	Ecdysozoa Crustacea Branchiapoda	*Dpu*SLO-1	1028	115.7	5.60	EFX85873
Pediculus humanus corporis	Ecdysozoa Insecta Anoplura	*Phu*SLO-1	1141	127.9	5.28	XP_002425826
Anopheles gambiae	Ecdysozoa Insecta Diptera	*Aga*SLO-1	1154	129.1	5.19	XP_313505.5
Drosophila melanogaster	Ecdysozoa Insecta Diptera	DmeSLO-1Q	1175	130.3	5.13	NP_001014651

Drosophila melanogaster	Ecdysozoa Arthropoda Diptera	*Dme*SLO-1S	1210	134.4	5.48	NP_001163712
Aplysia californica	Lophotrochozoa Mollusca Gastropoda	*Acal*SLO-1	1070	120.2	6.34	AAR27959
Gallus gallus	Vertebrata Aves	*Gga*SLO-1	1140	128.1	6.60	AAD16633
Bos taurus	Vertebrata Mammalia Ruminantia	*Bta*SLO-1	1166	130.1	6.31	NP_777105
Homo sapiens	Vertebrata Mammalia Primates	*Hsa*SLO-1	1236	135.6	6.64	NP_001154824

[a]If not further specified protein accession numbers from GenBank are given. [b]In WormBase. [c]In Sanger *S. ratti* genome database. [d]In INRA *M. incognita* genome database. [e]A full-length consensus sequence was built from these entries.

Supplementary Tab. 6-3. Primer sequences, which were used to amplify full-length sequences of parasitic nematode slo-1 cDNAs and to analyse alternative splicing of *T. muris* slo-1.1

Target sequence	Primer name	Primer sequence
B. malayi slo-1	Bm slo-1 Full for	5′- GAT AAA TAC ATC GAA GGA TGA GCG -3′
Full-length sequence	Bm slo-1 Full rev	5′- CAG GTA AGC ACA ATA GGT CAT AAG -3′
D. immitis slo-1a/b	Di slo-1 Full for	5′- ATG AGC GAT GTA TAC CAT CCT GGA TCC GGT -3′
Full-length sequence	Di slo-1 Full rev	5′- TAC AGG TAG AGC ATT CTT GAG CTA CAT CAT -3′
O. gutturosa slo-1	Og slo-1 Full for	5′- ATT ACC CAA GTT TGA GGT ATT ATT TTA TTG -3
Full-length sequence	Og slo-1 Full rev	5′- GTA GAG CAT TTT GAG CTA CAT CAT TTT TA -3′
A. suum slo-1	As slo-1 Full for	5′- CAT CGC CAA TCC GCC GAG GAT -3′
Full-length sequence	As slo-1 Full rev	5′- AGC AAT TTT CAT CGG GTC AT -3′
P. equorum slo-1	Pe slo-1 Full for	5′- CGC ACC ACT AGT GCC TAT CA -3′
Full-length sequence	Pe slo-1 Full rev	5′- AGC AAT TTT CGT TGG GTC AT -3′
T. canis slo-1	Tc slo-1 Full for	5′- TCA GAG CTG ACC GTG TCT TG -3′
Full-length sequence	Tc slo-1 Full rev	5′- CGA ATA GGG TAG CGG TTT CTC -3′
T. muris slo-1.1a/b/c/d	Tm slo-1.1 Full for	5′- AAC TTC TTC TTC CCG CGT TGG ACC TTC CCC AGT T -3′
Full-length sequence	Tm slo-1.1 Full rev	5′- ATC GTG GTC ATG TGC TAC TGT TCA ATC TGT TTC GTT-3′
T. muris slo-1.2	Tm slo-1.2 Full for	5′- CCT TAG CCC ATC GTC ACA GTT CAT -3′
Full-length sequence	Tm slo-1.2 Full rev	5′- TCA TCG TTT CGC AGG TTC ATA GGG TAT ACC -3′
T. muris slo-1.1	Tm slo-1.1 IEA for	5′- ACC TGT TTG CTA TGC GCT CG -3′
Splice sequence	Tm slo-1.1 IEA rev	5′- CAT AGG TAG TAA CTG AGC CA -3′



Supplementary Fig. 6-8. Currents obtained from *T. muris* slo-1.1a injected oocytes

Currents obtained from oocytes injected with water or *Tmu*slo-1.1a cRNA in the absence of any drug (basal) or in the presence of 10 µM emodepside (emo) (n=6). The asterisk highlights a significant difference between water injected oocytes and oocytes injected with *Tmu*slo-1.1a either in the absence or presence of emodepside.

Chapter 7

Comprehensive discussion

7 Comprehensive discussion

Parasitic helminths have an enormous, but often underestimated, impact on human and animal health and cause tremendous financial losses in agriculture and livestock production [1].

In humans the most prevalent platyhelminths are the food-borne trematodes *C. sinensis, Fasciola* spp., *Paragonimus* spp. and *O. viverrini* [2] as well as the cestodes *T. saginata*, *T. solium, H. nana* and *D. latum* [3]. The majority of human nematode infections are caused by gastrointestinal species including the giant roundworm *A. lumbricoides*, the two hookworm species *A. duodenale* and *N. americanus,* the whipworm *T. trichiura* and the threadworm *S. stercoralis* [4] as well as by the filariae *O. volvulus, W. bancrofti*, *B. malayi* and *B. timori* [5].

In sharp contrast to the safe and usually highly reliable treatment of infections with platyhelminths, mainly using either praziquantel or triclabendazole [6], the drugs listed on the WHO list of essential medicines for the treatment of nematode infections are insufficiently effective. The three listed drugs for the treatment of filariosis, albendazole, diethylcarbamazine and ivermectin, are reported to be highly effective against microfilariae, whereas poor macrofilaricidial efficacies were observed when administering in up to two doses [7]. In addition, co-endemicity of *O. volvulus* and LF with *L. loa* prohibits treatment with ivermectin due to the risk of encephalopathy caused by migrating microfilariae of *L. loa* [8]. The benzimidazoles, albendazole and mebendazole, as well as the cholinergic drugs, levamisole and pyrantel, targeting the gastrointestinal nematodes, are usually highly effective against *A. lumbricoides* in a single dose regimen, whereas at least multiple dosages are required to cure hookworm, threadworm and particularly whipworm infections [9,10].

In veterinary medicine, infections with helminths of major relevance are generally caused by species belonging to the same taxa as the human pathogens. Thus, in companion animals and livestock the most prevalent cestodes belong to the Cyclophyllidea and the most common nematodes belong to the Strongylida, Ascaridida, Strongyloididae, Onchocercidae and Trichocephalida. Since almost all anthelmintics were initially developed for the lucrative veterinary market [10], one might think that the arsenal of anthelmintics should be sufficient to control helminthosis of animals. But the current status is heterogeneous. In human medicine, the treatment of platyhelminthosis can be considered as adequate [11]. The treatment options against gastrointestinal nematodes in cats and dogs is even better than in human medicine, especially with the introduction of a product line containing the cyclo-octadepsipeptide emodepside, eliminating all gastrointestinal species including hookworms

and whipworms in a single dose regimen. However, treatment options and especially prevention of patent *D. immitis* infections are largely limited to macrocyclic lactones and due to recently increasing reports of resistance [12], the sustainable control of heartworm disease in dogs might be at risk in the near future. In contrast, in grazing animals including cattle, sheep, goat and horses anthelmintic resistance is widespread, not only affecting a large number of nematode species but also all commonly used drug classes [13-16].

Thus, the urgent demand for new anthelmintics is obvious. Besides a small number of others, the cyclooctadepsipeptides [9, 14] and the aminophenylamidines [17] are considered as very prominent drug classes for further evaluation as broad-spectrum anthelmintics and for the admission on the WHO list of essential medicines. Therefore, the present thesis further investigates and compares the potential of the aminophenylamidines amidantel, tribendimidine and dAMD and of the cyclooctadepsipeptides emodepside and particularly PF1022A.

In addition to the discovery of new drug classes, the use of drug combinations is considered as powerful tool for the treatment against nematode infections, especially of livestock [17-19] and humans [20-23]. Drug combinations can be used to (i) increase the anthelmintic spectrum (*e.g.* nematicidal drug plus cestodicidal drug) (ii) improve the efficacy of the respective drugs and/or (iii) decelerate the development of resistance [19]. Thus, besides the evaluation of the single drugs in monotherapy, a range of drug combinations containing aminophenylamidines and cyclooctadepsipeptides were tested for their combinatory effects.

However, before interpreting the range of data sets obtained regarding the efficacy of both drug classes and the combinations containing them and finally fitting these results into current literature, some attention will be drawn to the models, which were used to collect these data. The selection of methods and models for the evaluation of efficacy, especially when targeting such a diverse group such as helminths, is essential. Therefore, I would like to discuss each of the consciously chosen assays at first.

7.1 Evaluation of cestodicidal *in vivo* efficacy of aminophenylamidines

Activity of the aminophenylamidines amidantel and tribendimidine against cestodes has already been published. However, these reports were somewhat informal. For amidantel, efficacy against certain cestodes were claimed by listing the ED_{99} values without any worm counts or procedures [24], whereas for tribendimidine only a review mentioning efficacy against various chicken cestodes [25] and a clinical field trial reporting some effects on

Taenia spp. [26] are available. In addition to the isolated reports of cestodicidal effects, efficacies of aminophenylamidines against trematodes and nematodes have frequently been observed (for review [25,27]). A monotherapy against all three taxa of helminths, especially in areas where they are all co-endemic, would have significant advantages in comparison to individual treatment of only one taxon or a drug combination targeting all three taxa, including (i) the decreasing risk of side effects or toxicity due to the absence of any drug interactions, (ii) the reduction of selection for resistance in cases where both drugs share their mode of action and (iii) reduction of costs and management efforts in terms of MDAs, particular in poor communities lacking efficient public health infrastructure [10].

Therefore, the first publication (chapter 2) aimed on the first evaluation of the efficacy of the aminophenylamidines tribendimidine and dAMD against *H. microstoma* in a controlled laboratory trial. *H. microstoma* in mice is a thoroughly characterised and well-established *in vivo* model, being largely used to investigate several aspects of helminthology including the evaluation of anti-cestodal effects of chemotherapeutics [28]. Furthermore, *H. microstoma* belongs to the Cyclophyllidea, the order of tapeworms containing the majority of relevant species in human and veterinary medicine. The effects of the aminophenylamidines will be discussed in section 7.8, whereas the effects of the administered combinations will be analysed in section 7.10.

7.2 Evaluation of nematicidal *in vitro* efficacy

The current gold standard for the assessment of the efficacy of new chemical entities against helminths is the utilisation of *in vitro* assays based on the visual evaluation of worm motility using a microscope [29]. For some drugs tested here, different *in vitro* data already exist in the literature (for review see [25,27] for aminophenylamidines and [30,31] for cyclooctadepsi-peptides). However, Smout *et al.* [29] pointed out, that the visual assessment of worm motility is subjective and difficult to standardise. To allow comparisons between the efficacies of respective drugs, the second publication (chapter 3) reports on the efficacy evaluation of the drugs using two motility assays, targeting the rat hookworm *N. brasiliensis* L3 and *T. spiralis* L1, respectively. The assays were used to obtain dose-response curves for amidantel, tribendimidine, dAMD, levamisole, PF1022A and emodepside as well as for the assessment of additive or synergistic effects using different combinations. Admittedly, these data sets were collected in a subjective manner, but since the same person read out all assays and replicates, the interpretation of data was performed uniformly. The species were chosen

by consideration of the diversity of the phylum Nematoda. Thus, the clade I nematode *T. spiralis* and clade V rat hookworm *N. brasiliensis* were selected. *T. spiralis* belongs to the Trichocephalida and *N. brasiliensis* belongs to the Strongylida. Besides their phylogenetic distance, both species also represent taxa of major importance for human and veterinary parasitology. In addition, a fully objective *in vitro* assay was performed, which was evaluated by using colourimetric determination of AChE activity [32], which is secreted by adult *N. brasiliensis*. Dose-response curves of all test compounds and drug combinations were calculated in three *in vitro* assays, targeting L1 of *T. spiralis* as well as L3 and adult worms of *N. brasiliensis*. While all compounds and combinations showed dose-dependent inhibition of worm activity, the maximal inhibition in the AChE secretion assay was much lower than in the motility assays. Again, effects of the aminophenylamidines will be discussed in section 7.8, the effects of the cyclooctadepsipeptides in section 7.9 and the effects of the combinations in section 7.10.

7.3 Evaluation of nematicidal *in vivo* efficacy against *N. brasiliensis*

To reduce the variables from *in vitro* to *in vivo* efficacy evaluation as much as possible, the third publication (chapter 4) reports on the *in vivo* efficacy of the test compounds and combinations against *N. brasiliensis* in rats, which is the same nematode species chosen for two of the *in vitro* assays.

N. brasiliensis shows the typical life cycle of hookworms. L3 penetrate the skin and migrate via the blood system into the lungs, where the moult to L4 takes place. After approximately two days *p.i.,* L4 leave the lungs via the trachea. After being swallowed, the L4 finally reach the small intestine where the final moult occurs between days four and five *p.i.* [33]. Treatments were performed on days four to six *p.i.* to ensure gastrointestinal phase of *N. brasiliensis*. Treatment against migrating larval stages would require systemic availability of drugs. However, this would significantly affect the comparison between treatments using either tribendimidine or dAMD, since in plasma only dAMD is present following tribendimidine treatments (discussed in detail in section 7.5).

A major advantage of the *N. brasiliensis* rat model is that sensitivity against all available nematicidal drugs was confirmed. Therefore, *N. brasiliensis* is referred to as “good drug responder” and consequentially the *in vivo* model is well accepted and widely used to determine anthelmintic efficacy against gastrointestinal nematodes [34].

In the context of efficacy evaluation, the reproducibility of inoculum size and worm recovery was investigated. A high variability between individual animals within each block was shown; however, differences between blocks were not statistically significant. This underlines the requirement of using relatively high numbers of animals when testing anthelmintic drugs to obtain meaningful data. In addition, high variability was also observed for intermediate efficacy rates in the drug treated groups, highlighting the fact that the number of animals essentially contributes to the goodness of fit (dose-response curve) and the related determination of the ED_{50} values. If only intermediate efficacy rates are available, interpretation of data will remain vague, which will increase the risk of misinterpretation. Consequentially, at least some dosages, resulting in full and no anthelmintic efficacy against *N. brasiliensis* were also administered.
Again, effects of the aminophenylamidines will be discussed in section 7.8, the effects of the cyclooctadepsipeptides in section 7.9 and the effects of the combinations in section 7.10.

7.4 Evaluation of nematicidal *in vivo* efficacy against *T. muris*

As pointed out earlier, *Trichuris* is the dose-limiting gastrointestinal nematode genus in dogs, swine and humans for all relevant anthelmintic drug classes. To cure whipworm infections of humans or swine at least a multiple dose regimens or administration of drug combinations are required [9,10]. Additionally, within the increased utilisation of MDAs, persistent underdosing of *T. trichiura* may favour selection of resistant genotypes [10] as already described [35]. Furthermore, due to the long prepatent period of *Trichuris* spp. and the lack of efficacy of most drugs against histotropic larvae, multiple treatment blocks each with multiple doses are usually necessary [36].
For the investigation and comparison of the anthelmintic efficacy of test compounds against whipworms, the *T. muris* mouse model is highly suitable [37]. Thus, for publication 4 (chapter 5), a *T. muris* life cycle was successfully established and maintained in mice. However, it has been shown that the course of infection strongly depends on the respective mouse strain [38] and *T. muris* isolate [39]. In addition, the evaluation of drug effects against histotropic stages of *T. muris* is highly desirable, requiring an even more detailed knowledge of the time course of development within the host. Since data in the literature differ in many aspects, especially regarding the number and the time course of moults, the established isolate was subjected to an in-depth parasitological analysis, which is also described in publication 4.

The observations on larval development within the host mostly agreed with findings of Panesar and Croll [40]. First larvae extruding their caudal ends into the lumen of the gut were found on day 21 *p.i.* here and on day 20 *p.i.* by Panesar and Croll [40]. However, Panesar and Croll found no histotropic larvae after day 21 *p.i.*, whereas in our study small but significant numbers of histotropic larvae were found until day 29 *p.i.* Interestingly, findings on the female/male ratio, steadily developing towards more males and male *T. muris* surviving longer than females [41] were also observed in our study, which is in marked contrast to other parasitic nematode species, where females survive longer than males [41].

Following the parasitological characterisation, publication 4 (chapter 5) also investigated the *in vivo* potency of the aminophenylamidines amidantel, tribendimidine and dAMD (discussed in section 7.8) as well as of the cyclooctadepsipeptides emodepside and in particular of PF1022A (discussed in section 7.9) against patent *T. muris* infections using three consecutive dosages by different routes of administration. Due to the surprisingly high efficacy of PF1022A following oral administration, this drug was further evaluated in single drug administrations targeting patent *T. muris* infections as well as in single and multiple oral treatment schemes targeting developmental stages including histotropic larvae (also discussed in section 7.9).

7.5 Investigations on the *in vivo* pharmacokinetics of aminophenylamidines in rats

Profound knowledge on the pharmacology of anthelmintics is essential for an effective treatment, especially when efficacy against extra intestinal species is desired [42]. However, literature on the pharmacokinetics of the aminophenylamidine tribendimidine is highly controversial.

Yuan *et al.* [43] reported that tribendimidine is rapidly metabolised into two molecules of dAMD and one molecule of TPAL. The latter one was also found to be immediately metabolised to TPAC. Therefore, in plasma samples, only dAMD and TPAC are detectable using high performance liquid chromatography followed by mass spectrometry [43]. Additionally, it was reported that tribendimidine, in the absence of any enzymatic processes, spontaneously and rapidly disintegrates into the above-mentioned molecules in an aqueous environment [44]. On the contrary, Xiao *et al.* [25] summarised unpublished data with peak plasma concentrations of 318 ng/ml tribendimidine and an AUC of 1674 ng×h/ml after oral dosage of 150 mg/kg tribendimidine to rats. Furthermore, they reported that small amounts of tribendimidine have also been detected in human faeces after oral intake of a tablet containing

400 mg tribendimidine [25]. Due to the striking differences in published observations, chapter 4 (publication 3) also reports on *in vivo* plasma pharmacokinetic analysis after oral treatments with tribendimidine and dAMD in rats. The observations were in line with the findings of Yuan *et al.* [43] and Xue *et al.* [44]. Tribendimidine was neither detected in the plasma after oral treatment with tribendimidine, nor in plasma samples spiked with tribendimidine. The massive differences to the reported observations by Xiao *et al.* [25] remain unknown, especially due to the fact that they did not report any experimental details of their procedure.

7.6 Investigations on the mode of action of anthelmintics by electron microscopy

It is generally accepted, that cyclooctadepsipeptides boost the inhibitory system of nematodes, which results in hyperpolarisation of cells and finally induces flaccid paralysis of the worms [31]. For aminophenylamidines activation of the excitatory system has been demonstrated, particularly by activation of distinct subsets of AChRs, depending on the nematode species [45]. Boosting the excitatory system results in depolarisation of cells, finally inducing spastic paralysis [46]. Since it has been demonstrated that the (most-likely) targets of both drug classes are expressed in somatic muscle cells along the whole body of *C. elegans* and assuming that this is also the case in *N. brasiliensis*, strong morphological changes would be expected following treatment with AChR agonists. Thus publication 3 (chapter 4) also reports investigations on the mode of action of cyclooctadepsipeptides and aminophenylamidines against *N. brasiliensis* using scanning electron microscopy.

Interestingly, no morphology changes of *N. brasiliensis* following treatment with PF1022A in comparison to untreated worms where observed, except for several small cuticular knobs next to the longitudinal ridges. However, this was not unexpected, since flaccid paralysis only results in muscle relaxation. In sharp contrast, treatments with either aminophenylamidines or levamisole resulted in significant effects on the total body shape, more precisely in strongly contracted *N. brasiliensis*. Since the aminophenylamidines share their mode of action with levamisole, at least in *C. elegans* [47], the observed homogenous phenotypes were plausible. Differences in the intensity of contractions might be due to different intrinsic drug concentrations or activation of different subsets of AChRs. This might also be the explanation for extrusion of the two twisted spicules only following tribendimidine treatment. Since combinations had no further effects on the morphology of *N. brasiliensis* in comparison to the effects of the single drugs alone, interpretation of data obtained via scanning electron microscopy will not be included in the discussion of drug combinations in section 7.10.

7.7 Investigations on the mode of action of cyclooctadepsipeptides by heterologous expression of potential target genes in *X. laevis* oocytes

The mode of action of the cyclooctadepsipeptides is associated with three potential targets, including $GABA_A$ receptors [48,49], the latrophilin like G protein coupled receptor LAT-1 [50,51] and the voltage-gated calcium activated potassium channel SLO-1 [52-54]. During recent years, SLO-1 was generally considered to be the most important of the three candidates (for review see [31]), however, direct activation of the SLO-1 channel by cyclooctadepsipeptides has not been demonstrated. Therefore, the fifth publication (chapter 6) reports on the identification and characterisation of SLO-1 channels in nematodes.

To investigate SLO-1 and its potential activation by cyclooctadepsipeptides, slo-1 cRNAs were heterologously expressed in *X. laevis* oocytes. The *X. laevis* oocyte model is frequently used for the pharmacological characterisation of ion channels of helminths [45,55-60]. The system is robust and allows the simultaneous expression of several genes at the same time, as shown by the expression of heteromeric AChRs, which also require three auxiliary proteins with chaperone function for successful expression [45]. Additionally, oocytes are 1 mm in diameter and can be conveniently injected with cRNA and easily perfused with media or drugs of choice. However, the size of the oocyte also caused a major disadvantage by investigating the physiological properties of SLO-1. SLO-1 channels have been shown to require depolarisation of the membrane to at least +0 mV in combination with high, transient increase of intracellular calcium to open [61]. Since no changes in currents in SLO-1 injected oocytes in comparison to water injected oocytes were observed, the required calcium concentrations were obviously not achieved, presumably due to the comparatively enormous volume of the oocytes. Even preincubation of the oocytes with a calcium ionophore in calcium-free medium, followed by the perfusion with high calcium concentrations did not result in any increased currents. However, emodepside unequivocally induced a significant increased current in SLO-1 injected oocytes, demonstrating direct activation of the SLO-1 channel by cyclooctadepsipeptides, which will be further discussed in section 7.9.

7.8 Evaluation of aminophenylamidines as broad-spectrum anthelmintics

The *in vitro* and *in vivo* efficacies of the aminophenylamidines amidantel, dAMD and tribendimidine against the different species and life cycle stages tested were very diverse with amidantel being constantly the derivative with the lowest potency, whereas the route of administration strongly influenced the order of potency of dAMD and tribendimidine.
Investigations on the cestodicidal effects of the aminophenylamidines resulted in a very surprising finding. Treatments with dosages of up to 100 mg/kg dAMD on three consecutive days against *H. microstoma* did not result in any significant reduction of the worm burdens. In marked contrast, three treatments with 50 mg/kg and 100 mg/kg tribendimidine resulted in almost complete and complete parasite clearance, respectively.
Comparing the efficacy of the aminophenylamidines in the *in vitro* assays against nematodes, amidantel showed only minimal or at best partial reduction of nematode activity even at high concentrations resulting in EC_{50} values of 66.9 ppm and 141.5 ppm against *N. brasiliensis* adults and L3, respectively. The efficacy against L1 of *T. spiralis* was even so low, that a calculation of an EC_{50} value was not possible. However, these results are not surprising, since amidantel is considered to act as a prodrug, being rapidly metabolised to the anthelmintically active dAMD only *in vivo* [62]. Partial anthelmintic *in vitro* efficacy of amidantel might be attributed to (i) spontaneous decomposition to dAMD in an aqueous environment as known for tribendimidine [43], (ii) metabolisation by the parasite to dAMD and/or (iii) minimal anthelmintic activity of amidantel itself. In clear contrast, dAMD as well as tribendimidine showed concentration-dependent efficacy in all three *in vitro* assays, resulting in EC_{50} values of 1.1 ppm and 3.7 ppm against adult *N. brasiliensis*, of 0.06 ppm and 0.2 ppm against *N. brasiliensis* L3 and of 0.8 ppm and 1.2 ppm against *T. spiralis* L1, respectively. The constantly lower EC_{50} values for dAMD in comparison to the EC_{50} values for tribendimidine can be explained by the fact, that tribendimidine rapidly disintegrates into two molecules dAMD and one molecule TPAL in aqueous environments [43]. Since it was shown, that TPAL itself has no anthelmintic efficacy against *N. brasiliensis* [44], the absolute number of anthelmintically active molecules is approximately 25% lower in 100 ppm tribendimidine than in 100 ppm dAMD. However, the very obvious explanation for the differences in the *in vitro* assays, complicate the interpretation of the differences observed in the *H. microstoma in vivo* assay. As already pointed out, tribendimidine rapidly disintegrates into dAMD and TPAL. The latter one is also very unstable *in vivo* and is metabolised to TPAC. Since neither TPAL nor TPAC showed any anthelmintic efficacy against nematodes and trematodes [44,63]

and dAMD itself was completely ineffective against *H. microstoma* using 3× 100 mg/kg, interpretation of these findings remains vague. However, either TPAL or TPAC exert cestodicidal effects or, more likely, tribendimidine not completely disintegrates into dAMD and TPAL. Tribendimidine, which is a highly hydrophobic compound, might remain at least partially stable in the used formulation (dispersion containing Cremophor EL/deionised water), meaning that tribendimidine can be suspected to diffuse only slowly into the aqueous environment. This again corresponds to observations, where small amounts of tribendimidine have been detected in human faeces after oral intake of a tablet containing 400 mg tribendimidine [25]. However, this would implicate that tribendimidine is not only a prodrug, but exerts anthelmintic, at least cestodicidal, efficacy itself. Therefore, *in vitro* experiments using dAMD against *H. microstoma* could unequivocally show the presence or absence of its cestodicidal effects.

The data obtained from the *in vivo* study targeting *N. brasiliensis* are in line with the previous interpretation. Only high dosages of 200 mg/kg amidantel in three consecutive treatments resulted in an almost complete elimination of the worm burden. Thus, oral treatments with amidantel were effective indicating that metabolic activity at least partially released the anthelmintically active dAMD. Using the same treatment scheme, three treatments using 250 mg/kg amidantel resulted in complete elimination of the worm burden in a previous publication [24], showing that rather high amidantel dosages are required to cure infections with the rat hookworm. In a previous study, *in vivo* efficacies of dAMD and tribendimidine against *N. brasiliensis* using a single-treatment regimen were evaluated, resulting in ED_{90} values of 5.104 mg/kg and 8.435 mg/kg respectively [44]. Interestingly, the potencies of dAMD and tribendimidine against *N. brasiliensis* were much lower and reversed in the present study. Three consecutive treatments with 10 mg/kg dAMD or 5 mg/kg tribendimidine were required to achieve almost complete elimination of the worm burden. These findings agree with results published by Xiao *et al*. [25]. Again, oral administration of tribendimidine showed higher *in vivo* efficacy as oral administration of dAMD. The formulation of Cremophor EL/deionised water might also cause a slower release of the larger tribendimidine molecules in comparison to the smaller dAMD molecules, potentially preventing further metabolisation of dAMD to anthelmintically inactive metabolites, before reaching the parasite in the distal third of the small intestine.

The results of the *in vivo* studies on *T. muris* further support the interpretation of the pervious data. For amidantel, no efficacy was detected against patent *T. muris* infections in mice even by oral administration of 500 mg/kg on three consecutive days, which roughly corresponds to

a publication, where amidantel was also found to be only moderately active against *T. muris* [24]. Therefore, amidantel was not further evaluated using different routes of administration. In sharp contrast, complete eliminations of the worm burden, was observed when treating patent *T. muris* infections with three consecutive oral doses of either 25 mg/kg tribendimidine or 100 mg/kg dAMD. In contrast to our data, oral treatments using 1× 400 mg tribendimidine [64] or 3× 400 mg tribendimidine [25] resulted in cure rates of only 76.8% and 33.3% against *T. trichiura* in humans, respectively. Interestingly, intraperitoneal treatments with 3× 50 mg/kg of either tribendimidine or dAMD also resulted in complete elimination of the worm burden, which is a twofold lower dosage for dAMD but a twofold higher dosage for tribendimidine in comparison to the oral treatments. Subcutaneous administrations on three consecutive days using 500 mg/kg of either tribendimidine or dAMD did not result in any significant reduction of the parasites. However, this observation might depend on the very basic formulation used in the study. The difference between required dosages of dAMD versus tribendimidine after oral administration to achieve full efficacy is further increased against *T. muris* in comparison to *N. brasiliensis*. But since *T. muris* inhabits the colon and caecum, which is distal from the location of *N. brasiliensis,* the magnification of those dose differences is in agreement with the hypothesis that tribendimidine is slower released from the formulation in comparison to dAMD, preventing further metabolisation to anthelmintically inactive metabolites, before reaching the parasite. Finally, the equal efficacy of tribendimidine and dAMD after intraperitoneal treatment might be attributed to the more rapid release of dAMD in the digestive track under mechanical mixing in the presence of bile salts than in the peritoneum and that passive diffusion is of minor importance in the gut than in the peritoneum.

Studies using loss-of-function mutants of *C. elegans* suggest that the aminophenylamidines share their mode of action with levamisole and pyrantel acting as agonists of the L-subtype of AChRs [47]. These findings were in line with the observed morphological changes of *N. brasiliensis* treated with dAMD, tribendimidine or levamisole. All worms showed very similar effects in terms of contractions and swellings, which are most likely attributed to the activation of AChRs, resulting in spastic paralysis. In fact, if aminophenylamidines share their mode of action with levamisole, the value of these drugs for the potential use in humans and livestock is very low, since they would not have resistance-breaking properties. However, several recently published data show substantial differences regarding the compositions of AChRs and their sensitivity to anthelmintics when comparing different nematode species. In the body muscle of *O. dentatum* four subtypes of AChRs with distinct pharmacological

properties have been identified using electrophysiology [45]. The loss of subtype G 35 pS was shown to be associated with levamisole resistance [45]. By heterologous expression of different combinations of AChR subunits in *X. laevis* oocytes, the four pharmacologically different types of AChRs were reconstituted, which all showed effects in the presence of tribendimidine but not in the presence of levamisole indicating that tribendimidine may still be effective in levamisole resistant worms [45].

To summarise the further potential of the aminophenylamidines as broad-spectrum anthelmintics in veterinary medicine and for the admission on the WHO list of essential medicines following oral administration constantly lower dosages of tribendimidine were required in comparison to dAMD to achieve a complete elimination of the worm burden. In the tested rodent models similar dose ranges of tribendimidine were almost fully effective against *H. microstoma* (3× 50 mg/kg) as well as against *T. muris*. (3× 25 mg/kg). The dosages required to cure *N. brasiliensis* infections were significantly lower (3× 5 mg/kg), indicating that tribendimidine might indeed have a high potential as broad-spectrum anthelmintic. Importantly, only a very basic formulation was used for the evaluation, therefore, a much higher potency can be expected after optimisation of the formulation. In addition, if the resistance-breaking properties of aminophenylamidines, that have been demonstrated on *O. dentatum* distinct AChRs subtypes [45], can also be confirmed in other nematode species, aminophenylamidines might be of major interest for further evaluation as broad-spectrum anthelmintics. However, since even in multiple treatment regimens relatively high dosages were required to cure infections, a single dose regimen with tribendimidine appears to be unrealistic. Multiple dose regimens are in line with Steinmann *et al.* [26], suggesting the evaluation of the efficacy of multiple-dose treatments with tribendimidine to cure human intestinal taeniosis and infections with *S. stercoralis* although it must be taken into account that this would of course significantly increase costs and management efforts in the context of MDAs. More importantly for suitability in MDAs as well as for the treatment of livestock, drugs need reasonable safety profiles. Since the class of aminophenylamidines is still considered to be potentially hazardous [65], a detailed toxicological analysis needs to be performed and to be disclosed before any further efficacy studies in humans are conducted.

7.9 Evaluation of cyclooctadepsipeptides as extended broad-spectrum anthelmintics

The *in vitro* and *in vivo* efficacies of the cyclooctadepsipeptides emodepside and PF1022A against the different tested nematode species and life cycle stages were highly homogenous, with emodepside constantly showing higher efficacy in comparison to PF1022A. However, depending on the route of administration, the differences between the two were variably pronounced.

Emodepside and PF1022A showed very high concentration-dependent anthelmintic potency in the three *in vitro* assays. The EC_{50} values were determined at 0.06 ppm and 0.07 ppm against adult *N. brasiliensis*, at 0.06 ppm and 0.15 ppm against *N. brasiliensis* L3 and at 0.03 ppm and 0.06 ppm against *T. spiralis* L1 for emodepside and PF1022A, respectively. The high *in vitro* potency of both compounds was also previously observed in an *in vitro* motility assay targeting adult *H. contortus* [66]. Despite significantly higher efficacy of emodepside in both motility assays and observations, where a 40-fold higher single oral dosage of PF1022A (100 mg/kg) in comparison to emodepside (2.5 mg/kg) was required to cure *N. brasiliensis* infections in rats [67,68], it was decided to further evaluate the *in vivo* efficacy of PF1022A against *N. brasiliensis* in rats in more detail. Three treatments with PF1022A showed a high efficacy against *N. brasiliensis*, illustrated by an ED_{50} value of 1.2 mg/kg and by complete elimination of worm burdens at 2.5 mg/kg. These results agree with a previous study, where 3× 2.5 mg/kg PF1022A resulted in an almost complete elimination of *N. brasiliensis* [69]. The striking difference to the study where 100 mg/kg PF1022A were required [66] remains unknown. However, Conder *et al.* [66] tested only a single dose of PF1022A instead of three consecutive dosages and potentially a mixture of PF1022 isomers was used, which results in a significant reduction of the anthelmintic efficacy. Thus, the efficacy of PF1022A against *N. brasiliensis* appears to be in the same dose range as emodepside (1× 2.5 mg/kg) [68]. Not testing emodepside against *N. brasiliensis* in the same treatment regimen can be seen as a weak point of the study, but due to the principle of the three Rs – reduction, refinement an replacement of animal testings – it was decided to waive further testing of emodepside against *N. brasiliensis* and perform investigations on the morphology of worms after treatment with PF1022A by scanning electron microscopy instead. However, the morphology of *N. brasiliensis* after PF1022A treatments was largely unchanged, except for several small cuticular knobs next to the longitudinal ridges (cause remains unknown). Since PF1022A is known to cause flaccid paralysis, it was not unexpected to find only minimal changes following PF1022A treatment.

The outcome of the comparison of the *in vivo* efficacy of emodepside and PF1022A against patent *T. muris* infections was somewhat unexpected. Three dosages of 10 mg/kg, 25 mg/kg and 50 mg/kg emodepside or 50 mg/kg, 200 mg/kg and 400 mg/kg PF1022A using an oral, intraperitoneal and subcutaneous route of administration were required to achieve eliminations of *T. muris* (>99%). Whereas the differences between emodepside and PF1022A using intraperitoneal or subcutaneous administrations were highly significant, no significant difference between the two drugs was observed when comparing the efficacy following oral treatments. This is demonstrated by the ED_{90} values 24.5 mg/kg and 36.5 mg/kg for emodepside and PF1022A, respectively.

For suitability in MDA campaigns and for the treatment against helminthosis of livestock, drugs need to have low production costs. It has been reported, that production costs for PF1022A are much lower in comparison to emodepside, since no synthetic derivatisation is required [70]. Furthermore, oral formulation is the gold standard for all anthelmintics used against human helminthosis [71]. With respect to the lower costs and the relatively small differences in oral efficacy, PF1022A and not emodepside was further evaluated against *T. muris*.

In the triple dose regimen, PF1022A was able to completely eliminate all developmental stages of *T. muris*. However, the required dosages inversely correlated with the time span after infection. Thus, histotropic L1, histotropic L2, further developed larvae and adults had to be treated with 100 mg/kg, 100 mg/kg, 50 mg/kg and 50 mg/kg PF1022A to completely cure the infections, respectively. In single administrations of PF1022A between 100 mg/kg and 500 mg/kg were required to achieve complete elimination. However, L1 were not even significantly reduced following treatment with 500 mg/kg PF1022A. This can be explained by the localisation of the larvae. After invasion of the intestinal epithelium, larvae are found in the basal parts of the crypts of Lieberkühn before migrating closer to the surface of the epithelium, followed by the extruding their caudal ends into the lumen of the caecum, where most likely higher drug concentrations are present [40].

One might think that these high dosages of PF1022A could prevent its further development as trichuricidal drug. However, further significant improvement of the efficacy by optimisation of the formulation can most likely be achieved. The strong influence of the formulation is shown by the efficacy of emodepside against *T. vulpis* using the Profender® tablet. A single dose of 1 mg/kg emodepside resulted in almost complete elimination of immature and mature stages of *T. vulpis* in dogs (>99%) [72]. Similar observations have been made by Mehlhorn *et al.* [73], demonstrating that a single dose of 7.16 mg/kg emodepside (Profender® spot on

formulation for cats) was sufficient to clear *T. muris* infections of mice. In sharp contrast, 3× 75 mg/kg emodepside using a Cremophor EL/water formulation were required. The more than 10-fold increase comparing three doses of a Cremophor/water formulation and a single dose of the optimised Profender® formulation emphasises that dramatic decreases in required drug dosages are possible when using an optimised formulation.

Besides the efficacy evaluation, the mode of action of the cyclooctadepsipeptides was further investigated. As pointed out in chapter 7.7, $GABA_A$ receptors [48,49], the latrophilin like LAT-1 receptor [50,51] as well as the SLO-1 receptor [52-54] were found to be involved in the mode of action of the cyclooctadepsipeptides. Since *slo-1* deficient *C. elegans* are fully resistant against emodepside, SLO-1 is generally accepted to be the most crucial element. However, direct activation of the SLO-1 channel by any cyclooctadepsipeptide has not been reported.

Whereas SLO-1 was already identified in clade V nematodes, orthologs in three filariae (clade III), three ascarids (clade III) and *T. muris* (clade I) were described for the first time. In *A. suum*, *P. equorum*, *T. canis*, *D. immitis*, *B. malayi* and *O. gutturosa* a single *slo-1* gene was identified and corresponding cDNAs were cloned and sequenced. Surprisingly, in *T. muris* two paralogs were identified. BLAST search revealed that this gene duplication is also present in the trichocephalids *T. suis* and *T. spiralis*. In addition to the identification of paralogs, the heterogeneity of SLO-1 is further increased by the observation of alternative splice forms in *D. immitis* and *T. muris*. In *T. muris* even two variants were found, encoding for truncated forms, which might have an important influence on emodepside susceptibility or even on the development of resistance as shown for truncations in genes encoding for AChR subunits, associated with cholinergic drug resistance [74]. All SLO-1 sequences were aligned with orthologs from other nematodes as well as other phyla, serving as outgroups, identified by BLAST search of the several genome projects found on GenBank and WormBase, to analyse the phylogenetic relationship of SLO-1 in the diverse phylum Nematoda. In general, the phylogenetic tree of SLO-1 correlates with the clade system supposed by Blaxter *et al.* [75]. Additionally, the analysis revealed that trichocephalid SLO-1 channels are clustered in a monophyletic group, showing that duplication of SLO-1 occurred after divergence of Enoplea and Chromadorea. *C. elegans* and *T. muris* SLO-1 were expressed in *X. laevis* oocytes and analysed using voltage clamp experiments. Since SLO-1 requires depolarisation of the membrane and high, transient increase of intracellular calcium ions to open [61], no control was available to demonstrate functional expression of SLO-1 in the membrane under physiological conditions. Whereas voltage steps achieved depolarisation of the membrane,

calcium concentrations could not be increased in a sufficient manner to observe currents via SLO-1. Not even preincubation of the oocytes with the calcium ionophore A23187 in calcium-free medium, followed by the perfusion with high calcium concentrations did result in any increased currents. However, incubation of *C. elegans*, but not *T. muris,* SLO-1 injected oocytes showed significantly increased currents in the presence of 1-10 µM emodepside over a wide range of holding potentials. Since these observations were in the absence of high calcium, emodepside presumably opens SLO-1 in a calcium independent manner. Furthermore, significantly increased currents were also observed at highly negative potentials indicating that emodepside opens SLO-1 also voltage-independently. Investigations on the reversibility of the emodepside-induced effects showed that even after a washout of up to 25 min currents remained unchanged. This indicates that emodepside causes virtually irreversible effects, most likely since emodepside remains in the membrane due to its hydrophobic structure and is therefore not washable. However, preincubation with the SLO-1 blocker verruculogen prevented emodepside induced currents, whereas incubation with verruculogen following activation by emodepside had no influence. This suggests that both drugs bind very tightly to SLO-1 and cannot replace each other once the other one has bound.

Here it was shown for the first time that emodepside directly opens SLO-1, which significantly improves the understanding of the mode of action of the cyclooctadepsipeptides. However, several questions remain open. The study identified several splice variants and even paralogs in parasitic nematodes suggesting that there are substantial differences in channel properties between nematode species. In order to investigate the differences and similarities of the different orthologs, including sensitivity to emodepside, they have to be heterologously expressed and characterised using electrophysiological methods. Furthermore, it has never been shown that different splice forms are able to form heteromeric SLO-1 channels and even more importantly, the influence of the observed truncated form of *T. muris* should be investigated in detail, to address the following questions (i) do the truncated forms build homomeric channels, (ii) if the truncated forms build homomeric channels, are these channels sensitive to emodepside, (iii) do the truncated forms participate in heteromeric channels (iv) if the truncated forms participate in heteromeric channels, are these channels sensitive to emodepside and finally (v) do the truncated forms have dominant negative effects? However, as long as channels can be expressed and opened by emodepside, the *X. laevis* oocyte model is suitable, but since the intracellular calcium concentration cannot be controlled appropriately no physiological analysis of channel function is possible. Therefore, expression of SLO-1 in smaller cells, such as HEK293 cells, would improve the situation significantly.

7.10 Evaluation of drug combinations as broad-spectrum anthelmintics

Two types of drug combinations were tested within the studies: First, a combination of two cholinergic drugs (tribendimidine and levamisole) was evaluated against *H. microstoma in vivo*, *T. spiralis* L1 *in vitro*, *N. brasiliensis* L3 and adults *in vitro* and *N. brasiliensis in vivo*. Secondly, a cholinergic drug (dAMD or tribendimidine) were used in combination with an agonist of SLO-1 (PF1022A) in the same assays except of the *H. microstoma in vivo* assay.
Since several reports showed that levamisole exerts no anthelmintic effects against trematodes or cestodes [76-79]. Therefore, the absence of efficacy against *H. microstoma* using three consecutive administrations of 100 mg/kg levamisole was expected. However, two findings caused the decision to combine levamisole with tribendimidine against *H. microstoma*. First, synergism between levamisole and mebendazole against *M. corti* has been reported [79] and second, tribendimidine and levamisole share their mode of action against *C. elegans* [47]. However, treatments with combinations of tribendimidine and levamisole did not result in any synergistic, additive or antagonistic effects. The differences in efficacy between levamisole and tribendimidine can be explained by (i) the presence of AChRs that are sensitive to tribendimidine but not to levamisole, (ii) poor uptake of levamisole by cestodes or (iii) a completely different mode of action of tribendimidine in cestodes than in nematodes. However, since pyrantel is known to be active against the equine cestodes *Anoplocephala* spp. [80,81], combinations of tribendimidine with pyrantel should be evaluated in future experiments. The diverse effects of cholinergic drugs are again in agreement with recent findings by Buxton *et al.* [45], showing that tribendimidine and pyrantel are agonists of distinct AChR subtypes of *O. dentatum*, which are insensitive to levamisole.
In the *in vitro* assays against nematodes, levamisole constantly showed dose-dependent effects, resulting in EC_{50} values of 2.2 ppm, 0.2 ppm and 2.1 ppm against L1 of *T. spiralis* as well as against adults and L3 of *N. brasiliensis*. However, combinations of levamisole and tribendimidine did not result in any additive or synergistic effects. In contrast, in both assays targeting *N. brasiliensis* significantly higher EC_{50} values of the combinations in comparison to the EC_{50} values of one of the single drugs were observed indicating antagonistic effects. Interestingly, combining the same drugs in an *in vivo* assay targeting the same species as well as the same life cycle stage, higher efficacies in comparison to the individual drugs were observed. However, since a Wilcoxon rank sum test against the expected values of the sum of

the single drug medians was not significantly different from the efficacy of the combinations, additive, but no synergistic, effects can be presumed.

It has already been shown, that combinations of levamisole plus tribendimidine result in very diverse effects. Synergistic effects of tribendimidine and levamisole have been reported against *A. ceylanicum* L3 *in vitro* and against adult *A. ceylanicum in vivo*, whereas significant antagonistic effects have been observed against L3 of *H. bakeri in vitro* and adult *H. bakeri in vivo* [82]. Since the composition of AChRs varies between different nematode species, different sets of subunits might explain different sensitivities to individual cholinergic anthelmintics as already observed in *O. dentatum* [45]. In addition, it has been shown for *A. caninum,* that the AChR subunit populations vary during different lifecycle stages, resulting in different susceptibilities to cholinergic drugs as well [83]. However, in both experiments, adult *N. brasiliensis* were targeted and therefore, the same subset of AChRs should have been present. Surprisingly, antagonistic effects were observed *in vitro*, whereas additive effects were detected *in vivo*. The reason for this striking difference remains unknown, but obviously the *in vitro* evaluation of cholinergic drug combinations is not suitable model for conclusions on their combinatory behaviour *in vivo*. Therefore, combinations of cholinergic drugs should only be investigated *in vivo* in the life cycle stage of interest; otherwise wrong conclusions could be drawn.

The effects of combinations of cyclooctadepsipeptides with aminophenylamidines have not been reported previously. The aminophenylamidines activate AChRs meaning that they stimulate the excitatory system [47], whereas the cyclooctadepsipeptides cause muscle relaxation via SLO-1 [31]. Since both drugs have entirely different modes of action, additive effects of drugs from both drug classes might be expected. Interestingly, combinations of PF1022A with tribendimidine as well as with dAMD resulted in very diverse effects *in vitro*, whereas the same combinations showed a very homogenous picture *in vivo*. *In vitro*, slight antagonistic effects of PF1022A plus dAMD against adult *N. brasiliensis* as well as of PF1022A plus tribendimidine against *T. spiralis* L1 were detected. No additive or synergistic effects were observed for PF1022A plus dAMD against *T. spiralis* L1 and for PF1022A plus tribendimidine against adult *N. brasiliensis,* whereas slightly agonistic effects of PF1022A plus dAMD as well as of PF1022A plus tribendimidine were observed against *N. brasiliensis* L3. However, *in vivo* both combinations were found to act additively. These results demonstrate that additive effects are possible *in vivo*, although the drug classes act on neurological systems antagonising each other and again, the effects observed during the *in vitro* evaluation did not correspond to those made during *in vivo* testing.

7.11 Summary and outlook

To conclude, the cyclooctadepsipeptides PF1022A and emodepside and the aminophenyl-amidines dAMD and tribendimidine all showed high anthelminthic efficacy.

Tribendimidine is the only drug that was highly effective against all tested species, including *in vivo* efficacy against *H. microstoma* (3× 50 mg/kg), *T. muris* (3× 25 mg/kg) and *N. brasiliensis* (3× 5 mg/kg), indicating that tribendimidine might have potential as extended broad-spectrum anthelmintic. In addition, if the levamisole resistance-breaking properties of tribendimidine, as shown for *O. dentatum* [45], can be confirmed in nematode species of major relevance, the benefits of tribendimidine for human and veterinary use would even increase. But despite the broad-spectrum efficacy, required dosages in multiple dose regimens seem to be too high to achieve complete worm clearance in a single treatment using safe doses. Furthermore, serious concerns regarding aminophenylamidine toxicity have been expressed [65]. Thus, the next step should be the preparation of a detailed toxicological profile, before any further efficacy studies in humans, using multiple dose regimens, are conducted.

The cyclooctadepsipeptide PF1022A also showed high efficacy against all nematode species tested, with *in vivo* efficacy against *N. brasiliensis* (3× 2.5 mg/kg) and patent *T. muris* infections (3× 25 mg/kg; 1× 300 mg/kg). In addition to effects against adult *T. muris*, 1-3 high dosages of PF1022A were also effective against immature stages including histotropic larvae. Indeed, only a very basic formulation was used for all test compounds, hence further reduction of the dosages can be expected by optimising the formulation. Due to the obtained insights regarding their mode of action, the to date unique direct activation of SLO-1, and their potential to cure helminthoses in a single-treatment regimen, the cyclooctadepsipeptides might currently be the most interesting class for the treatment against helminthoses of human and livestock. However, since production costs need to be low, PF1022A, as the much cheaper derivative (due to omission of synthetic derivatisation), appears to be the more suitable candidate [70].

Additionally to the single drugs, combinations of cyclooctadepsipeptides and tribendimidine should also be further investigated. The combinations were shown to have increased efficacy against the rat hookworm, the spectrum would be broadened to include extraintestinal nematodes due to the cyclooctadepsipeptides [31] and by cestodes and trematodes due to tribendimidine [27]. Furthermore, the risk of selection for resistance would be decreased in comparison to combinations of two cholinergic drugs, since two completely independent

targets are affected. However, safety and pharmacology of both drug classes, especially in combination, need to be urgently investigated in a satisfactory manner for rodent models as well as for humans and livestock.

7.12 References

1. Manzanilla-Lopés RH, Evans K, Bridge J, 2004, Plant diseases caused by nematodes, In: Chen ZX, Chen WY, Chen SY, Dickson DW (Eds.), Nematology: Advances and Perspectives Vol 2: Neamtode Management and Utilization. CABI Publishing, Wallingford.
2. Keiser J, Utzinger J, 2009, Food-borne trematodiases. Clin Microbiol Rev 22: 466-483.
3. Craig P, Ito A, 2007, Intestinal cestodes. Curr Opin Infect Dis 20, 524-532.
4. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ, 2006, Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367, 1521-1532.
5. World Health Organization, 2013, Lymphatic filariasis, In: World Health Organization (Ed.) Fact sheet No 102, updated March 2013. World Health Organization, Geneva.
6. Keiser J, Utzinger J, 2010, The drugs we have and the drugs we need against major helminth infections. Adv Parasit, 73, 197-230.
7. Geary TG, Mackenzie CD, 2011, Progress and challenges in the discovery of macrofilaricidal drugs. Expert Rev Anti Infect Ther 9, 681-695.
8. Hoerauf A, Pfarr K, Mand S, Debrah AY, Specht S, 2011, Filariasis in Africa - treatment challenges and prospects. Clin Microbiol Infect 17, 977-985.
9. Suputtamongkol Y, Premasathian N, Bhumimuang K, Waywa D, Nilganuwong S, Karuphong E, Anekthananon T, Wanachiwanawin D, Silpasakorn S, 2011, Efficacy and safety of single and double doses of ivermectin versus 7-day high dose albendazole for chronic strongyloidiasis. PLoS Negl Trop Dis 5, e1044.
10. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, Yang GJ, Sripa B, Lustigman S, 2012, A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6, e1549.
11. Harder A, 2002, Chemotherapeutic approaches to trematodes (except schistosomes) and cestodes: current level of knowledge and outlook. Parasitol Res 88, 587-590.
12. Bowman DD, 2012, Heartworms, macrocyclic lactones, and the specter of resistance to prevention in the United States. Parasit Vectors 5, 138.
13. Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC, 2004, Drug resistance in veterinary helminths. Trends Parasitol 20, 469-476.

14. Wolstenholme AJ, Kaplan RM, 2012, Resistance to macrocyclic lactones. Curr Pharm Biotechnol 13, 873-887.
15. Sutherland IA, Leathwick DM, 2011, Anthelmintic resistance in nematode parasites of cattle: a global issue? Trends Parasitol 27, 176-181.
16. Peregrine AS, Molento MB, Kaplan RM, Nielsen MK, 2014, Anthelmintic resistance in important parasites of horses: Does it really matter? Vet Parasitol 201, 1-8.
17. Leathwick DM, Hosking BC, 2009, Managing anthelmintic resistance: modelling strategic use of a new anthelmintic class to slow the development of resistance to existing classes. N Z Vet J 57, 203-207.
18. Leathwick DM, Hosking BC, Bisset SA, McKay CH, 2009, Managing anthelmintic resistance: is it feasible in New Zealand to delay the emergence of resistance to a new anthelmintic class? N Z Vet J 57, 181-192.
19. Geary TG, Hosking BC, Skuce PJ, von Samson-Himmelstjerna G, Maeder S, Holdsworth P, Pomroy W, Vercruysse J, 2012, World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Guideline: Anthelmintic combination products targeting nematode infections of ruminants and horses. Vet Parasitol 190, 306-316.
20. Beach MJ, Streit TG, Addiss DG, Prospere R, Roberts JM, Lammie PJ, 1999, Assessment of combined ivermectin and albendazole for treatment of intestinal helminth and *Wuchereria bancrofti* infections in Haitian schoolchildren. Am J Trop Med Hyg 60, 479-486.
21. Knopp S, Mohammed KA, Speich B, Hattendorf J, Khamis IS, Khamis AN, Stothard JR, Rollinson D, Marti H, Utzinger J, 2010, Albendazole and mebendazole administered alone or in combination with ivermectin against *Trichuris trichiura*: a randomized controlled trial. Clin Infect Dis 51, 1420-1428.
22. Speich B, Ame SM, Ali SM, Alles R, Hattendorf J, Utzinger J, Albonico M, Keiser J, 2012, Efficacy and safety of nitazoxanide, albendazole, and nitazoxanide-albendazole against *Trichuris trichiura* infection: a randomized controlled trial. PLoS Negl Trop Dis 6, e1685.
23. Namwanje H, Kabatereine NB, Olsen A, 2011, Efficacy of single and double doses of albendazole and mebendazole alone and in combination in the treatment of *Trichuris trichiura* in school-age children in Uganda. Trans R Soc Trop Med Hyg 105, 586-590.

24. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, Thomas H, 1979, Amidantel, a potent anthelminthic from a new chemical class. Arzneimittelforschung 29, 31-32.
25. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W, 2005, Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94, 1-14.
26. Steinmann P, Zhou XN, Du ZW, Jiang JY, Xiao SH, Wu ZX, Zhou H, Utzinger J, 2008, Tribendimidine and albendazole for treating soil-transmitted helminths, *Strongyloides stercoralis* and *Taenia* spp.: open-label randomized trial. PLoS Negl Trop Dis 2, e322.
27. Xiao SH, Utzinger J, Tanner M, Keiser J, Xue J, 2013, Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. Acta Trop 126, 115-126.
28. Siles-Lucas M, Hemphill A, 2002, Cestode parasites: application of *in vivo* and *in vitro* models for studies on the host-parasite relationship. Adv Parasitol 51, 133-230.
29. Smout MJ, Kotze AC, McCarthy JS, Loukas A, 2010, A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. PLoS Negl Trop Dis 4, e885.
30. Harder A, Schmitt-Wrede HP, Krücken J, Marinovski P, Wunderlich F, Willson J, Amliwala K, Holden-Dye L, Walker R, 2003, Cyclooctadepsipeptides - an anthelmintically active class of compounds exhibiting a novel mode of action. Int J Antimicrob Agents 22, 318-331.
31. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, Welz C, von Samson-Himmelstjerna G, 2012, Anthelmintic cyclcooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28, 385-394.
32. Ellman GL, Courtney KD, Andres V, Feather-Stone RM, 1961, A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7, 88-95.
33. Kassai T, 1982, Handbook of *Nippostrongylus brasiliensis*. Akadémiai Kiadó, Budapest.
34. Mehlhorn H, Düwel D, Raether W, 1993, Diagnose und Therapie der Parasitosen von Haus-, Nutz- und Heimtieren. Gustav Fischer, Stuttgart.

35. Diawara A, Drake LJ, Suswillo RR, Kihara J, Bundy DA, Scott ME, Halpenny C, Stothard JR, Prichard RK, 2009, Assays to detect beta-tubulin codon 200 polymorphism in *Trichuris trichiura* and *Ascaris lumbricoides*. PLoS Negl Trop Dis 3, e397.
36. Traversa D, 2011, Are we paying too much attention to cardio-pulmonary nematodes and neglecting old-fashioned worms like *Trichuris vulpis*? Parasit Vectors 4, 32.
37. Keeling JE, 1961, Experimental trichuriasis. II. Anthelmintic screening against *Trichuris muris* in the albino mouse. J Parasitol 47, 647-651.
38. Wakelin D, 1967, Acquired immunity to *Trichuris muris* in the albino laboratory mouse. Parasitology 57, 515-524.
39. Johnston CE, Bradley JE, Behnke JM, Matthews KR, Else KJ, 2005, Isolates of *Trichuris muris* elicit different adaptive immune responses in their murine host. Parasite Immunol 27, 69-78.
40. Panesar TS, Croll NA, 1980, The location of parasites within their hosts: site selection by *Trichuris muris* in the laboratory mouse. Int J Parasitol 10, 261-273.
41. Pike EH, 1969, Egg output of *Trichuris muris* (Schrank, 1788) . J Parasitol 55, 1046-1049.
42. Geary TG, Woo K, McCarthy JS, Mackenzie CD, Horton J, Prichard RK, de Silva NR, Olliaro PL, Lazdins-Helds JK, Engels DA, Bundy DA, 2010, Unresolved issues in anthelmintic pharmacology for helminthiases of humans. Int J Parasitol 40, 1-13.
43. Yuan G, Xu J, Qu T, Wang B, Zhang R, Wei C, Guo R, 2010, Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs R D 10, 83-90.
44. Xue J, Xiao SH, Xu LL, Qiang HQ, 2010, The effect of tribendimidine and its metabolites against *Necator americanus* in golden hamsters and *Nippostrongylus braziliensis* in rats. Parasitol Res 106, 775-781.
45. Buxton SK, Charvet CL, Neveu C, Cabaret J, Cortet J, Peineau N, Abongwa M, Courtot E, Robertson AP, Martin RJ, 2014, Investigation of acetylcholine receptor diversity in a nematode parasite leads to characterization of tribendimidine- and derquantel-sensitive nAChRs. PLoS Pathog 10, e1003870.
46. Martin JM, Purcell J, Robertson AP, Valkanov MA, 2002, Neuromuscular organisation and control in nematodes, In: Lee DL (Ed.), The biology of nematodes. CRC Press, Boca Raton.

47. Hu Y, Xiao SH, Aroian RV, 2009, The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3, e499.
48. Chen W, Terada M, Cheng JT, 1996, Characterization of subtypes of gamma-aminobutyric acid receptors in an *Ascaris muscle* preparation by binding assay and binding of PF1022A, a new anthelmintic, on the receptors. Parasitol Res 82, 97-101.
49. Miltsch SM, Krücken J, Demeler J, Janssen IJ, Krüger N, Harder A, von Samson-Himmelstjerna G, 2012, Decreased emodepside sensitivity in unc-49 gamma-aminobutyric acid (GABA)-receptor-deficient *Caenorhabditis elegans*. Int J Parasitol 42, 761-770.
50. Willson J, Amliwala K, Davis A, Cook A, Cuttle MF, Kriek N, Hopper NA, O'Connor V, Harder A, Walker RJ, Holden-Dye L, 2004, Latrotoxin receptor signaling engages the UNC-13-dependent vesicle-priming pathway in *C. elegans*. Curr Biol 14, 1374-1379.
51. Saeger B, Schmitt-Wrede HP, Dehnhardt M, Benten WP, Krücken J, Harder A, Von Samson-Himmelstjerna G, Wiegand H, Wunderlich F, 2001, Latrophilin-like receptor from the parasitic nematode *Haemonchus contortus* as target for the anthelmintic depsipeptide PF1022A. FASEB J 15, 1332-1334.
52. Crisford A, Murray C, O'Connor V, Edwards RJ, Krüger N, Welz C, von Samson-Himmelstjerna G, Harder A, Walker RJ, Holden-Dye L, 2011, Selective toxicity of the anthelmintic emodepside revealed by heterologous expression of human KCNMA1 in *Caenorhabditis elegans*. Mol Pharmacol 79, 1031-1043.
53. Welz C, Krüger N, Schniederjans M, Miltsch SM, Krücken J, Guest M, Holden-Dye L, Harder A, von Samson-Himmelstjerna G, 2011, SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in *Caenorhabditis elegans* slo-1 loss of function mutants. PLoS Pathog 7, e1001330.
54. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, Harder A, Holden-Dye L, Hopper NA, 2007, The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclooctadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. Int J Parasitol 37, 1577-1588.
55. Dufour V, Beech RN, Wever C, Dent JA, Geary TG, 2013, Molecular cloning and characterization of novel glutamate-gated chloride channel subunits from *Schistosoma mansoni*. PLoS Pathog 9, e1003586.

56. Charvet CL, Robertson AP, Cabaret J, Martin RJ, Neveu C, 2012, Selective effect of the anthelmintic bephenium on *Haemonchus contortus* levamisole-sensitive acetylcholine receptors. Invert Neurosci 12, 43-51.
57. McCavera S, Rogers AT, Yates DM, Woods DJ, Wolstenholme AJ, 2009, An ivermectin-sensitive glutamate-gated chloride channel from the parasitic nematode *Haemonchus contortus*. Mol Pharmacol 75, 1347-1355.
58. Forrester SG, Prichard RK, Dent JA, Beech RN, 2003, *Haemonchus contortus*: HcGluCla expressed in *Xenopus* oocytes forms a glutamate-gated ion channel that is activated by ibotenate and the antiparasitic drug ivermectin. Mol Biochem Parasitol 129, 115-121.
59. Rufener L, Bedoni N, Baur R, Rey S, Glauser DA, Bouvier J, Beech R, Sigel E, Puoti A, 2013, acr-23 encodes a monepantel-sensitive channel in *Caenorhabditis elegans*. PLoS Pathog 9, e1003524.
60. Siddiqui SZ, Brown DD, Rao VT, Forrester SG, 2010, An UNC-49 GABA receptor subunit from the parasitic nematode *Haemonchus contortus* is associated with enhanced GABA sensitivity in nematode heteromeric channels. J Neurochem 113, 1113-1122.
61. Prakriya M, Lingle CJ, 1999, BK channel activation by brief depolarizations requires Ca^{2+} influx through L- and Q-type Ca^{2+} channels in rat chromaffin cells. J Neurophysiol 81, 2267-2278.
62. Woods RA, Malone KMB, 1985, The effects of amidantel (BAY d 8815) and its deacylated derivative (BAY d 9216) on wild-type and resistant mutants of *Caenorhabditis elegans*. Can J Zool 64, 1310-1316.
63. Xiao SH, Xue J, Xu LL, Zheng Q, Qiang HQ, Zhang YN, 2009, The *in vitro* and *in vivo* effect of tribendimidine and its metabolites against *Clonorchis sinensis*. Parasitol Res 105, 1497-1507.
64. Wu ZX, Fang YY, Liu YS, 2006, Effect of a novel drug - enteric coated tribendimidine in the treatment of intestinal nematode infections. Chin J of Parasitol and Parasit Dis 24: 23–26.
65. Epe C, Kaminsky R, 2013, New advancement in anthelmintic drugs in veterinary medicine. Trends Parasitol 29, 129-134.
66. Conder GA, Johnson SS, Nowakowski DS, Blake TE, Dutton FE, Nelson SJ, Thomas EM, Davis JP, Thompson DP, 1995, Anthelmintic profile of the cyclodepsipeptide PF1022A in *in vitro* and *in vivo* models. J Antibiot 48, 820-823.

67. von Samson-Himmelstjerna G, Harder A, Schnieder T, Kalbe J, Mencke N, 2000, *In vivo* activities of the new anthelmintic depsipeptide PF 1022A. Parasitol Res 86, 194-199.
68. Harder A, von Samson-Himmelstjerna G, 2002, Cyclooctadepsipeptides - a new class of anthelmintically active compounds. Parasitol Res 88, 481-488.
69. Wang M, Watanabe N, Shomura T, Ohtomo H, 1995, Effects of PF1022A on *Nippostrongylus brasiliensis* in rats and *Hymenolepis nana* in mice. Jpn J Parasitol 44, 306-310.
70. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, Don R, Keiser J, 2011, Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5, e1138.
71. Vercruysse J, Albonico M, Behnke JM, Kotze AC, Prichard RK, McCarthy JS, Montresor A, Levecke B, 2011, Is anthelmintic resistance a concern for the control of human soil-transmitted helminths? Int J Parasitol Drugs Drug Resist 1, 14-27.
72. Schimmel A, Altreuther G, Schroeder I, Charles S, Cruthers L, Kok DJ, Kraemer F, Krieger KJ, 2009, Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature adult *Trichuris vulpis* infections in dogs. Parasitol Res 105, 17-22.
73. Mehlhorn H, Schmahl G, Frese M, Mevissen I, Harder A, Krieger K, 2005, Effects of a combinations of emodepside and praziquantel on parasites of reptiles and rodents. Parasitol Res 97, 65-69.
74. Martin RJ, Robertson AP, Buxton SK, Beech RN, Charvet CL, Neveu C, 2012, Levamisole receptors: a second awakening. Trends Parasitol 28, 289-296.
75. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas WK, 1998, A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71-75.
76. Sturchler D, 1982, Chemotherapy of human intestinal helminthiases: a review, with particular reference to community treatment. Adv Pharmacol Chemother 19, 129-154.
77. Smith MC, Sherman DM, 2009, Goat medicine. Wiley-Blackwell, Ames, 454-456.
78. Rabbani GH, Gilman RH, Kabir I, Mondel G, 1985, The treatment of *Fasciolopsis buski* infection in children: a comparison of thiabendazole, mebendazole, levamisole, pyrantel pamoate, hexylresorcinol and tetrachloroethylene. Trans R Soc Trop Med Hyg 79, 513-515.

79. Bennet EM, Behm C, Bryant C, 1978, Effects of mebendazole and levamisole on tetrathyridia of *Mesocestoides corti* in the mouse. Int J Parasitol 8, 463-466.

80. Reinemeyer CR, Hutchens DE, Eckblad WP, Marchiondo AA, Shugart JI, 2006, Dose-confirmation studies of the cestocidal activity of pyrantel pamoate paste in horses. Vet Parasitol 138, 234-239.

81. Marchiondo AA, White GW, Smith LL, Reinemeyer CR, Dascanio JJ, Johnson EG, Shugart JI, 2006, Clinical field efficacy and safety of pyrantel pamoate paste (19.13% w/w pyrantel base) against *Anoplocephala* spp. in naturally infected horses. Vet Parasitol 137, 94-102.

82. Tritten L, Nwosu U, Vargas M, Keiser J, 2012, *In vitro* and *in vivo* efficacy of tribendimidine and its metabolites alone and in combination against the hookworms *Heligmosomoides bakeri* and *Ancylostoma ceylanicum*. Acta Trop 122, 101-107.

83. Kotze AC, Lowe A, O'Grady J, Kopp SR, Behnke JM, 2009, Dose-response assay templates for *in vitro* assessment of resistance to benzimidazole and nicotinic acetylcholine receptor agonist drugs in human hookworms. Am J Trop Med Hyg 81, 163-170.

Chapter 8

Summary / Zusammenfassung

8 Summary/ Zusammenfassung

8.1 Summary

Parasitic helminths including cestodes, trematodes and nematodes, have an enormous impact on human and animal health and cause tremendous financial losses. Therefore, the urgent demand for modern and safe treatment options against helminthoses is obvious. In addition to the optimisation of existing drug classes, two major objectives are in the focus of research to increase treatment options against helminth infections: (i) the evaluation of combinations, to increase the anthelmintic spectrum, to improve the efficacy of the respective drugs and to decelerate the development of resistance and (ii) the identification and development of safe and highly efficacious new drug classes with novel, resistance-breaking modes of action.

The present cumulative thesis, consisting of five publications, describes investigations addressing both of the above mentioned aspects by comparing the potential of the aminophenylamidines amidantel, tribendimidine and deacylated amidantel (dAMD) and of the cyclooctadepsipeptides emodepside and PF1022A for the treatment against helminth infections of humans and animals in monotherapy as well as in several combinations.

Since activitiy of the nematicidal aminophenylamidines against cestodes has already been published, but respective reports were somewhat informal, publication 1 aimed on the evaluation of the efficacy of tribendimidine and dAMD against the tapeworm *Hymenolepis microstoma* in a controlled laboratory trial. Whereas treatments with three dosages of up to 100 mg/kg dAMD did not result in any significant reduction of the worm burdens, in marked contrast, treatments with 3× 50 mg/kg tribendimidine, which is the parent compound of dAMD, resulted in high parasite clearance.

In publication 2, substantial concentration-dependent anthelmintic potencies of aminophenylamidines and cyclooctadepsipeptides as well as of some drug combinations were detected against larval and adult stages of the rat hookworm *Nippostrongylus brasiliensis* and against first larval stages of *Trichinella spiralis* using three *in vitro* assays. Consequently, in publication 3 the same derivatives were found to be highly efficacious using the *N. brasiliensis in vivo* model in rats. Moreover, tested drug combinations of two cholinergic drugs or an aminophenylamidine plus a cyclooctadepsipeptide resulted in additive effects, making not only the individual drugs but also combinations interesting for further evaluation. Furthermore, a pharmacokinetic analysis revealed that after oral administration of tribendimidine, only its metabolite dAMD was detectable in rat blood samples. Finally, electron microscopic analysis of hookworms treated with the test compounds revealed

information about their mode of action. Worms treated with PF1022A showed signs of flaccid paralysis, whereas individuals treated with cholinergic drugs including aminophenylamidines and levamisole, showed strong contraction along the whole body of the worm suggesting spastic paralysis.

Following the establishment and parasitological characterisation of a *Trichuris muris* mouse model, publication 4 investigated the *in vivo* potency of the aminophenylamidines and the cyclooctadepsipeptides against patent whipworm infections using three consecutive dosages by different routes of administration. Only treatments with relatively high dosages of tribendimidine or dAMD resulted in complete elimination of the worm burden whereas treatments with fairly low dosages of the cyclooctadepsipeptides emodepside and PF1022A, especially after oral administration, resulted in full parasite clearance. Due to the high efficacy, PF1022A was further evaluated in single and multiple oral treatment schemes targeting developmental stages including histotropic larvae. Again, dose-dependent efficacies were observed. However, using a single dose regimen, only high dosages cured the larval whipworm infections completely. But since only a very basic formulation was tested, reduction of the required dosages can likely be achieved by optimisation of the formulation.

The cyclooctadepsipeptides showed high efficacy against all nematode species tested. However, their mode of action is not fully understood. The calcium-gated and voltage-dependent potassium channel SLO-1 is generally accepted to be crucial for the nematicidal effects of cyclooctadepsipeptides in *Caenorhabditis elegans* but direct activation of SLO-1 channels by any cyclooctadepsipeptide has not been demonstrated. Therefore, manuscript 5 reports the identification and characterisation of SLO-1 channels from various nematodes. After identification and cloning of slo-1 orthologs from a wide phylogenetic range of parasitic nematodes, SLO-1 and its potential activation by cyclooctadepsipeptides were analysed by heterologous expression in *Xenopus laevis* oocytes. Accordingly, it was demonstrated for the first time, that emodepside directly induces currents via *C. elegans* SLO-1.

Due to the obtained insights regarding their mode of action and their potential to cure helminthoses in a single-treatment regimen, the cyclooctadepsipeptides might currently be the most interesting new class for the treatment against helminthoses of human and livestock. In addition to monotherapies, the use of combinations containing cyclooctadepsipeptides and cholinergic drugs such as tribendimidine should also be further investigated. The combinations were shown to have increased efficacy against the rat hookworm and presumably the risk of selection for resistance would be decreased, since two completely independent targets are affected.

8.2 Zusammenfassung

Helminthosen, hervorgerufen durch Cestoden, Trematoden und Nematoden, verursachen enorme gesundheitliche Schäden bei Menschen und Tieren und sind für gewaltige ökonomische Schäden verantwortlich. Der immense Bedarf an modernen chemotherapeutischen Möglichkeiten zur Bekämpfung von Wurmerkrankungen ist daher offensichtlich. Neben der Optimierung der vorhandenen Präparate, stehen zwei weitere zentrale Herausforderungen im Fokus der Wissenschaft: (i) Die Evaluierung von Kombinationspräparaten, welche das Wirkungsspektrum erweitern sowie die Wirksamkeit gegen spezifische Spezies erhöhen, aber auch der Entwicklung von hochresistenten Wurmpopulation entgegenwirken sollen, und (ii) die Erforschung von hochwirksamen und sicheren neuen Präparaten, welche mit alternativen Wirkmechanismen resistenzbrechende Eigenschaften haben und folglich die therapeutischen Möglichkeiten erweitern können.

Die vorliegende kumulative Dissertation, bestehend aus fünf wissenschaftlichen Artikeln, beinhaltet Untersuchungen und Erkenntnisse zu beiden genannten Zielen, indem Verbindungen aus den Substanzklassen der zyklischen Oktadepsipeptide (PF1022A und Emodepsid) und der Aminophenylamidine (Amidantel, deacyliertes Amidantel und Tribendimidin) alleine und in diversen Kombinationen für eine mögliche Entwicklung zur Bekämpfung von Wurmerkrankungen von Menschen und Tieren untersucht wurden.

Da den Aminophenylamidinen neben einer Wirkung gegen Rundwürmer auch eine Wirksamkeit gegen Bandwürmer zugesprochen wird, hierzu jedoch keine experimentellen Ergebnisse publiziert waren, wurde in Artikel 1 die Wirksamkeit von zwei Aminophenylamidinen gegen den Bandwurm *Hymenolepis microstoma in vivo* evaluiert. Während für Tribendimidin eine hohe Wirksamkeit nachgewiesen werden konnte, zeigte dessen nematizider Metabolit, deacyliertes Amidantel, unter Verabreichung vergleichbarer Dosierungen überraschenderweise keine Wirkung gegen *H. microstoma*.

In Artikel 2 wurden solide dosisabhängige Wirksamkeiten sämtlicher getesteter Verbindungen beider Substanzklassen sowie diverser Kombinationen sowohl gegen larvale und adulte Stadien des Hakenwurms der Ratte, *Nippostrongylus brasiliensis,* als auch gegen erste Larvenstadien der humanpathogenen Trichinen *Trichinella spiralis in vitro* festgestellt. In Ergänzung dazu, zeigt Artikel 3, dass entsprechende Vertreter beider Wirkstoffklassen auch *in vivo* im verwendeten Hakenwurmmodell eine hohe Wirksamkeit besitzen. Aufgrund additiver Effekte von Kombinationen aus beiden Substanzklassen ist neben der Evaluierung der Einzelverbindungen auch eine weitergehende Untersuchung der Kombinationen in Betracht zu ziehen. Ferner wurde in Artikel 3 mittels einer pharmakokinetischen

Untersuchung festgestellt, dass nach oraler Gabe von Tribendimidin ausschließlich dessen Metabolit, deacyliertes Amidantel, im Blut der Ratte nachweisbar ist. Darüber hinaus wurden durch elektronenmikroskopische Untersuchungen Hinweise bezüglich der Wirkmechanismen beider Substanzklassen gewonnen. Während Hakenwürmer, die mit PF1022A behandelt wurden, morphologische Anzeichen einer schlaffen Paralyse aufwiesen, zeigten mit Levamisol oder Aminophenylamidinen behandelte Würmer, stark kontrahierte Hautmuskelschläuche, was auf eine spastische Paralyse schließen lässt.

Basierend auf der Etablierung und einer detaillierten parasitologischen Charakterisierung eines *T. muris*-Maus-Modells behandelt Artikel 4 die Untersuchung der Wirksamkeit von Aminophenylamidinen und zyklischen Oktadepsipeptiden gegen den Peitschenwurm der Maus unter Verwendung verschiedener Applikationsschemata. Während nach dreimaliger Behandlung mit deacyliertem Amidantel oder Tribendimidin nur unter Verwendung relativ hoher Dosierungen vollständige Wirksamkeit gegen *T. muris* erzielt wurden, wurden von Emodepsid und PF1022A nur niedrige orale Dosen für denselben Effekt benötigt. Folglich wurde PF1022A in mehrfacher und einfacher oraler Gabe auch gegen larvale Stadien getestet. Auch hier wurde eine signifikante Reduktion der Wurmbürden, allerdings bei erhöhten Dosierungen, erzielt. Durch Verbesserung der Formulierung kann jedoch vermutlich eine deutliche Reduktion der benötigten Dosis erzielt werden.

Die zyklischen Oktadepsipeptide zeigten in sämtlichen Untersuchungen eine durchgängig hohe Wirksamkeit, wobei deren Wirkmechanismus nicht vollständig aufgeklärt ist. Daher wurde in Artikel 5 der kalziumaktivierte und spannungsgesteuerte Kaliumkanal SLO-1 verschiedener Nematoden isoliert und phylogenetisch analysiert. SLO-1 wurde in *Caenorhabditis elegans* als essentiell für die Wirksamkeit von Emodepsid beschrieben, jedoch konnte bisher keine direkte Interaktion zwischen SLO-1 und einem zyklischen Oktadepsipeptide gezeigt werden. Nach Identifizierung und Klonierung diverser slo-1 Orthologe, konnte durch Studien an *Xenopus laevis* Oozyten, welche *C. elegans* SLO-1 heterolog exprimierten, gezeigt werden, dass durch Emodepsid Ströme über SLO-1 entstehen.

Aufgrund des einzigartigen Wirkmechanismus und der Möglichkeit, Helminthosen in einer Einzelgabe zu kurieren, können zyklische Oktadepsipeptide als vielversprechende Kandidaten zur Behandlung humaner und tierischer Helminthosen betrachtet werden. Zusätzlich sollten Kombinationen, bestehend aus einem zyklischen Oktadepsipeptid und einer cholinergen Verbindung wie Tribendimidin aufgrund additiver Effekte im Hakenwurm Modell und der wohlmöglichen Reduktion der Resistenz-Selektion, aufgrund der unterschiedlichen Wirkungsweise, als weitere Behandlungsoption in Betracht gezogen werden.

Chapter 9

List of publications

9 List of publications

The present Inaugural-Dissertation was submitted as a cumulative thesis. The following articles were taken as a basis for this dissertation:

I.)

Kulke D., Krücken J., Welz C., von Samson-Himmelstjerna G., Harder A.

***In vivo* efficacy of the anthelmintic tribendimidine against the cestode *Hymenolepis microstoma* in a controlled laboratory trial**

Acta Tropica 2012, 123 (2): 78-84.

II.)

Kulke D., Krücken J., Demeler J., Harder A., Mehlhorn H., von Samson-Himmelstjerna G.

In vitro* efficacy of cyclooctadepsipepdtides and aminophenylamidines alone and in combination against third stage larvae and adult worms of *Nippostrongylus brasiliensis* and first stage larvae of *Trichinella spiralis

Parasitology Research 2013, 112 (1): 335-345.

III.)

Kulke D., Krücken J., Harder A., Krebber R., Fraatz, K., Mehlhorn H., von Samson-Himmelstjerna G.

In vivo* efficacy of PF1022A and nicotinic acetylcholine receptor agonists alone and in combination against *Nippostrongylus brasiliensis

Parasitology 2013, 140 (10): 1252-1265.

IV.)

Kulke D., Krücken J., Harder A., von Samson-Himmelstjerna G.

Efficacy of cyclooctadepsipeptides and aminophenylamidines against larval, immature and mature adult stages of a parasitologically characterized trichurosis model in mice

PLoS Neglected Tropical Diseases 2014, 8 (2): e2698.

V.)

Kulke D., von Samson-Himmelstjerna G., Miltsch S., Wolstenholme A.J., Jex A.R., Gasser R.B., Ballesteros C., Geary T., Keiser J., Townson S., Harder A., Krücken J.

Characterisation of the calcium-gated and voltage-dependent potassium channel SLO-1 of nematodes and its interaction with emodepside

Submitted to PLoS Pathogens

Parts of this thesis have been presented and published previously:

I.)

Kulke D.

Investigations on the effects of nicotinergic drugs and cyclooctadepsipeptides alone or in combination against *Nippostrongylus brasiliensis* in rats

Diploma thesis (Heinrich-Heine-Universität Düsseldorf): Düsseldorf, 2009

II.)

Kulke D., Harder A.

Efficacy of tribendimidine against the cestode *Hymenolepis microstoma* in experimentally infected mice

Biannual Meeting of the German Society of Parasitology (DGP): Düsseldorf, 2010

International Congress of Parasitology (ICOPA): Melbourne, 2010

III.)

Kulke D., Harder A.

Efficacy of tribendimidine in comparison to levamisole against the nematode *Nippostrongylus brasiliensis* in experimentally infected rats

Biannual Meeting of the German Society of Parasitology (DGP): Düsseldorf, 2010

International Congress of Parasitology (ICOPA): Melbourne, 2010

IV.)

Kulke D., Krücken J., Harder A., Townson S., von Samson-Himmelstjerna G.

Identifikation von SLO-1 Kanälen in *Onchocerca gutturosa* und *Trichuris muris*: Einheitlicher Wirkmechanismus von Emodepsid gegen parasitische Nematoden?

Annual meeting of the German Veterinary Medical Society (DVG), professional group on Parasitology and Parasitic Diseases: Berlin, 2011

V.)

Kulke D., Krücken J., Harder A., Townson S., von Samson-Himmelstjerna G.

Identification of SLO-1 calcium- and voltage-activated potassium channels within different clades of parasitic nematodes: hints for a consistent mode of action of the anthelmintic emodepside?

International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP): Buenos Aires, 2011

VI.)

Kulke D., Krücken J., Welz C., Harder A., von Samson-Himmelstjerna G.

The *Trichuris muris* genome encodes two homologs of SLO-1 calcium- and voltage-activated potassium channels: hints for a different sensitivity to the anthelmintic emodepside?

Symposium on Membrane Ion Channels in Helminth Parasites, Resistance and Sites of Action for Anthelmintics: Philadelphia, 2011

VII.)

Kulke D., Krücken J., Dufour V., Geary T., Welz C., Harder A., von Samson-Himmelstjerna G.

More diverse than expected: The putative emodepside target SLO-1 shows considerable heterogeneity between different clades of nematodes

Annual meeting of the German Veterinary Medical Society (DVG), professional group on Parasitology and Parasitic Diseases: Hannover, 2012

VIII.)

Kulke D., von Samson-Himmelstjerna G., Miltsch S., Wolstenholme A., Townson S., Ballesteros C., Geary T., Keiser J., Krücken J.

Considerable heterogeneity of the Ca^{2+}-activated and voltage-gated K^{+}-channel SLO-1 between different clades of nematodes and the potential impact on the interaction of SLO-1 and emodepside

International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP): Perth, 2013

IX.)

Kulke D., Miltsch S., Wolstenholme A., Townson S., Ballesteros C., Geary T., Keiser J., von Samson-Himmelstjerna G., Harder A., Krücken J.

Phylogenetic analysis of nematode SLO-1 and electrophysiological characterisation of SLO-1 in Xenopus oocytes

Anthelmintics: From Discovery to Resistance: San Francisco, 2014

The individual contributions of Daniel Kulke are stated:

	Paper I	Paper II	Paper III	Paper IV	Paper V
Idea	75 %	25 %	50 %	50 %	25 %
Theory	75 %	50 %	75 %	75 %	50 %
Literature	90 %	90 %	90 %	90 %	75 %
Study design	75 %	25 %	25 %	100 %	50 %
Implementation	100 %	100 %	90 %	100 %	80 %
Analysis of data	50 %	50 %	50 %	75 %	50 %
Manuscript	75 %	75 %	75 %	75 %	50 %

Implementation of studies for paper II:
D. Kulke performed and published two replicates of each of the *in vitro* assays in his diploma thesis.

Implementation of studies for paper III:
D. Kulke performed and published several treatment regimens of cyclooctadepsipeptides and cholinergic drugs alone and in combination against *N. brasiliensis* in rats in his diploma thesis. D. Kulke also performed and published the scanning electron microscopy studies in his diploma thesis.

Implementation of studies for paper III:
R. Krebber performed the analysis of the plasma samples for the pharmacokinetic study by turbulent flow chromatography coupled with a tandem mass spectrometry detector.

Implementation of studies for paper V:
S. Miltsch performed the amplification, cloning and sequencing of ascarid slo-1 cDNAs.
C. Ballesteros performed isolation of *B. malayi* RNA and transcription of the respective cDNA.

The co-authors of the five publications confirm in all conscience, that the stated contributions of Daniel Kulke are declared truthfully.

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Chapter 10

Curriculum vitae

10 Curriculum vitae

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Chapter 11

Appendices

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PLOS | NEGLECTED TROPICAL DISEASES

Efficacy of Cyclooctadepsipeptides and Aminophenylamidines against Larval, Immature and Mature Adult Stages of a Parasitologically Characterized Trichurosis Model in Mice

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Abstract

Background: The genus *Trichuris* includes parasites of major relevance in veterinary and human medicine. Despite serious economic losses and enormous impact on public health, treatment options against whipworms are very limited. Additionally, there is an obvious lack of appropriately characterized experimental infection models. Therefore, a detailed parasitological characterization of a *Trichuris muris* isolate was performed in C57BL/10 mice. Subsequently, the *in vivo* efficacies of the aminophenylamidines amidantel, deacylated amidantel (dAMD) and tribendimidine as well as the cyclooctadepsipeptides emodepside and in particular PF1022A were analyzed. This was performed using various administration routes and treatment schemes targeting histotropic and further developed larval as well as immature and mature adult stages.

Methodology/Principal Findings: Duration of prepatent period, time-dependent localization of larvae during period of prepatency as well as the duration of patency of the infection were determined before drugs were tested in the characterized trichurosis model. Amidantel showed no effect against mature adult *T. muris*. Tribendimidine showed significantly higher potency than dAMD after oral treatments (ED_{50} values of 6.5 vs. 15.1 mg/kg). However, the opposite was found for intraperitoneal treatments (ED_{50} values of 15.3 vs. 8.3 mg/kg). When emodepside and PF1022A were compared, the latter was significantly less effective against mature adults following intraperitoneal (ED_{50} values of 6.1 vs. 55.7 mg/kg) or subcutaneous (ED_{50} values of 15.2 vs. 225.7 mg/kg) administration. Only minimal differences were observed following oral administration (ED_{50} values of 2.7 vs. 5.2 mg/kg). Triple and most single oral doses with moderate to high dosages of PF1022A showed complete efficacy against histotropic second stage larvae (3×100 mg/kg or 1×250 mg/kg), further developed larvae (3×10 mg/kg or 1×100 mg/kg) and immature adults (3×10 mg/kg or 1×100 mg/kg). Histotropic first stage larvae were only eliminated after three doses of PF1022A (3×100 mg/kg) but not after a single dose.

Conclusions/Significance: These results indicate that the cyclooctadepsipeptides are a drug class with promising candidates for further evaluation for the treatment of trichurosis of humans and livestock animals in single dose regimens.

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Competing Interests: Daniel Kulke, PhD student of the Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, is employed by Bayer HealthCare, Global Drug Discovery, Animal Health, developing veterinary pharmaceuticals including dewormers. Furthermore, Achim Harder was an employee of Bayer HealthCare when the study was conducted. Except of Achim Harder and Daniel Kulke, Bayer HealthCare was not involved in study design, data collection, data analysis or preparation of the manuscript. The decision to publish the manuscript was jointly taken. This does not alter our adherence to all PLOS policies on sharing data and materials.

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Introduction

About 20 major human helminthoses have a significant impact on global public health [1]. Since a highly disproportionate share of the burden occurs in developing areas of sub-Saharan Africa, Asia and the Americas, helminth infections belong to both, the "neglected tropical diseases" and the "neglected infections of poverty" [2,3]. In these regions more than a billion people are infected with one or more worm species [2]. An important part of human helminth infections worldwide is caused by soil-transmitted nematodes, including the roundworm *Ascaris lumbricoides* with 800 million infections, the whipworm *Trichuris trichiura* with 600 million infections, and the hookworms *Ancylostoma duodenale* and *Necator americanus* with 600 million infections [4]. An estimated 1.6–6.4 million disability adjusted loss of life years are a direct result of trichurosis [4]. In 2010 an estimated 5023 million people lived in

Author Summary

Treatment options against whipworm infections of humans and livestock are very limited and even anthelmintics recently introduced into the market do not significantly improve the situation. Here, we evaluated members of two relatively new drug classes, the aminophenylamidines (amidantel, deacylated amidantel, tribendimidine) and the cyclooctadepsipeptides (PF1022A, emodepside) in a murine trichurosis model. While tribendimidine is licensed for the treatment of human helminthosis caused by hookworms, pinworms and roundworms in China, emodepside is the nematocidal component of dewormers for cats and dogs. With the exception of amidantel, all drugs showed good efficacies against adult whipworms using three consecutive doses. Due to considerations regarding drug safety and price, PF1022A was further evaluated against histotropic first and second stage larvae, further developed larvae, immature and mature adults using a single or three consecutive doses. Three doses eliminated all stages while a single dose was inefficient against histotropic first stage larvae. In general, higher doses were required for early stages in comparison to stages protruding into the gut lumen. Since only a very basic formulation of drugs was tested, further improvement can be expected from optimized formulations. Cyclooctadepsipeptides should therefore be considered as candidates for evaluation to treat *Trichuris* spp. infections in livestock and humans.

areas stable for transmission of *Trichuris trichiura*, plus another 284 million lived in areas of unstable transmission of whipworms, globally [5]. High prevalence often comes along with high abundance of protein energy malnutrition and anemia as well as limited access to medical care and educational opportunities [6]. Mild *T. trichiura* infections are often asymptomatic, but severe and chronic infections can result in the *Trichuris* dysentery syndrome including chronic inflammation of the intestine, rectal prolapse, anemia, poor growth, and clubbing of the fingers [6].

Despite the strong impact of helminthoses on public health, only four anthelmintics (albendazole, mebendazole, levamisole, and pyrantel) with only two different modes of action are listed on the WHO list of essential medicines to treat soil-transmitted nematode infections [7] with mebendazole and albendazole being by far the most commonly used drugs [8]. Whereas both drugs are highly effective against adult *A. lumbricoides* in a single dose, only albendazole is used for the treatment against tissue migrating larvae – mebendazole is poorly absorbed from the gastrointestinal tract thus its therapeutic activity is largely confined to adult/luminal worms [8]. Furthermore, the efficacy of both drugs is unsatisfactory against hookworms and *T. trichiura* in single dose regimen [9]. Higher efficacies against whipworms and hookworms were observed when albendazole or mebendazole were administered using multiple drug administration [10]. However, treatments using multiple doses significantly increase costs and management efforts in particular in poor communities lacking efficient public health infrastructure. Moreover, persistent underdosing of *A. duodenale*, *N. americanus* and *T. trichiura* within recently increased large-scaled mass drug administration campaigns against filariosis and soil-transmitted helminthosis may favor selection of highly resistant genotypes [9] as already described for *T. trichiura* [11].

In addition to its relevance in human medicine, the genus *Trichuris* also has an enormous impact on veterinary medicine. For instance, *Trichuris vulpis*, the dog whipworm, causes an intestinal parasitosis of clinical relevance and is also suspected to be zoonotic [12]. However, several anthelmintics registered for use in dogs such as diethylcarbamazine, piperazine, ivermectin and pyrantel lack efficacy against *T. vulpis* severely limiting the choice of drug for deworming [12]. In swine, infections with *Trichuris suis*, the dose-limiting nematode for all relevant anthelmintic drug classes, lead to reduced growth rates and therefore result in significant economic losses [13]. Finally, due to the long period of prepatency of *Trichuris* spp. and the lack of efficacy of most drugs against histotropic larval forms, two blocks with one to three doses each are usually necessary to completely eliminate the parasites [12].

It is therefore obvious, that the development of new, safe and highly efficacious drugs to treat soil-transmitted nematode infections is urgently required. In particular, new drugs for the treatment of *Trichuris* spp. using a single dose would significantly increase treatment options in both, human and veterinary medicine. Therefore, the evaluation of the efficacy of promising drug candidates against whipworms is an essential step towards improvement of anthelmintic treatment opportunities.

To investigate and compare the anthelmintic profiles of new drug candidates against whipworm infections, the *Trichuris muris* mouse model is highly suitable [14]. *Trichuris* L1 hatch in the small intestine of their host and migrate rapidly to the caecum and colon [15], where they invade the epithelium [16] and undergo a histotropic phase with two molts lasting several days (duration depends on the particular species and isolate). Then, larvae migrate to the surface of the epithelium extruding their caudal ends freely into the lumen of the intestine (further developed larvae or free larvae) [16]. In general anthelmintics have been reported to be less effective against histotropic larvae, which might be attributed to the poor accessibility of drugs to these larvae within the tissue [12].

In order to eliminate parasites using a single dose or at least a single treatment block, it is desirable to evaluate drug candidates not only against mature adult worms but also against histotropic larvae and further developed immature stages. Since duration of development and timespan of infection depend on both, the host strain [17] and whipworm isolate [18], a detailed characterization of the respective host-parasite relationship is essential. Thus, localization of larvae in the course of the prepatent period and onset of patency of the infection have to be analyzed carefully before *in vivo* assays against specific stages of *T. muris* can be conducted meaningfully with the respective isolate.

The cyclooctadepsipeptides [19] and the aminophenylamidines [20] are promising anthelmintic classes for further development of broad-spectrum drugs to treat intestinal nematode infections. The semi-synthetic cyclooctadepsipeptide emodepside has been shown to have an almost complete efficacy against immature and mature stages of *T. vulpis* in dogs [21] and *T. muris* in mice [22,23] while the aminophenylamidines amidantel and tribendimidine showed only low to moderate efficacy against *T. muris* in mice [24] and *T. trichiura* in humans [25–27].

Both drug classes have completely different target molecules. It is clear that the aminophenylamidines are agonists of acetylcholine receptors and have a very similar mode of action as levamisole [28,29] whereas several targets have been suggested for the cyclooctadepsipeptides with the voltage-gated, calcium-activated potassium channel SLO-1 as most important candidate [19,30,31]. However, the G-protein coupled receptor LAT-1 [32] and ionotropic $GABA_A$ receptors [33,34] might also contribute to susceptibility to cyclooctadepsipeptides.

Therefore, the present study investigated and compared the *in vivo* anthelmintic properties of the semi-synthetic cyclooctadepsipeptide emodepside, its parental natural fermentation product

PF1022A and the aminophenylamidines amidantel, deacylated amidantel and tribendimidine against *T. muris*. Since tribendimidine has previously been reported to have insufficient activity after oral administration in humans [25–27], drugs were also administered intraperitoneally and subcutaneously. In addition to the evaluation of adulticidal efficacy, PF1022A was further tested against histotropic larvae and further developed immature stages of whipworms, using single and three consecutive doses.

Materials and Methods

2.1 Ethical statement

All studies presented were conducted at the laboratories of Bayer HealthCare, Global Drug Discovery, Animal Health in Monheim, Germany. The experiments were registered and approved by the State Office for Nature, Environment, Agriculture, and Consumer Protection, North Rhine-Westphalia, Germany (reference number 200/V14), in accordance with §8a, Section 1 and 2 of the German Protection of Animals Act and the European Union directive 2010/63/EU.

2.2 Drugs

Amidantel, dAMD, emodepside and PF1022A were available at Bayer HealthCare AG, Global Drug Discovery Animal Health in Monheim, Germany. Tribendimidine was obtained from Shandong Xinhua Pharmaceutical Company Limited (Zibo, People's Republic of China). All drugs were stored at 4°C until further use. Individual drug concentrations were prepared separately as dispersions in Cremophor EL (BASF, Ludwigshafen, Germany) and deionized-water [1:3] on the days of treatment.

2.3 Animals and parasites

Female SPF inbred mice of the strain C57BL/10 ScSnOlaHsd (C57BL/10) were purchased from Harlan UK Limited, at four weeks of age. They were housed in Macrolon cages under environmentally controlled conditions and kept in groups of five animals unless otherwise indicated. Water and Sniff rodent food pellets were available *ad libitum*. Mice were allowed to acclimate for exactly seven days before starting any experiments. The *T. muris* isolate was kindly provided by Heinz Mehlhorn (Düsseldorf, Germany). A detailed history regarding isolation and passage is not available.

Mice were orally infected with a gavage using 0.2 ml fresh tap water with 200 eggs containing fully developed L1 of *T. muris*. Murine feces were collected on days 49, 56 and 63 p.i., euthanasia was performed by carbon dioxide suffocation.

Isolation of the eggs was performed as described in section 2.4.1. The development of L1 in the eggs was performed in stender dishes in an incubator at 27°C and 95% humidity for approximately 8 weeks. Progress of embryonation was controlled weekly. After development of L1 in >90% of the eggs was completed, eggs were stored at 4°C until further usage for a maximum of 6 months. Before infection of mice, the egg suspension was washed with fresh tap water at room temperature.

2.4 Parasitological characterization of a *T. muris* life cycle in C57BL/10 mice

2.4.1 Determination of the periods of prepatency and patency. To assess the duration of prepatent period, ten mice were infected. Starting from day 7 p.i., all ten mice were housed on grids to collect feces for 24 h once a week. During these periods, the bottom of the cage was covered with 300 ml tap water. Feces and water were collected in a 1 l beaker and homogenized with a hand-held blender. Using a wooden spatula, fine components of the feces were separated from remaining debris by filtration through a 200 µm sieve and collected in a clean 1 l beaker. The residues were rinsed with tap water until the filtrate reached a volume of 600 ml. After sedimentation for 1 h, the supernatants (approximately 500 ml) were removed. The sediment was centrifuged at 2,000×g and room temperature for 10 min. The pellet was resuspended in 200 ml tap water and centrifuged under the same conditions. After another washing step, the pellet was resuspended in 200 ml saturated sodium chloride solution. Then, samples were centrifuged at 2,000×g and room temperature for 5 min, the top 25 ml were filled into a 300-ml beaker and 225 ml tap water were added. After at least 2 h of sedimentation the supernatant was decanted and the sediment was washed in tap water another four times. After decanting the supernatant, the sediment (approximately 20 ml) was examined for the presence of eggs. Examination of feces was continued until three consecutive samples were found to be negative. Three independent experiments with ten mice each were performed.

2.4.2 Variation in egg output in the course of patency of the infection. To determine the variation in egg output in the course of patency of the infection, a fecal egg count method was adapted from Stoll [35]. In brief, 10 mice were infected. Only animals, positive for eggs in their feces on day 35 p.i., were included in the study. Starting from day 35 p.i., mice were housed individually on grids in Macrolon cages to collect individual feces for 12 h periods once a week. Fecal samples (0.5 g) were weighed from each mouse, 7.0 ml water were added and incubated for 15 min. Feces were roughly macerated with a wooden spatula followed by an extensive homogenization using a magnetic stirrer at low speed until samples were analyzed. For each sample, three 75 µl aliquots were pipetted on microscope slides and eggs were counted. To obtain the number of eggs per gram feces, the arithmetic mean of the three counts was multiplied by 200 to calculate the number of eggs per gram feces (epg). Feces were analyzed until 15 weeks p.i., since status of patency of the infection became quite variable afterwards (see 2.4.1 and 3.1.1).

2.4.3 Time course of localization of larvae in the course of prepatent period. To analyze the time course of the localization of larvae during prepatent period the following experiment was adapted from Panesar [36]. For this experiment 120 mice were infected. During the first 40 days p.i., three mice were euthanized daily and their duodena, caeca and colons were removed and split open. The luminal content was removed and inspected for any stages of *T. muris*. Then, the mucosa of the guts was examined for the presence of worms extruding into the lumen of the guts. Finally, duodena, caeca and colons were cut into small squares and separately incubated in 0.85% physiological sodium chloride solution at 37°C for 24 h. By carefully scraping the mucosa the histotropic larvae became visible using a dissecting microscope. Seven mice, in which not a single stage of *T. muris* was found, were excluded from the study.

2.4.4 Female/male ratio in the course of infection. On day 35 p.i., fecal examinations were performed for each of the 60 infected mice individually to confirm patency of the infection. Only animals found positive for eggs in their feces were included in the study. Weekly, starting from day 35 until day 152 p.i., three mice were euthanized and dissected. Female and male whipworms in caecum and colon were counted. Two independent experiments with 60 mice in each experiment were performed.

2.4.5 *In vitro* embryonation of *T. muris* eggs. The embryonation of eggs was analyzed and compared under several different conditions. Freshly isolated and purified eggs were suspended in (i) 0.5% formaldehyde in physiological sodium chloride solution, (ii) physiological sodium chloride solution or

(iii) tap water and transferred into 40 ml stender dishes (see [37]) to compare the rate and speed of development. The progress of embryonation was assessed weekly by microscopic analysis of three 10 µl aliquots. Eggs were counted and categorized as (i) unembryonated, (ii) partially embryonated, (iii) fully embryonated or (iv) degraded. The latter category was chosen according to the following criteria: a) vesicular appearance of unsegmented eggs or b) deformed larval structures within the eggs.

Furthermore, incubation temperatures of 4°C, 19°C, 27°C and 37°C as well as the influence of the presence of antibiotic (i.e. 10 µg/ml sisomycin plus 1 µg/ml clotrimazole), relative humidity (75%, 85% and 95%) and light conditions (light versus no light) were evaluated in tap water using the same method.

Finally, the influence of storage at 4°C after full embryonation of eggs was compared to storage at 27°C to determine the best storage condition. After embryonation at 27°C, eggs were stored at 27°C or at 4°C for 70 days. For each incubation temperature, 5 mice were infected. On day 45 p.i., mice were euthanized and worm counts were determined.

2.5 *In vivo* efficacy against *T. muris* in mice

In 24 consecutive experimental blocks, 655 mice were randomized into 132 groups, each consisting of five animals. One group of 5 mice was used for each dosage and for each administration route tested. In each block, 5 infected mice served as untreated control and received the vehicle only.

2.5.1 *In vivo* efficacy against mature adult stages of *T. muris*. On day 42 p.i., a fecal examination was performed for each mouse to confirm patency of the infection. Only animals positive for *T. muris* eggs in their feces were included in the study. Based on the individual body weight on day 45 p.i., exact dosages were calculated. In case of multiple dose regimens, three doses of the respective drug were administered orally, intraperitoneally or subcutaneously (nuchal fold) on days 46–48 p.i. Dosages used are summarized in Table 1. For single doses, 50, 75, 100, 150, 200, 250, 300 or 500 mg/kg PF1022A were administered on day 48 p.i. On day 49 p.i., mice were euthanized. Subsequently, necropsy was performed and worms in colons and caeca were counted.

2.5.2 *In vivo* efficacy against larval and immature adult stages of *T. muris*. Based on the parasitological characterization (duration of prepatent period and time-dependent localization of larvae during period of prepatency), the *in vivo* efficacy of PF1022A was also investigated against larval and immature adult stages of *T. muris*. According to the time course of localization of developmental stages in the present study and the analysis on the molting pattern in *T. muris* [36], the following time points for drug administration were chosen:

Individual body weight was determined on the day of infection for L1, on day 11 p.i. for L2 and on day 25 p.i. for further developed stages. PF1022A dosages of 10, 100, 250, and 500 mg/kg or 1.0, 10, 50 and 100 mg/kg were administered on day 3 p.i. or on days 1–3 p.i. to target L1. For the evaluation of efficacy against the histotropic L2, treatments were carried out with PF1022A dosages of 10, 100, 250, and 500 mg/kg or 1.0, 10, 50 and 100 mg/kg on day 14 p.i. or on days 12–14 p.i., respectively. Since the following molts of males and females are less synchronous [36], treatments were directed against further developed immature stages in general. Treatments with 1.0, 10, 50 and 100 mg/kg PF1022A were performed on three consecutive days (26–28 p.i.) and treatments with 10, 100, 250, and 500 mg/kg PF1022A only on day 28 p.i. Independently of the targeted stage euthanasia of mice and worm counts were performed on day 49 p.i.

2.6 Calculation of dose-response curves and statistical analysis

For parasitological characterization of the *T. muris* isolate used in C57BL/10 mice, descriptive statistics were performed using GraphPad Prism 5.03. Differences in worm counts between different weeks of infection and in sex ratio were determined by a One Way ANOVA followed by Dunnet's post hoc test using the first week of the patent period as the control against which all other time points were tested.

Table 1. Single and multiple drug dosages evaluated *in vivo* against mature adults of *T. muris*, classified by route of administration.

Dosage (mg/kg)		0.5	1.0	2.5	5.0	7.5	10	15	20	25	50	75	100	150	200	250	300	400	500
PF1022A	**3×oral**		X	X	X	X	X			X	X		X						
	1× oral										X	X	X	X	X	X	X		X
	3× subcutaneous										X		X	X	X	X	X	X	X
	3× intraperitoneal						X			X	X	X	X	X	X	X			
Emodepside	**3× oral**	X	X	X	X	X	X				X		X						
	3× subcutaneous		X				X	X	X	X	X	X	X						
	3× intraperitoneal		X		X		X	X	X	X	X		X						
Amidantel	**3× oral**																		X
	3× subcutaneous																		
	3× intraperitoneal																		
dAMD	**3× oral**		X		X		X			X	X		X						
	3× subcutaneous												X						X
	3× intraperitoneal				X		X			X	X		X						
Tribendimidine	**3× oral**		X		X		X			X	X		X						
	3× subcutaneous												X						X
	3× intraperitoneal				X		X			X	X		X						

'X' indicated that the respective drug was evaluated in the given dose using the indicated route of administration.
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For all drugs and routes of administration tested against patent *T. muris* infections, the reduction of the worm burden expressed in percent of the corresponding control groups of 5 mice was plotted against the $\log_{10}$ of the drug dosages. Efficacies were set to zero if mean of the worm counts was higher than the mean of the corresponding control group. Furthermore, the corresponding SEM values of the affected groups start from 0 (all figures showing dose- response curves). Four-parameter-logistic curves were fitted using GraphPad Prism 5.03 [38]. The top was constrained to values between 0 and 100%. The no-drug controls were set to 10^{-4} mg/kg to allow $\log_{10}$ transformation of dosages. Calculated ED_{50} and ED_{95} values were compared using the extra sum of squares F test. If multiple tests were performed, p values were corrected using the Bonferroni-Holmes procedure.

The absolute number of recovered mature adult worms after treatment against larval and immature adult stages was compared to the number of worms isolated from the negative controls by using the non-parametric Kruskal-Wallis test with Dunn's *post hoc* test for identification of significant differences between individual groups.

Results

3.1 Time course of *T. muris* development in C57BL/10 mice

3.1.1 Periods of prepatency and patency of the infection. In each of the three independent experiments determining presence of eggs in feces in weekly intervals, first eggs were found on day 35 p.i. Therefore, prepatent period lasted for at least four but not longer than five weeks. None of the mice that became patent stopped shedding eggs before week 14 p.i. However, starting from week 15 p.i., samples were much more heterogeneous. Mice in experiment 1 remained patent at least until week 16 p.i., whereas animals in experiment 2 stopped shedding eggs in week 15 p.i. In experiment 3, fecal examination was negative in week 16 p.i., but was positive in week 17 p.i., again. Patency of the infection ended in week 18 p.i.

3.1.2 Detailed analysis on egg shedding. Nine out of ten mice were found positive for eggs in their feces on day 35 p.i. Therefore, only a single mouse was excluded from the study. Furthermore, one individual mouse died on day 67 p.i. and was only included in the statistics until day 63 p.i. Variation in egg shedding in the course of patency of the infection is summarized in Figure 1. Strong variation in individual egg counts was observed, indicated by the relatively large standard deviations (Figure 1). In general, the mean eggs per gram feces (epg) increased during the first three weeks of patency of the infection starting with a mean epg of 2919±1182 (range 800–5600) at day 35 p.i. The peak in egg output was observed in week 8 p.i. (epg = 12200±3813, range 4200–19800). Starting from week 9 p.i., a gradual reduction of the average egg count was observed. In week 14 p.i. the first mouse was found to be negative for eggs in its feces. In another two mice patency of the infection ceased in week 15 p.i. For the remaining five mice the mean (± SD) of eggs per gram feces was calculated to be 1120±1110.5 (range 200–3600) on week 15 p.i., being the final week of this analysis.

3.1.3 Localization of developmental stages throughout infection. The analysis of the time course of the migration of *T. muris* stages during the period of prepatency revealed distinct phases of localization. Figure 2 summarizes the trend in absolute numbers of recovered stages in the course of the prepatent period. Supplementary Table S1 shows the individual counts divided by duodenum, caecum, colon and luminal debris.

On days 1–4 p.i. a small number (2.8±1.9, mean ± SD) of embryonated eggs was recovered from the intestinal debris of duodenum, caecum and colon. After day 5 p.i. no embryonated eggs were found in the gastrointestinal tract. Free larvae were identified in the debris of guts also for a very limited period during the first days after infection. Whereas 9.0±6.2 free larvae were recovered between day 1 and day 5 p.i. only one sample on day 8 was found positive for two free larvae. However, starting from day

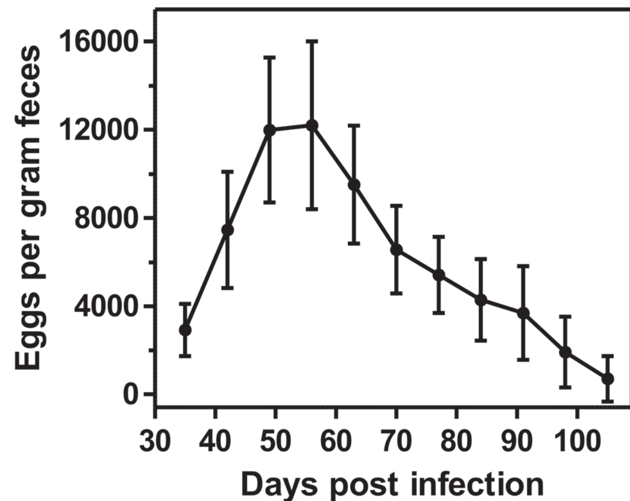

Figure 1. Analysis on egg shedding in the course of patency of the infection. The graph shows the arithmetic mean values with standard deviations of the absolute numbers of eggs per gram feces between days 35 and 105 p.i. with a group size of nine animals. Due to the death of one mouse, group size was reduced to eight starting from day 70 p.i.
doi:10.1371/journal.pntd.0002698.g001

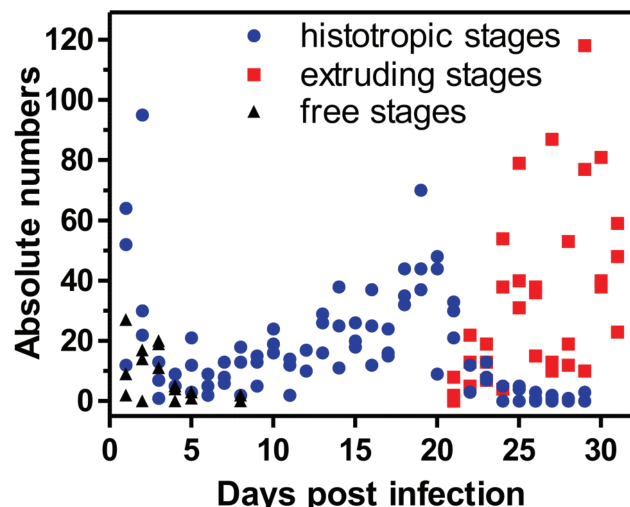

Figure 2. Analysis on the occurrence of specific stages of *T. muris* in the course of the period of prepatency. Presented is the occurrence of first stage larvae in the luminal content of the guts (free stages), of histotropic first, second and third stage larvae (histotropic stages) and of third and fourth stage larvae as well as immature and mature adults attached to the epithelium while extruding their posterior parts into the lumen of the guts (extruding stages) between days 1 and 31 p.i. Based on dissection of three mice per time point, the graph shows three data points for each stage and time point. If the count was found to be zero for a specific stage in each of the three independent counts, data points are not shown.
doi:10.1371/journal.pntd.0002698.g002

27 an increasing number of immature and mature adult worms in the debris was counted (see Supplementary Table S1).

Histotropic larvae were recovered almost throughout the whole evaluation period. However, during the period of prepatency two relative maxima in histotropic larval counts were observed. A high number of histotropic larvae was detected on days 1 and 2 p.i. (45.8±4.5), while only a small number was recovered between days 3 and 12 p.i. (10.3±4.1). Starting from day 13 the number steadily increased until day 19 p.i., where 50.3±17.4 larvae were counted (see Supplementary Table S1). From day 20 p.i. on, the number of histotropic larvae decreased again and finally, starting from day 24 p.i., the majority of the guts was found to be negative. Further developed stages were not found before day 21 p.i. The number of these stages then increased until day 24 and remained stable (42.6±13.9) until the end of the evaluation period (see Figure 2). As expected, neither histotropic larvae nor any further developed stages were found in the duodenum (Supplementary Table S1). On days 30 and 31, the intestinal debris became positive for unembryonated eggs, indicating the start of patency of the infection (Supplementary Table S1).

3.1.4 Worm counts and sex ratio. On day 35 p.i., 107/120 mice harbored a patent infection (infection rate of 89.17%). 13 uninfected mice and 4 mice which had died in the course of the experiment were exclude from the analysis.

The absolute worm counts per infected host are summarized in Figure 3A. Mean worm counts were not significantly different from those on day 35 p.i. up to day 70 p.i. (One Way ANOVA followed by Dunnet's post hoc test, $p > 0.05$) although a tendency to lower and steadily decreasing mean worm counts was observed already at earlier time-points. Thereafter, mean worm counts were significantly lower than on day 35 ($p < 0.01$) and a continuous decrease in recovered worms was observed (Figure 3A). On day 112 p.i. only five worms per mouse where recovered on average and finally on days 145 and 152 p.i. only two whipworms were found in one of the necropsied mice. In addition to the absolute worm counts, Figure 3B shows the relative sex distribution of the worms during the same evaluation period. The male/female ratio was progressively skewed towards male worms. The ratio was 1:1.27 5 weeks p.i, 1:0.97 9 weeks p.i., 1:0.56 14 weeks p.i., and 1:0.12 17 weeks p.i. Starting in week 9, the male/female ratio was significantly higher than on day 35 p.i. (One Way ANOVA followed by Dunnet's post hoc test, $p < 0.001$). From week 18 on, 100% of the recovered worms were males (see Figure 3B).

3.1.5 Optimized conditions for *in vitro* embryonation of *T. muris* eggs. The influence of different media on the rate and speed of embryonation were compared. No significant difference was observed between (i) 0.5% formaldehyde in physiological sodium chloride solution, (ii) physiological sodium chloride solution and (iii) tap water (data not shown). Therefore, tap water was used as medium for the following analyses. The incubation temperature (4°C, 19°C, 27°C or 37°C) had an enormous impact on both speed and embryonation rate (Supplementary Table S2). Speed of embryonation steadily increased with temperature. However, at 37°C the absolute number of degenerated eggs was also increased. Additives such as sisomycin plus clotrimazole or lighting conditions did not influence embryonation and were therefore neglected. However, relative humidity (75%, 85% and 95%) strongly affected the loss of medium by evaporation and therefore 95% humidity was chosen for routine purposes.

Finally, the influence on storage temperature on egg infectivity after full embryonation was tested. Mice infected with eggs stored at 27°C or at 4°C for at least 70 days were necropsied on day 45 p.i. The infection levels between both groups were not found to differ significantly, as illustrated by worm counts ranging between 28 and 45 or 12 and 59 ($p = 0.69$ using the Mann Whitney U test).

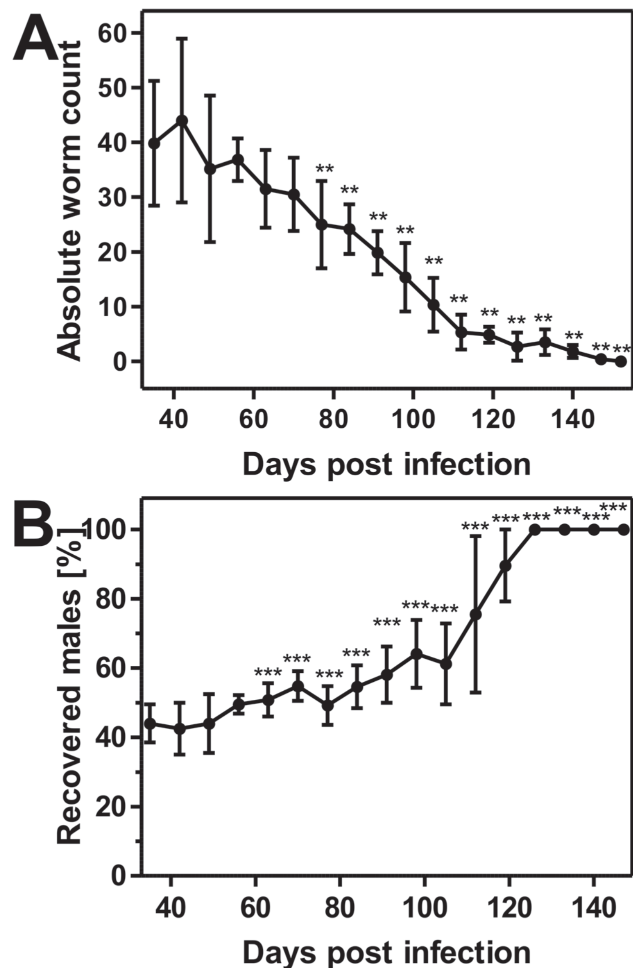

Figure 3. Analysis on the occurrence of *T. muris* in the course of patency of the infection. (A) Absolute worm counts in the course of patency of the infection. The graph shows the arithmetic mean values and standard deviations of the absolute number of recovered worms during time with a group size of six animals per time point. Mean worm counts were compared to day 35 p.i. using One-Way-ANOVA followed by Dunnet's post hoc test. **, $p < 0.01$ vs. day 35. (B) Sex ratio of *T. muris* in the course of patency of the infection. Graph shows the arithmetic means with standard deviations of the recovered male worms expressed as percentage of total recovered worms with a group size of six animals per time point. ***, $p < 0.001$ vs. day 35.
doi:10.1371/journal.pntd.0002698.g003

3.2 *In vivo* efficacy of cyclooctadepsipeptides and aminophenylamidines against *T. muris*

The average number of worms recovered from caecum and colon from untreated control mice on day 49 was 33.77±15.59. Worm counts after treatment against developmental stages were also determined on day 49 p.i. (see 2.5.2), while four mice died before evaluation and were, therefore, not included in the statistics. The highest worm count was 80, whereas no worms were recovered in two cases.

3.2.1 *In vivo* efficacy of aminophenylamidines against mature adult stages of *T. muris*. Three oral doses of 500 mg/kg of the aminophenylamidine amidantel led to no significant reduction of the worm burden. Since three high

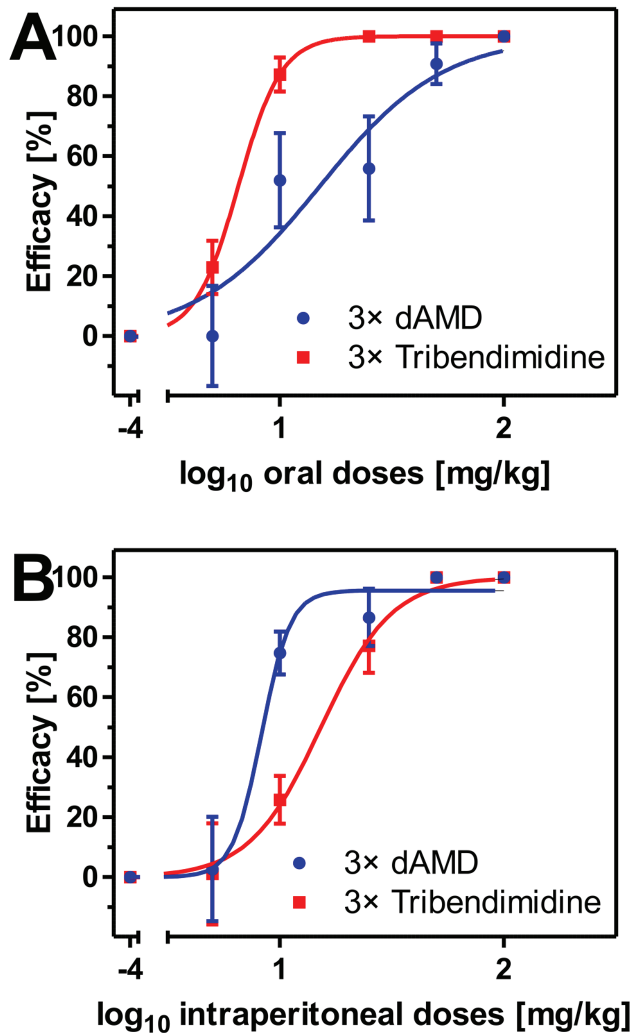

Figure 4. *In vivo* dose-response curves of dAMD (blue) and tribendimidine (red) after oral (A) and intraperitoneal (B) treatments against mature adults of *T. muris*. Dose-response curves show the arithmetic mean values and standard errors of the mean with a group size of five animals per drug and dose. Efficacy was calculated as relative number of recovered worms compared to the no-drug control in percentage. Dosages were $\log_{10}$ transformed and logistic regressions were calculated with top values constrained between 0 and 100%. Efficacies were set to zero if mean of the worm counts was higher than the mean of the corresponding control group. Furthermore, the corresponding SEM values of the affected groups start from zero. The no-drug controls were set to 10^{-4} mg/kg to allow $\log_{10}$ transformation of dosages.
doi:10.1371/journal.pntd.0002698.g004

consecutive doses of amidantel did not reduce worm counts in comparison to the no-drug control, this derivative was not further evaluated in the present study. In contrast to amidantel, both oral and intraperitoneal treatments with either tribendimidine or dAMD resulted in dose-dependent reductions of the *T. muris* burden. Dose-response curves for both drugs and both routes of administration are given in Figure 4. Furthermore, ED_{50} and ED_{95} values with 95% confidence intervals as well as p values from comparisons between the derivatives and R^2 values are summarized in Table 2.

The ED_{95} of tribendimidine was found to be approximately eight times lower than the ED_{95} of dAMD following three oral consecutive doses, whereas the ED_{95} of tribendimidine was approximately four times higher than the ED_{95} of dAMD after three intraperitoneal administrations (Table 2). However, three subcutaneous doses with 100 mg/kg or 500 mg/kg of either tribendimidine or dAMD had no effect on worm counts in comparison to the vehicle treated group (data not shown).

3.2.2 *In vivo* efficacy of cyclooctadepsipeptides against mature adult stages of *T. muris*. Three oral, intraperitoneal or subcutaneous doses of either emodepside or PF1022A on days 46–48 p.i., resulted in dose-dependent reductions of the *T. muris* burden. Table 2 summarizes ED_{50} and ED_{95} values as well as comparisons between them by administration route.

By comparing the three routes of administration, oral treatments diminished the worm burden at significantly lower doses than intraperitoneal or subcutaneous administrations (Figure 5A, B, C and Table 2). For emodepside, the ED_{50} values for intraperitoneal and subcutaneous treatments were approximately twofold and fivefold higher than for oral treatment (Table 2). The differences for PF1022A were even more pronounced. The ED_{50} values for intraperitoneal and subcutaneous treatments were approximately ten and 43-times higher in comparison to the ED_{50} values for the oral treatments.

By comparing the ED_{50} values of the two cyclooctadepsipeptides, the results were very diverse depending on the respective route of administration. However, the calculated ED_{50} value for PF1022A after three intraperitoneal doses was approximately nine times higher than the ED_{50} value of emodepside. A comparison of the two drugs after three subcutaneous doses resulted in an approximately 15-fold higher ED_{50} value for PF1022A. Surprisingly, the ED_{50} value of emodepside using three oral administrations was only twofold lower than that of PF1022A. Since the costs of PF1022A are much lower than those of emodepside and the difference between both drugs was only small for oral administration, a single oral dose against mature adult stages of *T. muris* was only evaluated for PF1022A.

A single oral administration of PF1022A on day 48 p.i. also resulted in dose-dependent reduction of the whipworm burden. A dose-response curve was calculated (Figure 5A) and ED_{50} and ED_{95} values with 95% CI as well as R^2 values are presented in Table 2. The ED_{50} value for PF1022A using a single oral dose was approximately 36-fold higher in comparison to the three oral administrations.

3.2.3 *In vivo* efficacy of PF1022A against developmental stages of *T. muris*. Both single and multiple PF1022A doses on day 28 and days 26–28 resulted in dose-dependent reductions in the number of recovered worms (Figure 6A, B and Supplementary Table S3). While a single administration of 10 mg/kg did not result in any apparent effects, 100 mg/kg or higher dosages already eliminated the worm burden completely. Three oral doses of PF1022A against developmental stages on days 26–28 also resulted in nearly complete or complete cure rates starting from 10 mg/kg. Therefore, an approximately 10-fold lower dosage of PF1022A was sufficient to cure the infection with further developed larval stages and immature adult worms with three doses in comparison to a single dose.

The efficacy of single and multiple PF1022A doses on day 14 and days 12–14 respectively, targeting the histotropic L2, also resulted in dose-dependent significant reductions of the worm burden (Figure 6C, D and Supplementary Table S3). In particular, three dosages of 100 mg/kg PF1022A or a single administration of 250 mg/kg PF1022A were required for complete elimination of whipworms.

In contrast to the efficacy against L2, the effects of PF1022A against L1 were not sufficient in the single dose regimen. While

Table 2. Comparison of the *in vivo* efficacies of the aminophenylamidines amidantel, dAMD and tribendimidine and the cyclooctadepsipeptides emodepside and PF1022A against patent *Trichuris muris* infections in mice.

Drug	Admin.	ED_{50} with 95%CI (in mg/kg)	p value[a]	ED_{95} with 95% CI (in mg/kg)	p value[b]	R^2
dAMD	3× oral	15.1 (9.9–22.9)	<0.0001 (vs. 3× tribendimidine oral)	97.3 (28.3–334.2)	0.0007 (vs. 3× tribendimidine oral)	0.8039
	3× i.p.	8.3 (7.3–9.5)	<0.0001 (vs. 3× tribendimidine i.p.)	12.8 (10.6–15.4)	<0.0001 (vs. 3× tribendimidine i.p.)	0.9349
Tribendimidine	3× oral	6.5 (6.0–7.2)	<0.0001 (vs. 3× dAMD oral)	12.6 (9.9–15.9)	0.0007 (vs. 3× dAMD oral)	0.9447
	3× i.p.	15.3 (13.2–17.7)	<0.0001 (vs. 3× dAMD i.p.)	44.8 (30.9–65.0)	<0.0001 (vs. 3× dAMD i.p.)	0.9279
Emodepside	3× oral	2.7 (1.9–3.9)	0.0009 (vs. 3× PF1022A oral)	24.5 (8.7–68.8)	0.3684 (vs. 3× PF1022A oral)	0.8368
	3× i.p.	6.1 (4.8–7.7)	<0.0001 (vs. 3× PF1022A i.p.)	40.0 (18.9–84.5)	<0.0001 (vs. 3× PF1022A i.p.)	0.9274
	3× s.c.	15.2 (13.0–17.7)	<0.0001 (vs. 3× PF1022A s.c.)	40.7 (24.5–67.4)	<0.0001 (vs. 3× PF1022A s.c.)	0.8481
PF1022A	3× oral	5.2 (4.0–6.8)	0.0009 (vs. 3× emodepside oral)	36.5 (14.9–89.8)	0.3684 (vs. 3× emodepside oral)	0.8681
	3× i.p.	55.7 (44.4–70.0)	<0.0001 (vs. 3× emodepside i.p.)	208.5 (99.2–438.2)	<0.0001 (vs. 3× emodepside i.p.)	0.8657
	3× s.c.	225.7 (180.2–282.6)	<0.0001 (vs. 3× emodepside s.c.)	515.0 (254.8–1041)	<0.0001 (vs. 3× emodepside s.c.)	0.7432
	1× oral	186.6 (111.0–313.5)	<0.0001 (vs. 3× PF1022A oral)	686.7 (168.5–2798)	<0.0001 (vs. 3× PF1022A oral)	0.6086

Presented are the ED_{50} and ED_{95} values with 95% confidence intervals (CI) and coefficients of determination (R^2) as well as p values, for determination of significant differences.
[a]Significant difference in ED_{50} to drug in brackets.
[b]Significant difference in ED_{95} to drug in brackets.
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three doses of 100 mg/kg PF1022A on days 1–3 p.i. were sufficient to completely cure the mice, a single dose on day 3 p.i., using even 500 mg/kg, was not able to significantly reduce worm burdens (Figure 6E, F and Supplementary Table S3).

Discussion

The majority of human gastrointestinal nematode infections are caused by *A. lumbricoides*, *A. duodenale*, *N. americanus*, *Strongyloides. stercoralis* and *T. trichiura* [8]. Whereas available drugs are usually highly effective against *A. lumbricoides* in a single dose regimen, at least multiple dosages of those drugs are required to cure hookworm, threadworm and particularly whipworm infections. [9,39].

In addition to the enormous impact on human medicine, the genus *Trichuris*, like *T. suis*, is also considered to be a dose-limiting nematode for most current anthelmintics in a variety of hosts of veterinary importance [13]. However, treatment options are often limited. For example, a large number of drugs (diethylcarbamazine, ivermectin, piperazine, pyrantel) registered to treat nematode infections in dogs are lacking sufficient efficacy against *T. vulpis* [12]. Among the new anthelmintics that entered the market in the recent past, especially the cyclooctadepsipeptides [21–23] and partially the aminophenylamidines [20,24,27] are active against *Trichuris* spp., whereas paraherquamide has only poor efficacy [40] and monepantel lacks efficacy [41]. For derquantel, only data describing a high efficacy of the combination with abamectin against *Trichuris ovis* have been published [42]. However, if these effects are attributed to derquantel, abamectin or only the combination of both needs to be clarified.

Persistent underdosing of *Trichuris* spp. in both humans (during the reinforced mass drug administration campaigns against lymphatic filariasis and soil-transmitted nematodes) and animals of veterinary importance may favor selection of highly resistant genotypes [9] as already described for *T. trichiura* [11]. Therefore, the urgent need for new drugs for the treatment against *Trichuris* spp., preferably in a single dose regimen, is obvious for both human and veterinary medicine.

Due to the long prepatent period of *Trichuris* spp. and the lack of efficacy of most drugs against the histotropic phase of larval forms, multiple blocks with one to three doses each are usually necessary to completely eliminate the infections [12]. Larvae of several gastrointestinal nematode species penetrate into the pits and glands of the mucosa (e.g. *Haemonchus* spp., *Ostertagia* spp., *Teladorsagia* spp.) or even penetrate and feed on individual cells (e.g. *Trichuris* spp., *Trichinella* spp.) to survive the lethargy associated with molting without losing their place in the gut [43]. These histotropic larvae are often difficult to eliminate and require higher or repeated doses when compared with luminal or mucosal stages.

In order to evaluate the effects of drugs against the histotropic stages of *T. muris*, a detailed knowledge of the time course of development within the host is required. Since data in the literature are often quite old and differ in many observations, especially regarding the number and the time course of molts (for review see [15]), the isolate used in the present study was subjected to an in-depth parasitological analysis. Furthermore, the course of infection strongly depends on the respective mouse strain [17] and *T. muris* isolate [18], making a detailed characterization even more crucial.

The parasitological data obtained here were in agreement with findings of Panesar and Croll [16]. They reported, that on day 20 p.i., all larvae were found embedded in the surface epithelium with their posterior ends extruding into the lumen of the gut. In the present study, this observation was made from day 21 onwards. In contrast to Panesar and Croll, we still found a small but significant number of histotropic stages until day 29 p.i. However, the period in which histotropic stages were exclusively present was almost the same. Interestingly, observations by Pike [37] were also in line with data shown here. They have shown, that the female/male ratio steadily develops towards more male worms and that male *T. muris* survive longer than females, which is in marked contrast to other parasitic nematode species, where females survive longer than males [37].

In the present study, no *in vivo* efficacy of the aminophenylamidine amidantel was found against patent *T. muris* infections in mice. Three oral doses of 500 mg/kg amidantel did not reduce the

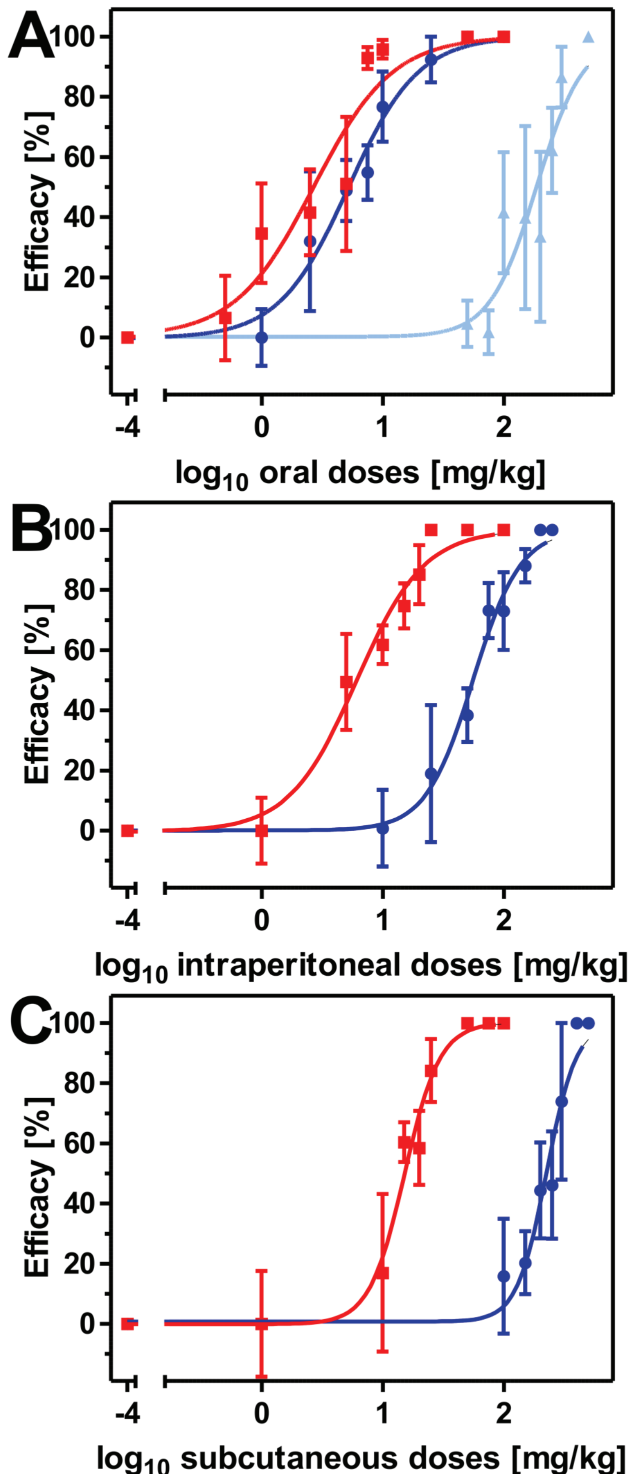

Figure 5. *In vivo* dose-response curves of emodepside (red) and PF1022A (blue) after oral (A), intraperitoneal (B) or subcutaneous (C) treatments against mature adults of *T. muris*. Dose-response curves show the arithmetic mean values with standard errors of the mean with a group size of five animals per drug and dose. Efficacy was calculated as relative number of recovered worms compared to the no-drug control in percentage. Dosages were $\log_{10}$ transformed and logistic regressions were calculated with top values constrained between 0 and 100%. Triangles indicate a single dose of PF1022A (light blue), circles three doses of PF1022A (dark blue) and squares three doses of emodepside (red). Efficacies were set to zero if mean of the worm counts was higher than the mean of the corresponding control group. Furthermore, the corresponding SEM values of the affected groups start from zero. The no-drug controls were set to 10^{-4} mg/kg to allow $\log_{10}$ transformation of dosages.
doi:10.1371/journal.pntd.0002698.g005

worm burden in comparison to the no-drug control. The efficacy of amidantel against *T. muris* was investigated previously and was also found to be only moderate [24]. Therefore, amidantel was not further evaluated in the presented study. In contrast, three consecutive oral doses with either tribendimidine or dAMD resulted in ED_{50} values of 6.5 mg/kg and 15.1 mg/kg, respectively. Complete elimination of the worm burden was achieved by three oral doses using either 25 mg/kg tribendimidine or 100 mg/kg dAMD. Oral doses of 1×400 mg [44] or 3×400 mg [26] tribendimidine have been shown to result in cure rates of 76.8% and 33.3%, respectively, against *T. trichiura* in humans. Intraperitoneal injections of the drugs, which to our knowledge were evaluated for the first time, resulted in reversed potency with ED_{50} values of 15.3 mg/kg for tribendimidine and 8.3 mg/kg for dAMD and complete elimination at dosages above 50 mg/kg in both cases. This is somewhat surprising since tribendimidine is known to rapidly disintegrate in aqueous environments releasing two molecules of dAMD [45]. Differences in release of the highly hydrophobic drugs from the used formulation (dispersion containing Cremophor EL/deionized water) are the most likely explanation for the observed phenomenon. The larger tribendimidine molecule can be suspected to diffuse more slowly into the aqueous environment. It can be assumed that release of drugs from the dispersion occurs more rapidly in the digestive track under mechanical mixing in the presence of bile salts than in the peritoneum and that passive diffusion is of minor importance in the gut. The absence of efficacy of tribendimidine and dAMD using subcutaneous administrations might also be due to the very basic formulation of the drugs. However, neither intraperitoneal nor subcutaneous administrations, using such a basic formulation, were able to significantly improve the efficacy of tribendimidine or dAMD against *T. muris* in mice.

In contrast to the aminophenylamidines, the cyclooctadepsipeptide, emodepside, has previously been shown to be completely effective against *T. vulpis* [21] and also *T. muris* [22,23]. A single dosage of 7.16 mg/kg emodepside in the Profender spot on formulation for cats was sufficient to clear patent *T. muris* infections of mice within 48 h [22] and even treatments of mice against immature stages using 6.0 mg/kg emodespide of the same formulation on day 3, day 20 or day 35 p.i., resulted in significantly reduced worm counts (>95% efficacy) [23]. Next to the oral tablet formulation of Profender for dogs with 1 mg/kg emodepside [21], also a single dose of 0.45 mg/kg emodepside of the oral Procox suspension was sufficient to completely eliminate immature and mature *T. vulpis* from dogs [46]. However, almost all investigations on the efficacy and safety of emodepside were conducted on nematodes of veterinary importance and only few *in vitro* data on important nematodes of humans are available [47], and PF1022A has not been evaluated against *Trichuris* spp. at all. However, while no clinical signs of intolerability were found, a high degree of efficacy against a large number of helminths in a variety of hosts including *Heligmosomoides bakeri* in mice [48], *Strongyloides ratti* and *Nippostrongylus brasiliensis* in rats, *Ancylostoma caninum* in dogs, cyathostomes in horses, *Trichostrongylus colubriformis* and *Haemonchus contortus* in sheep and *Dictyocaulus viviparus* in cattle using fairly low dosages of 1–10 mg/kg PF1022A were reported [49].

There were also differences in efficacy comparing emodepside and PF1022A in the present study, but the magnitude of these

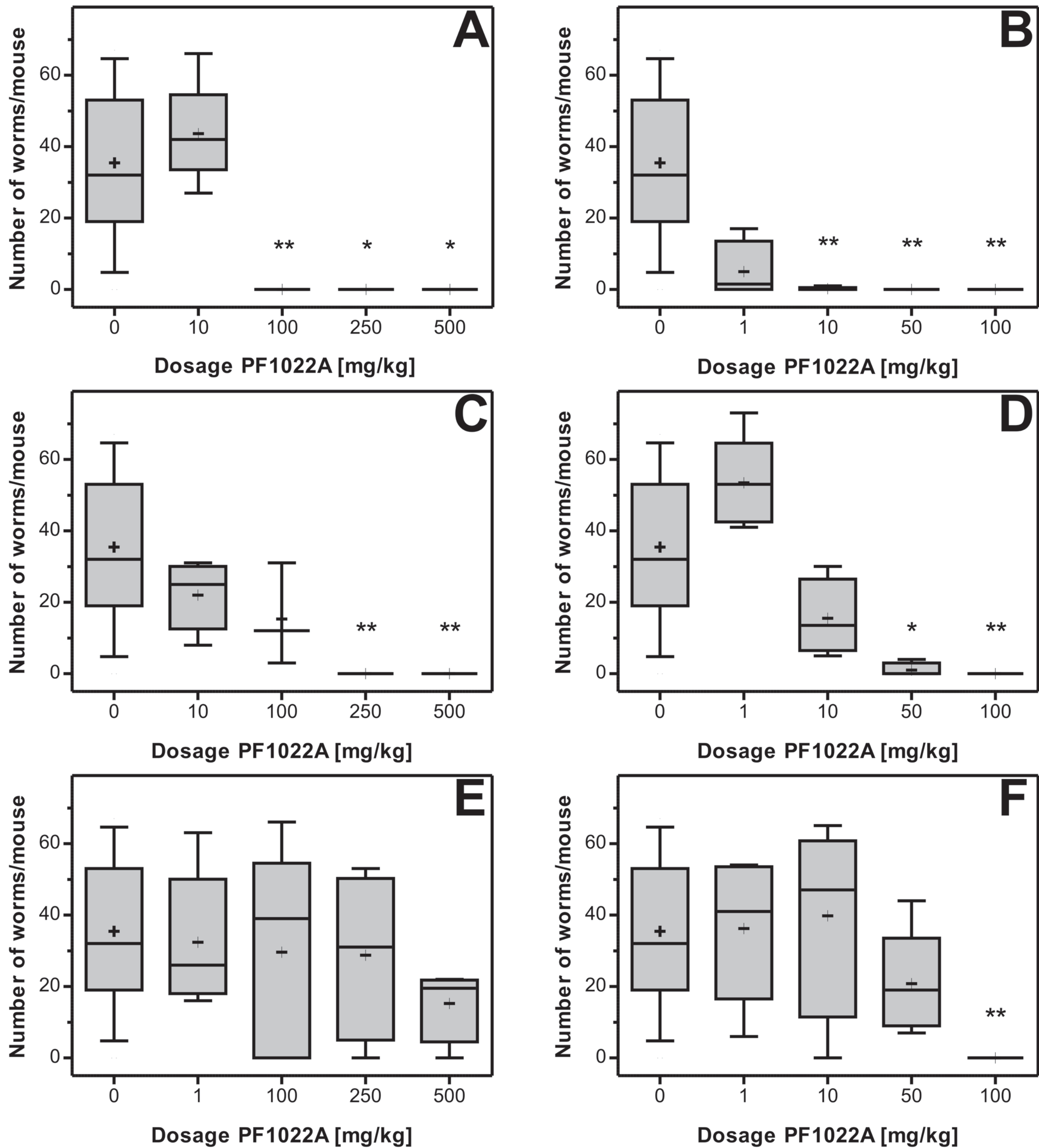

Figure 6. *In vivo* efficacy of PF1022A against L3, L4 and immature adults (A, B), histotropic L2 (C, D) and histotropic L1 (E, F) of *T. muris* using both, single (A, C, E) and triple (B, D, F) dose regimens. Box plots show the median numbers and quartiles of recovered adult *T. mu*ris after treatment against immature stages with wiskers representing minimal and maximal values. Group sizes were 5 mice per drug and dose. +, arithmetic mean; *, $p<0.01$ vs. control; **, $p<0.001$ vs. control.
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differences was dependent on the route of administration. However, emodepside always performed significantly better than PF1022A using the ED_{50} value as criterion. The difference between both drugs was particularly small for the oral administration, which also performed better than the intraperitoneal and the subcutaneous route. The ED_{50} and ED_{95} values for PF1022A were only 1.9 and 1.5 fold higher than those for emodepside, respectively.

For its suitability in mass-drug-treatment programs, drugs need a high safety and production costs should be as low as possible.

Due to the fact that the class of aminophenylamidines is still considered to be potentially hazardous [50] and PF1022A does have much cheaper production costs than emodepside (due to omission of semi-synthetic derivatization) [47], single dose experiments and treatments targeting developmental stages were only performed with PF1022A. In addition, no data regarding the effects of PF1022A on any stages of *Trichuris* spp. have been published previously. To examine whether PF1022A has the potential to replace the more expensive emodepside in therapy of *Trichuris* spp., it is important to determine the suitability of PF1022A as a broad-spectrum anthelmintic.

At least in the triple dose regimen, PF1022A was able to completely eliminate all developmental stages of *T. muris*. However, the required dosages inversely correlated with the time span after infection, i.e. the earlier stages had to be treated with higher dosages. Single drug administration needed 2.5 to 10-fold higher dosages to achieve complete resolution of the infections, and against L1 larvae no significant effect on worm burdens could be obtained using only a single dose. The most likely reason for this observation is the localization of the larvae. Larvae develop deep in the epithelium of the basal parts of the crypts of Lieberkühn until day 5 p.i., while they were found closer to the surface of the epithelium between days 5 and 10 p.i. On day 15 p.i., a large proportion of histotropic larvae was already found in the epithelial surface, where a higher drug concentration might be present [16].

One might think that the relatively high dosages of PF1022A required to completely eliminate developmental *T. muris* stages, especially in single dose regimens, could prevent its further development as trichuricidal drug. However, potential improvement of efficacy through optimized galenic formulations should be taken into account. The potential of cyclooctadepsipeptides for efficient treatment against *Trichuris* spp. has been shown using single oral administration of Profender tablets (Bayer Animal Health GmbH, Leverkusen, Germany). A dose rate of 1 mg/kg emodepside resulted in almost complete elimination of immature and mature stages of *T. vulpis* in dogs (>99%) [20], suggesting that optimized formulations can dramatically improve drug performance in this drug class. The formulation in Profender tablets is optimized to eliminate all relevant parasitic nematodes of dogs and optimization can be considered to improve drug efficacy. Emodepside is also the only nematocidal ingredient of Profender spot-on for cats and Procox suspension for puppies. Although using a different route of administration (dermal) Mehlhorn et al. [22] have shown that a single dosage of 7.16 mg/kg emodepside in the Profender spot on formulation for cats was sufficient to clear *T. muris* infections of mice within 48 h. In sharp contrast to that, three consecutive oral doses of 75 mg/kg emodepside using the Cremophor EL/water dispersion were required to achieve a complete elimination of patent *T. muris* infections in the present study. The more than 10-fold increase in efficacy between three doses using Cremophor/water and a single dose using the optimized Profender formulation emphasizes that every drug formulation has to be optimized for each drug and host species and that dramatic decreases in required drug dosages are possible when using an optimized formulation. In addition, optimization of formulations also decreases the risk of intoxications and the costs of treatment, in particular, if drugs can be targeted specifically towards the location of the parasite, e.g., the gut, avoiding high drug concentrations in tissues, e.g., the brain, which may be important for side effects.

In conclusion, *in vivo* treatments with relatively high doses of PF1022A resulted in complete elimination of *Trichuris muris*, including mature adult and immature adult worms as well as histotropic and further developed larval stages in a single-dose regimen. Since only non-optimized formulations were evaluated in this study, considerably lower dosages might be achievable, using formulations optimized for particular host species. Despite the fact that detailed safety and pharmacokinetic studies are still completely missing for humans, distinct effects of PF1022A against the usually dose-limiting genus *Trichuris* in the mouse model suggest that cyclooctadepsipeptides are useful candidates for development as agents against human soil-transmitted helminthoses and nematode infections of livestock animals.

Supporting Information

Table S1 Localization of *T. muris* stages throughout prepatency.
(PDF)

Table S2 Temperature dependency of *T. muris* larval development in eggs.
(PDF)

Table S3 Descriptive statistics for each treatment group (classified by individual drugs, dose regimen and route of administration).
(PDF)

Author Contributions

Conceived and designed the experiments: DK AH GvSH. Performed the experiments: DK. Analyzed the data: DK JK. Wrote the paper: DK JK GvSH.

References

1. Awasthi S, Bundy DA, Savioli L (2003) Helminthic infections. BMJ 327: 431–433.
2. Lustigman S, Prichard RK, Gazzinelli A, Grant WN, Boatin BA, et al. (2012) A research agenda for helminth diseases of humans: the problem of helminthiases. PLoS Negl Trop Dis 6: e1582.
3. Knopp S, Steinmann P, Keiser J, Utzinger J (2012) Nematode infections: soil-transmitted helminths and trichinella. Infect Dis Clin North Am 26: 341–358.
4. Hotez PJ, Fenwick A, Savioli L, Molyneux DH (2009) Rescuing the bottom billion through control of neglected tropical diseases. Lancet 373: 1570–1575.
5. Pullan RL, Brooker SJ (2012) The global limits and population at risk of soil-transmitted helminth infections in 2010. Parasit Vectors 5: 81.
6. Stephenson LS, Holland CV, Cooper ES (2000) The public health significance of Trichuris trichiura. Parasitology 121 Suppl: S73–95.
7. World Health Organization (2011) WHO model list of essential medicines : 17th list, March 2011. Geneva: World Health Organization. 45 p. p.
8. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, et al. (2006) Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet 367: 1521–1532.
9. Prichard RK, Basanez MG, Boatin BA, McCarthy JS, Garcia HH, et al. (2012) A research agenda for helminth diseases of humans: intervention for control and elimination. PLoS Negl Trop Dis 6: e1549.
10. Keiser J, Utzinger J (2010) The drugs we have and the drugs we need against major helminth infections. Adv Parasitol 73: 197–230.
11. Diawara A, Drake LJ, Suswillo RR, Kihara J, Bundy DA, et al. (2009) Assays to detect beta-tubulin codon 200 polymorphism in Trichuris trichiura and Ascaris lumbricoides. PLoS Negl Trop Dis 3: e397.
12. Traversa D (2011) Are we paying too much attention to cardio-pulmonary nematodes and neglecting old-fashioned worms like Trichuris vulpis? Parasit Vectors 4: 32.
13. Arends J, Vercruysse J (2002) The use of macrocyclic lactones to control parasites of pigs. In: Vercruysse J, Rew RS, editors. Macrocyclic lactones in antiparasitic therapy. Wallingford, UK and New York, USA: CABI Publishing.
14. Keeling JE (1961) Experimental trichuriasis. II. Anthelmintic screening against Trichuris muris in the albino mouse. J Parasitol 47: 647–651.
15. Anderson RC (2000) Nematode parasites of vertebrates : their development and transmission. Wallingford, Oxon, UK; New York, NY: CABI Pub. xx, 650 p. p.
16. Panesar TS, Croll NA (1980) The location of parasites within their hosts: site selection by Trichuris muris in the laboratory mouse. Int J Parasitol 10: 261–273.
17. Wakelin D (1967) Acquired immunity to Trichuris muris in the albino laboratory mouse. Parasitology 57: 515–524.

18. Johnston CE, Bradley JE, Behnke JM, Matthews KR, Else KJ (2005) Isolates of Trichuris muris elicit different adaptive immune responses in their murine host. Parasite Immunol 27: 69–78.
19. Krücken J, Harder A, Jeschke P, Holden-Dye L, O'Connor V, et al. (2012) Anthelmintic cyclcooctadepsipeptides: complex in structure and mode of action. Trends Parasitol 28: 385–394.
20. Xiao SH, Utzinger J, Tanner M, Keiser J, Xue J (2013) Advances with the Chinese anthelminthic drug tribendimidine in clinical trials and laboratory investigations. Acta Trop 126: 115–126.
21. Schimmel A, Altreuther G, Schroeder I, Charles S, Cruthers L, et al. (2009) Efficacy of emodepside plus praziquantel tablets (Profender tablets for dogs) against mature and immature adult Trichuris vulpis infections in dogs. Parasitol Res 105 Suppl 1: S17–22.
22. Mehlhorn H, Schmahl G, Frese M, Mevissen I, Harder A, et al. (2005) Effects of a combinations of emodepside and praziquantel on parasites of reptiles and rodents. Parasitol Res 97 Suppl 1: S65–69.
23. Schmahl G, Mehlhorn H, Harder A, Klimpel S, Krieger KJ (2007) Efficacy of combination of emodepside plus praziquantel against larval and adult stages of nematodes (Trichuris muris, Angiostrongylus cantonensis) in rodents. Parasitol Res 101: 77–84.
24. Wollweber H, Niemers E, Flucke W, Andrews P, Schulz HP, et al. (1979) Amidantel, a potent anthelminthic from a new chemical class. Arzneimittelforschung 29: 31–32.
25. Steinmann P, Zhou XN, Du ZW, Jiang JY, Xiao SH, et al. (2008) Tribendimidine and albendazole for treating soil-transmitted helminths, Strongyloides stercoralis and Taenia spp.: open-label randomized trial. PLoS Negl Trop Dis 2: e322.
26. Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W (2005) Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop 94: 1–14.
27. Xiao SH, Wu ZX, Zhang JH, Wang SQ, Wang SH, et al. (2007) Clinical observation on 899 children infected with intestinal nematodes and treated with tribendimidine enteric coated tablets. Chin J Parasit Dis 25: 372–375.
28. Hu Y, Xiao SH, Aroian RV (2009) The new anthelmintic tribendimidine is an L-type (levamisole and pyrantel) nicotinic acetylcholine receptor agonist. PLoS Negl Trop Dis 3: e499.
29. Miltsch SM, Krucken J, Demeler J, Ramunke S, Harder A, et al. (2013) Interactions of anthelmintic drugs in Caenorhabditis elegans neuro-muscular ion channel mutants. Parasitol Int 62: 591–8.
30. Welz C, Kruger N, Schniederjans M, Miltsch SM, Krucken J, et al. (2011) SLO-1-channels of parasitic nematodes reconstitute locomotor behaviour and emodepside sensitivity in Caenorhabditis elegans slo-1 loss of function mutants. PLoS Pathog 7: e1001330.
31. Guest M, Bull K, Walker RJ, Amliwala K, O'Connor V, et al. (2007) The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in Caenorhabditis elegans. Int J Parasitol 37: 1577–1588.
32. Saeger B, Schmitt-Wrede HP, Dehnhardt M, Benten WP, Krucken J, et al. (2001) Latrophilin-like receptor from the parasitic nematode Haemonchus contortus as target for the anthelmintic depsipeptide PF1022A. FASEB J 15: 1332–1334.
33. Chen W, Terada M, Cheng JT (1996) Characterization of subtypes of gamma-aminobutyric acid receptors in an Ascaris muscle preparation by binding assay and binding of PF1022A, a new anthelmintic, on the receptors. Parasitol Res 82: 97–101.
34. Miltsch SM, Krucken J, Demeler J, Janssen IJ, Kruger N, et al. (2012) Decreased emodepside sensitivity in unc-49 gamma-aminobutyric acid (GABA)-receptor-deficient Caenorhabditis elegans. Int J Parasitol 42: 761–770.
35. Stoll NR (1923) Investigations on the control of hookworm disease. XV. An effective method of counting hookworm eggs in feces. Am J Epidemiol 3: 59–70.
36. Panesar TS (1989) The moulting pattern in Trichuris muris (Nematoda: Trichuroidea). Can J Zool 67: 2340–2343.
37. Pike EH (1969) Egg output of Trichuris muris (Schrank, 1788). . J Parasitol 55: 1046–1049.
38. Motulsky H, Christopoulos A (2004) Fitting models to biological data using linear and nonlinear regression : a practical guide to curve fitting. Oxford; New York: Oxford University Press. 351 p. p.
39. Suputtamongkol Y, Premasathian N, Bhumimuang K, Waywa D, Nilganuwong S, et al. (2011) Efficacy and safety of single and double doses of ivermectin versus 7-day high dose albendazole for chronic strongyloidiasis. PLoS Negl Trop Dis 5: e1044.
40. Shoop WL, Eary CH, Michael BF, Haines HW, Seward RL (1991) Anthelmintic activity of paraherquamide in dogs. Vet Parasitol 40: 339–341.
41. Tritten L, Silbereisen A, Keiser J (2011) In vitro and in vivo efficacy of Monepantel (AAD 1566) against laboratory models of human intestinal nematode infections. PLoS Negl Trop Dis 5: e1457.
42. Little PR, Hodge A, Maeder SJ, Wirtherle NC, Nicholas DR, et al. (2011) Efficacy of a combined oral formulation of derquantel-abamectin against the adult and larval stages of nematodes in sheep, including anthelmintic-resistant strains. Vet Parasitol 181: 180–193.
43. Sutherland I, Scott I (2010) Gastrointestinal nematodes of sheep and cattle - biology and control. Oxford, United Kingdom, Ames, Iowa, United States of America: Blackwell Publishing.
44. Wu ZX, Fang YY, Liu YS (2006) Effect of a novel drug – enteric coated tribendimidine in the treatment of intestinal nematode infections. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 24: 23–26.
45. Yuan G, Xu J, Qu T, Wang B, Zhang R, et al. (2010) Metabolism and disposition of tribendimidine and its metabolites in healthy Chinese volunteers. Drugs R D 10: 83–90.
46. Petry G, Altreuther G, Wolken S, Swart P, Kok DJ (2013) Efficacy of emodepside plus toltrazuril oral suspension for dogs (Procox(R), Bayer) against Trichuris vulpis in naturally infected dogs. Parasitol Res 112 Suppl 1: 133–138.
47. Olliaro P, Seiler J, Kuesel A, Horton J, Clark JN, et al. (2011) Potential drug development candidates for human soil-transmitted helminthiases. PLoS Negl Trop Dis 5: e1138.
48. Nwosu U, Vargas M, Harder A, Keiser J (2011) Efficacy of the cyclooctadepsipeptide PF1022A against Heligmosomoides bakeri in vitro and in vivo. Parasitology 138: 1193–1201.
49. von Samson-Himmelstjerna G, Harder A, Schnieder T, Kalbe J, Mencke N (2000) In vivo activities of the new anthelmintic depsipeptide PF 1022A. Parasitol Res 86: 194–199.
50. Epe C, Kaminsky R (2013) New advancement in anthelmintic drugs in veterinary medicine. Trends Parasitol 29: 129–134.