Innovative options for the treatment of non-melanoma skin cancer

Investigations on the activity of antimicrobial peptides against topical diseases and study of peptide penetration into human skin *ex vivo*

DISSERTATION

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Presented by

Nhung Do

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1st Reviewer: Prof. Dr. Monika Schäfer-Korting
Institute of Pharmacy (Pharmacology and Toxicology)
Freie Universität Berlin
Königin-Luise-Straße 2+4
14195 Berlin

2nd Reviewer: Prof. Dr. Jens Rolff
Institute of Biology
Freie Universität Berlin
Königin-Luise-Straße 1-3
14195 Berlin

Date of defense: 19. November 2014

This thesis was done under the supervision of Prof. Dr. Monika Schäfer-Korting

Institute of Pharmacy (Pharmacology and Toxicology)

Freie Universität Berlin

For my family

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Abbreviations

AK	actinic keratosis
AMP	antimicrobial peptide
ATP	adenosine triphosphate
Balb/c mouse	albino, laboratory-bred mouse strain
BCC	basal cell carcinoma
BMAP	bovine myeloid antimicrobial peptide
CHO	chinese hamster ovary
CMS nanotransporter	core multishell nanotransporter
COX	cyclooxigenase
CPP	cell-penetrating peptide
Da	dalton
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DP	differential power
DS-Nh mouse	disorganized non-hair mouse
e.g.	exempli gratia, for example
EGF	epidermal growth factor
EMA	European Medicines Agency
FDA	Food and Drug Administration
5-FU	5-fluorouracil
HeLa cell	Henrietta Lacks, immortal cell line from cervical cancer
HIV	human immunodeficiency virus

HNP	human neutrophil peptide
HPLC	high-pressure liquid chromatography RP-HPLC: reverse phase-HPLC
ICC	indotricarbocyanine
i.e.	id est, that is
ITC	isothermal titration calorimetry
LPS	lipopolysaccharide
ml	millilitre
mg	milligram
mPEG	methoxypoly(ethylene glycol)
Mr	relative molecular mass
NHK	normal human keratinocyte
NMSC	non-melanoma skin cancer
PMSF	phenylmethanesulfonyl fluoride
SCC	squamous cell carcinoma SCC12: SCC cell lines derived from head and neck SCC25: SCC cell lines derived from tongue
siRNA	small interfering ribonucleic acid
SLN	solid lipid nanoparticle
tat peptide	transcription-transactivating peptide
TiO_2	titanium dioxide
TJ	tight junction
ODN	oligodeoxynucleotide
UVB	ultraviolet B

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1. INTRODUCTION

1.1 The barrier function of the skin

The human skin represents a fundamental barrier against the environment. Its function is versatile ranging from protection against microorganisms, physical or mechanical stress to the regulation of body temperature and water loss. Additionally, the skin is a sensory organ for the recognition of pressure, temperature and pain.

Three layers including the epidermis, dermis and hypodermis manage these essential functions. The epidermis is divided into the stratum corneum and the viable epidermis. The stratum corneum, the outermost layer, represents the main physical barrier. Dehydrated, anuclear keratinocytes (corneocytes), embedded in a complex lipid matrix, restrict the penetration of exogenous compounds and invasion of microorganisms, while the regulation of body water loss is possible. The viable epidermis provides additional stability. It is build up by viable keratinocytes in different stages of differentiation, which migrate from the basal layer outwards to the skin surface. The vascularized dermis offers elasticity and guarantees blood and nutrient supply by elastin fibers and collagen bundles. The hypodermis follows the dermis and represents an energy reservoir and cold protection system with its adipocytes.

Sufficient lipophilicity and low molecular weight are essential properties of compounds to surmount the stratum corneum, the major physical barrier of the skin. However, aqueous solubility is also necessary in particular for the permeation through the second physical barrier, the viable epidermis and the dermis [1]. Accordingly, entrance into the skin is possible, i.e. alongside the stratum corneum's lipid matrix (intercellular route) or across hair follicles, sebaceous glands and sweat glands (transappendageal route). Less clear is the uptake through corneocytes and lipid matrix (transcellular route, Figure 1).

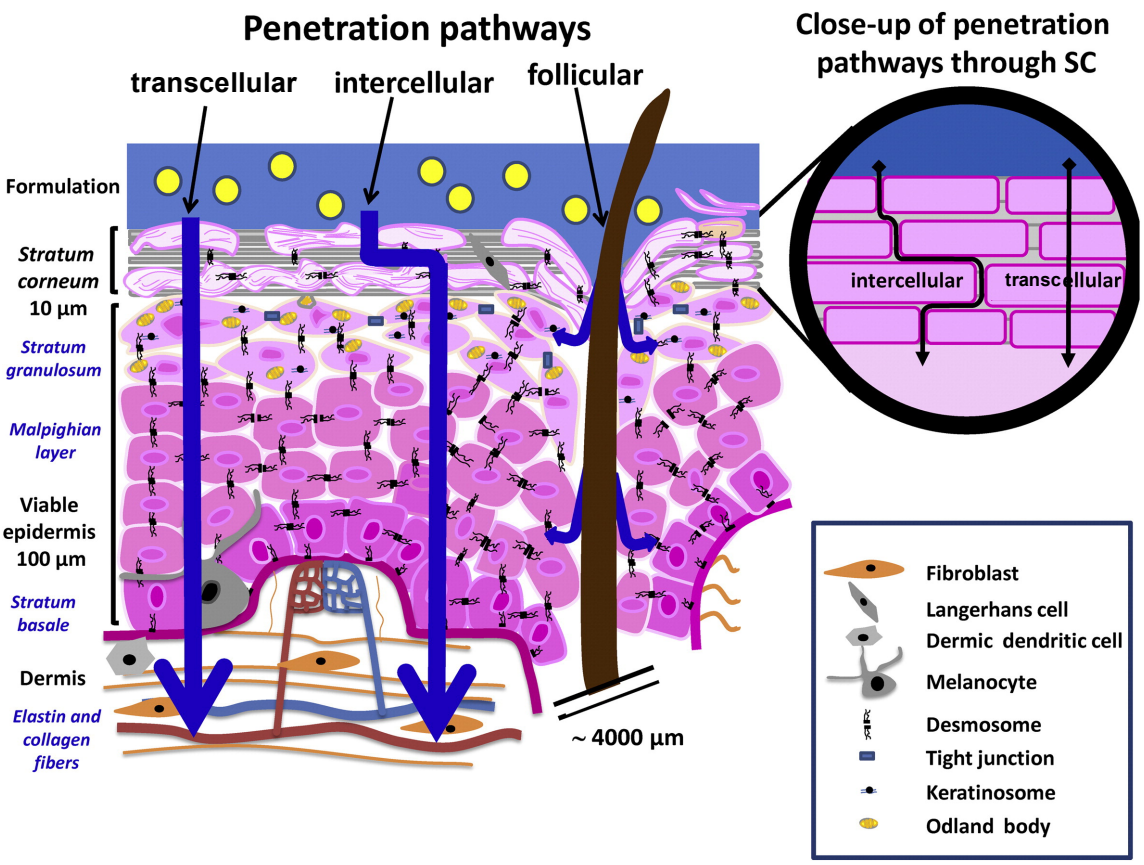

Figure 1: Penetration pathways across the skin [2].

In addition, tight junction (TJ) proteins such as the transmembrane proteins occludin and claudins, junctional adhesion molecules and TJ plaque proteins ZO-1 and ZO-3, exhibit barrier function in human skin. They regulate the paracellular pathway of molecules including water and solutes, restrict the entrance of pathogens but also mediate the transepidermal water loss [3-5]. Mainly located in the stratum granulosum between neighboring cells of the interfollicular epidermis and skin appendages, expression of TJ proteins is strongly influenced by the stratum corneum's condition. Up or down regulation and change in localization was observed in diseased skin with perturbed stratum corneum barrier function e.g. psoriasis vulgaris, ichthyosis vulgaris and skin infections [6,7]. Therefore, TJ proteins may influence skin penetration by influencing the barrier function of the skin.

In human skin, a broad variety of different enzymes exists. These enzymes belong to the skin's metabolic barrier and are situated especially in the viable epidermis, sebaceous glands and hair follicles [8,9]. They range from phase I drug metabolizing enzymes, such as cytochrome P450 enzymes, alcohol dehydrogenases, esterases and amidases, to phase II drug metabolizing enzymes including glutathione S-

transferases or glucuronyl-, sulfo- and acetyltransferases [10,11]. Metabolic activity is essential in the use of prodrugs, where biotransformation is crucial to generate the effective drug. This sophisticated strategy can be used to reduce adverse effects or enhance drug stability, selectivity and efficacy. For example, lipophilic glucocorticoid diester e.g. prednicarbate can penetrate into the skin very efficiently, but only show weak binding affinity to the glucocorticoid receptor. However, esterases in human skin can hydrolyse the diester at C-21 by to the very effective C-17 glucocorticoid monoester derivative, increasing glucocorticoid effects. Nonetheless, drug metabolism of active substances can result in loss of activity and quick clearance of the drug. Additionally, a change in penetration characteristics and altered toxic profile by biotransformation is possible. Although topically applied drugs are less affected by metabolism compared to oral administration resulting in initial access to the liver, knowledge about biotransformation profiles is crucial to guarantee sufficient efficiency and control toxic effects.

Next to the physical and metabolic barrier, the skin exhibits an extensive antibacterial barrier. Especially antimicrobial peptides (AMPs) possess essential functions against invaders from the environment. Two antimicrobial peptide families, α-helical cathelicidins and β-sheet defensins (for details see Table 1), have major roles in the human skin and are produced in keratinocytes, neutrophils and sebocytes [12]. They can either be constitutively expressed, especially at the sites of potential bacterial entry e.g. hair follicles, or their production might be induced in response to skin infections. In addition, both possibilities may occur. AMPs can act directly against microorganisms or they activate host defense cells by initiating inflammation and cytokine release [13]. Furthermore, they are involved in pathophysiological mechanisms of various skin diseases. Up-regulation of AMPs was observed in psoriasis, rosacea and acne vulgaris. In patients with Atopic Dermatitis, the expression of the cathelicidin LL-37 and β-defensins HBD-2 and 3 is decreased, while other AMPs such as psoriasin and RNase 7 are increased [13,14]. Moreover, AMPs can influence wound healing and angiogenesis [15].

1.2 Non-Melanoma Skin Cancer

1.2.1 General aspects of disease

Epidemiological studies show an increased incidence of cancerous diseases in Germany. Most frequent cancers are located in the intestine, lung and prostate for men or breast for women (Table 1). Until most recently, non-melanoma skin cancer (NMSC) is not included since it does not belong to the malicious emergent cancer diseases. However, when looking at incidence rates 101,100 and 89,500 new NMSC diseases were counted for men and women in Germany in 2010 (Krebs in Deutschland 2009/2010, chapter 3.28 [16]). These facts are most worrying as they supersede the most common malicious cancer diseases for women (70,340 breast) and men (65,830 prostate) from the same year. NMSC includes actinic keratosis (AK), squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), which prevalently establish on sun-exposed skin areas of older people with light-colored skin. Therefore, an increased incidence of NMSC needs to be expected in particular due to the demographic ageing population. Further risk factors include infection with human papilloma virus and chronically injured or diseased skin as well as immunodeficiency due to diseases e.g. HIV or medication such as glucocorticoids or other immunosuppressant agents. Importantly, if left untreated, tumor cells can invade into adjacent tissues of the body or metastasize.

Table 1: Overview of selected frequent common cancer locations compared with cancer occurrence in non-melanoma skin in Germany in 2010 [16].

localization	**incidence of new detected cancer disease**	
	men	women
lung	35,040	17,030
intestine	33,800	28,630
breast	610	70,340
prostate	65,830	-
non-melanoma skin	101,100	89,500
basal cell	77,800	73,800
squamous cell	22,000	14,700

Actinic keratosis (AK), also called solar keratosis, is a carcinoma in-situ, described by discrete lesions of keratinocyte dysplasia, which are restricted to the epidermis. First, focal areas of atypical keratinocytes develop at the stratum spinosum, which can progress to the stratum granulosum and to broad areas of the epidermis. AK lesions grow slowly and without treatment they can persist or may even regress spontaneously. However, progression into malignant squamous cell carcinoma (SCC) and invasion into the dermis can occur [17,18]. Treatment of AK is therefore essential to reduce risk of progression into SCC, since a prediction of possible outcomes of the individual lesion is not possible.

Squamous cell carcinoma (SCC) displays firm lesions, which are pink or skin colored with sometimes itchy or painful symptoms. The majority of SCC arise from existing AK-lesions. The earliest stage of SCC is called Bowen disease. Here, lesions tend to be larger, more reddish and scalier than AK-lesions. They can progress to invasive SCC, which spread as metastases in other parts of the body. Here, treatment is essential, especially in the early stages.

Basal cell carcinoma (BCC) lesions occur in the lowest layer of the epidermis, the basal cell layer. Although tumor growth is slow, the treatment of BCC is challenging due to frequent recurrence and if left untreated, an invasion into nearby tissues of the skin may happen.

1.2.2 Current therapeutic options

Management of NMSC starts with extensive patient education for an attentive behavior toward sun-exposition and the use of UV-protection creams. Furthermore, self-examination of the skin is essential for the detection of novel lesions. Current treatment options address individual lesions (lesion-directed therapy) or for patients with multiple lesions, additionally the surrounding skin (field-directed therapy).

Invasive methods include shave excision, dermabrasion and chemical peels for the removement of larger areas of diseased skin. Cryosurgery and curettage, which show high efficacy and good tolerability, are used in lesion-directed therapy, but are not favored by patients due to pain and scarring [19]. Especially for BCC, surgical excision belongs to the first line therapy in particular for the infiltrative subtype.

The photodynamic therapy is based on the production of reactive oxygen species. Illumination of the applied photosensitizers e.g. methyl aminolevulinate on lesions of NMSC results in induction of apoptosis or necrosis [20]. This therapy applies for AK and superficial BCC, as the photosensitizer does not penetrate into deeper tissues. Hence photodynamic therapy should not be used for the treatment of invasive SCC, nodular or thick BCC (>2 mm). The photodynamic therapy is not invasive, but effective (>90 % cure rates) and does not result in scarring. However, acute pain during time of light exposure and high recurrence rate limits its usage.

Topical pharmacotherapy is often preferred in the field-directed therapy to treat multiple lesions and reduce scarring, in particular when surgery is not possible. The anticancer agent 5-fluorouracil (5-FU) inhibits the thymidilate synthetase resulting in interference of DNA synthesis. Topical monotherapy with 5-FU ointment or – rarely – a combination with other therapeutic options is used for AK and superficial BCC. Depending on the used concentration and treatment duration, in general twice daily for up to 6 weeks, cure rates up to 90 % may be achieved for superficial BCC [21], 54-85 % for Bowen's disease [22] and up to 100 % for AK [23]. However, adverse effects such as severe erythema and scabbing as well as long treatment duration often limit patient compliance. Imiquimod acts as an immune response modifier via stimulating Toll-like receptor 7 of macrophages and dendritic cells resulting in release of proinflammatory cytokines [24]. Treatment duration depends on the effect and may take up to 16 weeks by using typically 5 % imiquimod cream for superficial BCC and actinic keratosis. Depending on the treatment regime and severity of disease, cure rates range between 43-94 % for superficial BCC [25-28]. Up to 56 % cure rates may be achieved for the 16-week treatment of AK using 5 % cream 3 times per week [29]. Hereby, similar strong adverse effects as by 5-FU were observed. Diclofenac gel is approved for the treatment of AK. The drug inhibits cyclooxigenase (COX). Particularly COX-2 regulates the production of prostaglandin E_2, which is often increased after extensive UVB exposure, one risk factor for the development of NMSC [30]. Yet, the efficacy of diclofenac against AK is weak, 60-80 % [31], and the treatment duration is very long (up to 90 days). However, adverse effects such as rash and pruritus are mild [19]. Recently, FDA and EMA have approved ingenol mebutate gel as a new topical treatment for AK. Derived from the plant extract of *Euphorbia peplus*, ingenol mebutate has two effective modes of action: induction of

necrotic cell death and antibody production against specific antigens on dysplastic epidermal cells, which attracts neutrophils [32]. Using ingenol mebutate 0.05 % gel, cure rates up to 71 % within 7 days treatment of AK lesions was observed [33]. Yet, efficacy is limited to the rare cases of not hyperproliferative epidermis. Ingenol mebutate gel may also represent a promising candidate for the treatment of superficial BCC, but experience is still limited.

Existing strategies against NMSC often show insufficient treatment success and severe adverse effects. In addition, long-term therapy can reduce patient compliance. Thus, there is still need for the development of new treatment options to have alternative therapeutics in case of treatment failure or intolerable adverse reactions.

1.3 Human DNA polymerase alpha

The DNA polymerase alpha belongs to the family of eukaryotic DNA polymerases and is essential for the nuclear DNA replication and repair. An inhibition of this enzyme can result in induction of apoptosis. This sophisticated strategy is currently successfully used for the treatment of infection with herpes and human immunodeficiency virus. Using molecular modeling on basis of known homologue structures for polymerase alpha, several potent guanosine-analog phosphonates have been designed [34,35]. Their antitumor effects against cancer cell lines have been tested *in vitro* and especially the promising guanosine-analog, OxBu (Figure 2), showed pronounced cytotoxic effects on different cancer cell lines and no toxic effects on keratinocytes [36,37]. Therefore, inhibition of the DNA polymerase alpha is a possible innovation in the treatment of NMSC.

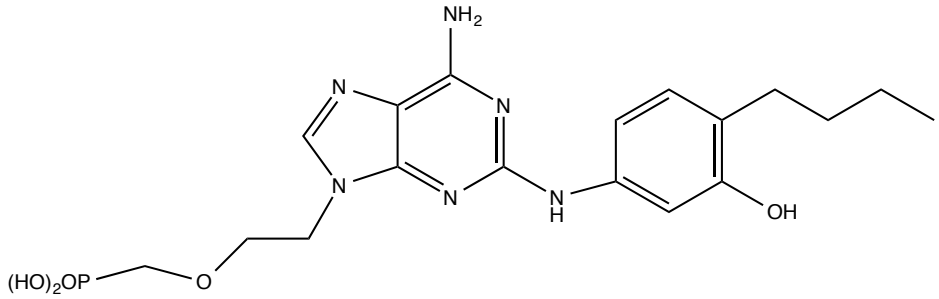

Figure 2: Chemical structure of the DNA polymerase alpha inhibitor OxBu.

1.4. Membrane-active Peptides

Membrane active peptides include cationic antimicrobial peptides (AMPs) and cell penetrating peptides (CPPs). Containing 12-50 amino acids, these small polypeptides have an amphipathic structure and a cationic net charge. Their cationic charge mainly derives from basic amino acids e.g. lysine and arginine, while the amphipathic nature develops from the arrangement of hydrophobic amino acid sequences and positively charged areas.

While AMPs exhibit a broad cytotoxic activity against various pathogens [38-40], CPPs became prominent due to their excellent translocation capacity across membranes without cell damaging effects [41,42].

1.4.1 Antimicrobial Peptides

Although AMPs have been discovered about 90 years ago, they came into focus only recently. As part from the innate immune system, AMPs exhibit cytotoxic effects against fungi, bacteria, viruses and/or parasites [43,44]. They occur in various natural sources including insects, mammals and amphibians. The different origins and structural diversity generates a broad variety of AMPs, which can be roughly classified according to their structure (Table 2):

- α-helical cationic AMPs
- β-sheet cationic AMPs
- Cationic AMPs enriched in specific amino acids

Table 2: Overview of selected AMPs.

peptide / name	source	structural characteristics	actions / suggested mode
α-helical cationic AMPs			
cathelicidins / LL-37, hCAP18, BMAP-28	human neutrophils, mast cells, epithelia (skin, lung, gastrointestinal, urogenital, oral), sweat	leucine- and lysine-rich, linear	antimicrobial, anticancer, chemotactic / membrane permeation, cellular uptake, apoptosis
cecropins / cecropin A, B	Hyalophora cecropia and other insects, mammals	hydrophobic C-, hydrophilic N-terminus, linear	anticancer, antimicrobial, antiprotozoa / transmembrane pores
melittin	venom of *Apis mellifera*	linear, amphipatic	antimicrobial, anticancer / membrane perturbation
β-sheet cationic AMPs			
α-defensins / HNP-1 to HNP-4; HD-5, HD-6	human neutrophils and epithelia (intestine, Paneth's cells, genital, oral)	cysteine- and arginine-rich; 3 disulfide bridges	antimicrobial and anticancer / membrane lysis inhibition of angiogenesis / binding to fibronectin and integrin α5β1
β-defensins / HBDs 1-4	human neutrophils and epithelia (skin, oral, mammary, lung, urinary, eccrine ducts, ocular)	cysteine- and arginine-rich; 3 disulfide bridges	antimicrobial, chemotactic, induces histamine release / membrane interaction, receptor activation
cathelicidins / protegrin-1-5	porcine leukocytes	cysteine-rich; 2 disulfide bridges	antimicrobial, anticancer, leishmanicidal/ membrane perturbation, intracellular receptors
cationic AMPs enriched in specific amino acids			
histatins / Histatin 5	human parotid saliva and submandibular glands	histidine-rich, linear, α-helical	antibacterial, antifungal / cell penetration and targeting mitochondria

Electrostatic interaction between negatively charged surfaces (e.g. compounds of the cell membrane) and the positively charged peptide is the basis for their activity. Depending on the peptide's individual structure, different target sites and modes of actions are possible. Especially shorter peptides form pores via the "carpet" model in the phospholipid membrane after reaching the threshold concentration. AMPs with higher peptide length oligomerize to "barrel stave" or "toroidal" pores (Figure 3)

[45,46]. This results in destabilization of the cell membrane and release of internal compounds followed by a quick necrotic cell death. Some AMPs can also penetrate cell membranes without damaging effects and thereafter, influence intracellular processes such as protein (enzyme) function and DNA synthesis. AMP permeation of the mitochondrial membrane can result in subsequent cytochrome c release and induction of apoptosis [46,47].

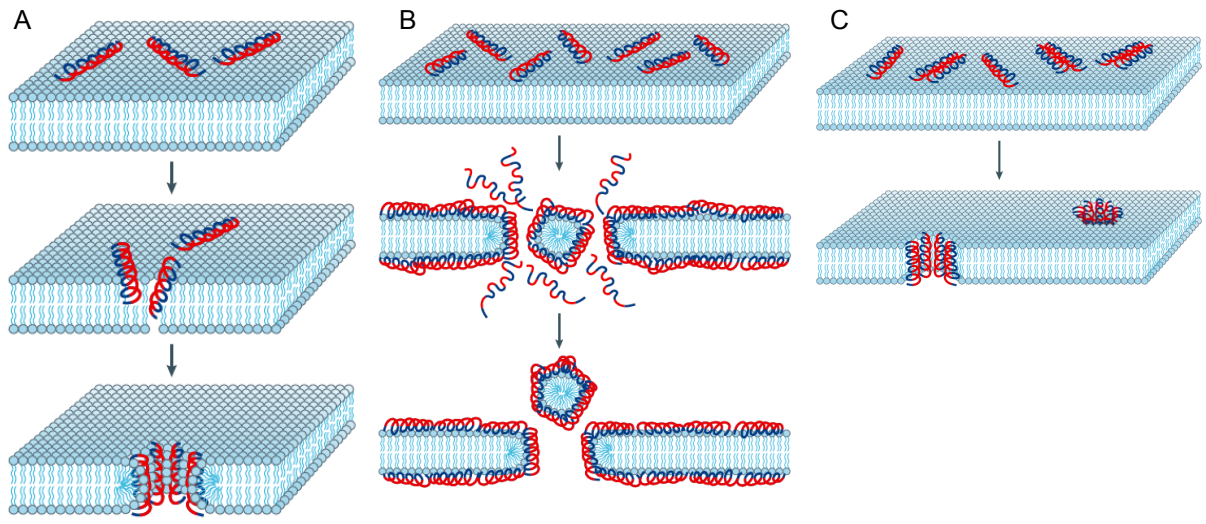

Figure 3: Pore formatting mechanisms of AMPs. (A) The lipid monolayers bend through the pores and build a water core with the peptide in the toroidal pore model. (B) In the carpet model AMPs cover the surface of membranes and extract parts out of the membrane. (C) The peptides insert into the hydrophobic core and build a pore in the barrel stave model (modified from [46]).

Next to antimicrobial effects, AMPs have also been extensively studied for their anticancer activity. Increased drug resistance and insufficient cure rates of cancer diseases with conventional chemotherapy ask for new treatment options. Especially AMP-induced fast response and reduced resistance occurrence has attracted researcher's attention. However, only a few studies also investigated the toxic effects on normal mammalian cells. Therefore, knowledge about their effects on and their selectivity for cancer cells is not clearly understood.

In particular the AMP families defensins and cathelicidins have multiple functions in human skin (Table 2). Regarding anticancer activity, human defensins HNP-1 and -3 exhibit anticancer effects by membrane perturbation and inhibition of angiogenesis via influencing signaling cascades during vascularization [48]. However, their use as

anticancer agents is limited due to lack of selectivity over cancer cells and loss of activity in serum excluding systemic administration [49]. Cathelicidin BMAP-28 destabilizes mitochondrial membranes and releases cytochrome c resulting in apoptosis, but strong toxicity to human lymphocytes limits its use as anticancer agent [50].

Focusing on non-melanoma skin cancer, the effects and side effects of several natural occurring AMPs have been evaluated in this work.

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) is the main component from venom of the honeybee *Apis mellifera*. Next to activity against human immunodeficiency virus 1 [51], melittin shows strong antibacterial, antifungal and anticancer effects [52-55]. In a human lymphoblastoid cell line, melittin causes maximal cell lysis after 90 min exposure [56]. Furthermore, melittin-linkage to perfluorocarbon nanoparticles specifically allows delivery to multiple tumor targets in mice after intravenous application, reducing tumor growth [57].

Cecropin A (KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK) was first isolated from the giant silk moth *Hyalophora cecropia* [58]. Next to effects against bacteria, viruses and protozoa, cecropin A exhibits anticancer activity on bladder cancer cells [59]. In combination with classical anticancer agents cecropin A shows strong synergistic effects against leukemia cells [60]. Synergistic activity is favorable in the treatment of cancerous diseases.

Protegrin-1 (RGGRLCYCRRRFCVCVGR) belongs to the protegrins, a sub-family of cathelicidins, which were isolated from porcine leukocytes [61]. Similar to melittin, protegrin-1 shows a broad activity against gram positive and gram negative bacteria, fungi and viruses via membrane perturbation by forming toroidal pores [62,63]. In addition, protegrin-1 exhibits anticancer activity against the human histiocytic lymphoma cell line U937 and the fibrosarcoma cell line HT1080 [64,65]. Three disulfide bridges are essential for the activity of protegrin-1 as they ensure structure stability in physiological environment e.g. in the presence of serum components and extracellular cations [66]. Hence, protegrin-1 combines characteristics of peptide stability and strong potency, which are important criteria in the investigations of novel peptide-based drugs.

Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) is part of the family of histatin-rich peptides and was found in human parotid saliva and submandibular glands [67,68]. Composing of 24 amino acids, histatin 5 exhibits antibacterial and especially fungicidal activity against *C. albicans* [69]. It does not only target the mitochondria of fungi but also the mitochondrial ATP production of leishmania and hence induces a collapse in the protozoan metabolism [70]. The histatin-derived AMP periondotix (Demgen, Pittsburgh, PA, USA, and Dow Pharmaceuticals Sciences, Patuloma, CA, USA) belongs to one of the most promising AMPs in clinical trial as mouth wash gels for the treatment of gingivitis, periodontal disease and oral candidiasis in HIV and chronic *Pseudomonas aeruginosa* infections [71,72]. Activity of histatin 5 against cancer cells has not yet been reported. However, as this AMP naturally occurs in human, histatin 5 is included as control in the experiments. If anticancer effects of histatin 5 occur, a strong selectivity over cancer cells can be expected.

1.4.2 Cell-Penetrating Peptides

Cell-penetrating peptides were first discovered and isolated from natural sources about 20 years ago [73,74]. CPPs are also called protein transduction domains since these small cationic peptide sequences, as part of large proteins, are responsible for translocation of the complete protein across membranes without harming effects. Therefore, CPPs have the ability to translocate and deliver linked cargoes, which may be up to 100 times larger than the CPP itself, across membranes [41,75]. Cargoes, such as proteins including antibodies, DNA as well as nanoparticles and liposomes have been successfully transported [78].

The entire uptake mechanism is not completely understood and may depend on the single CPP, the used concentration and the cargo. Attachment to the cargo can be achieved by covalent conjugation or by electrostatic interaction. CPP delivery into the cell occurs by endocytotic or energy-independent pathway (Figure 4a) [42]. Here, the electrostatic interaction with the cell membrane surface is important and can be increased by membrane surface sugars. Suggested modes of membrane translocation include the inverted micelle model (Figure 4b) and, similar to AMPs, pore formation mechanisms (Figure 3) [75,76].

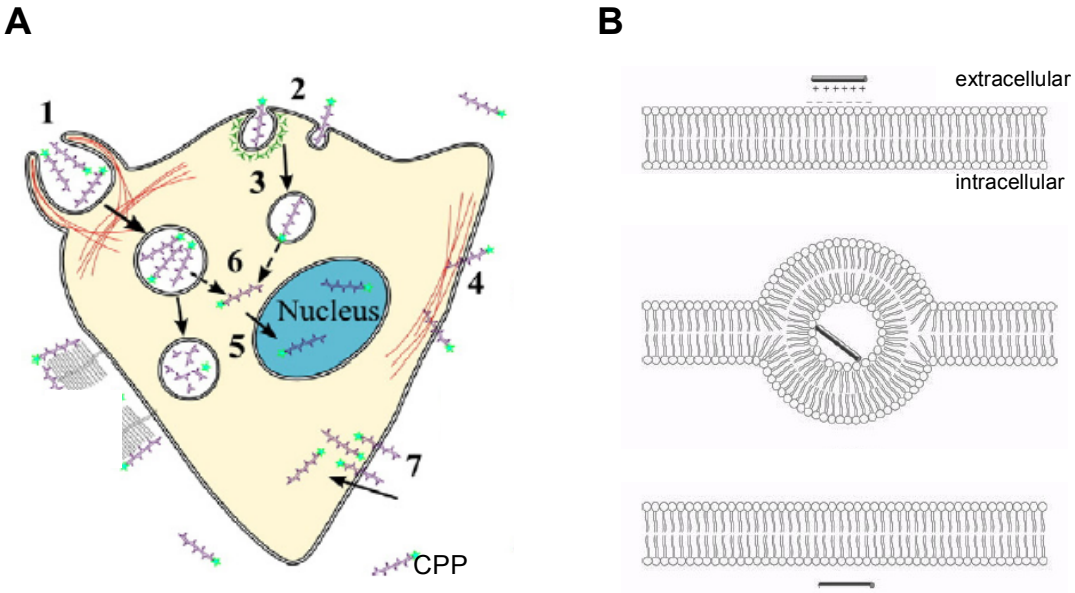

Figure 4: Possible mechanisms of CPP entrance into the cell. (A) CPPs are taken up by macropinocytosis (1) or other endocytotic pathways (2), which results in endosomal location (3). From this place they may enter the cytoplasm (6) but thereafter often accumulate into lysosomes or nucleus (5). Translocation across the plasma membrane may deliver CPPs directly into the cytoplasm (7). (B) CPP translocation across the cell membrane by the inverted micelle model (Modified after [75] and [76]).

While the first CPPs were derived from protein transduction domains, nowadays, chimeric or complete synthetic CPPs with optimized features regarding penetration and translocation properties have been developed. As their variety is very high, CPPs can be roughly classified into two classes: polycationic and amphipathic CPPs (Table 3).

Table 3: Overview of selected CPPs (modified after [41]).

peptides	sequence	origin	cargo types
amphipathic CPPs			
tat peptide	PGRKKRRQRRPPQ	HIV-tat protein	protein, peptide, siRNA, liposome, nanoparticles
penetratin	RQIKIWFQNRRMKWKK	Antennapedia homeodomain	peptide, siRNA, liposome
polycationic CPPs			
polyarginine	R_n	synthetic or chimeric	protein, peptide, siRNA, ODN
LMWP	VSRRRRRRGGRRRR	protamine	protein

The amphipathic transcription-transactivating (tat) peptide is the transduction domain of the HIV-tat protein. Tat peptide allows replication of the human immunodeficiency virus type 1 by translocation into the nucleus and transactivation of the viral genome [77]. Tat peptide is able to deliver various components into cells, e.g. caspase-3 into jurkat T-cells or nanoparticles into lymphocytes [78].

Penetratin was originally isolated from the 3rd helix of the antennapedia homeodomain of *Drosophila*. It is one of the first discovered and best characterized CPPs [73]. Penetratin delivers peptides, oligonucleotides as well as other chemical compounds into cells. Cell-type specifity as well as strict cargo size limit appear to be lacking [79]. Conjugation of penetratin to doxorubicin induced apoptosis of CHO cells at lower doses than free doxorubicin [80]. Surmounting the skin and improving penetration of even larger molecules into the deeper skin layers is another property of CPPs, which is not completely understood. In mice, linkage to penetratin enhanced transdermal delivery of interferon-γ without loss of activity [81].

Polyarginine structures, optimally containing 7 to 9 arginine clusters enter cells and deliver linked cargoes very efficiently [82]. Similar to tat peptide, polyarginine (R_8) e.g. delivers large covalently bond carbonic anhydrase (29 kDa) into macrophages [83]. Transportation of proteins into the skin is facilitated by polyarginines and additionally increased by the penetration enhancer oleic acid [84]. Cyclosporin skin penetration and anti-inflammatory activity is favored by R_7 linkage [42,85]. Low molecular weight protamine (LMWP), is another polyarginine, derived from

protamine by enzymatic digestion [86-88]. Covalent linkage of LMWP to albumin enhanced uptake by keratinocytes *in vitro* and penetration into Balb/c mouse skin *in vivo* [89]. LMWP conjugated to the growth factor EGF, a 53-mer polypeptide, resulted in deeper skin penetration and enhanced wound-healing efficacy in laser induced burn wounds of mice [90,91].

Although the stratum corneum illustrates a much stronger barrier for CPPs than the phospholipid cell membrane, CPPs have the ability to surmount this barrier and deliver linked cargoes into the skin. Since the mode of CPP-mediated cutaneous absorption is not clearly understood, LMWP and penetratin have been chosen for a closer evaluation of these properties as both peptides have a good translocation capacity and may penetrate the skin efficiently.

1.5. Nanocarrier delivery systems for controlled topical drug delivery

Structural properties for a good skin penetration are moderate lipophilicity and low molecular weight – to overcome the stratum corneum barrier – as well as sufficient water solubility – to cross the viable epidermis. In addition, the formulation can strongly influence skin absorption of the respective drug. Classical penetration enhancers such as alcohols, fatty acids or propylene glycols can intercalate with the stratum corneum lipid and influence their conformational order or interact with the drug itself, manipulating drug solubility [92]. However, modification of the skin surface may come along with irritation or damage of the skin barrier functions. Thus, highly efficient and well-tolerated drug delivery systems are looked for.

A broad spectrum of different nanocarriers such as liposomes, solid lipid nanoparticles, nanostructured lipid carriers, polymeric nanoparticles, nanoemulsion and quantum dots have been developed and studied for topical drug delivery [93]. They can reduce degradation and may enhance penetration of the drug to the target site. Furthermore, nanoparticles can control drug release from the formulation and therefore allow sustained drug delivery. Whether intact nanoparticles penetrate the human skin or enhance drug delivery via influencing the lipid composition of the stratum corneum or the drug solubility is still under debate. Suggested modes for the action of nanoparticles on the skin include [94,95]:

- The interaction of nanoparticles with stratum corneum lipids impairs the stratum corneum's barrier function. The drug released directly on the skin surface easily surmounts the disturbed skin barrier.
- The nanoparticles exhibit stronger permeability and allows skin penetration. Intact, loaded nanoparticles penetrate the skin and release the drug directly at the site of disease.
- Penetration of intact nanoparticles into hair follicles and sebaceous glands.

Special care needs to be addressed to particle toxicity. Exposure to nanoparticles, especially in combination with environmental factors such as UV radiation or allergens, can trigger hypersensitivity, atopic dermatitis and skin barrier defects. In particular, lesions similar to atopic dermatitis were detected by UV irradiation in

combination with TiO_2 nanoparticles in DS-Nh mice [96]. Furthermore, immunostimulation in mice was observed by carbon nanotubes [97].

Core multishell (CMS) nanotransporter are made of a central core, which controls the size, 3D shape and the branching direction of the particle. The polyglycerol or polyethylenamin core is linked to the inner shell, which is connected to the outer shell. The outer shell can contain reactive groups at the surface, e.g. for chemical transformations (Figure 5a). The void space within these regions allows entrance of the cargo and is therefore essential for the binding. Specific CMS nanotransporter with the empirical formula $PG_{10000}(-NH_2)_{0.7}(C_{18}mPEG_6)_{1.0}$, belong to novel and more sophisticated carrier systems (Figure 5b). They are made up by a hyperbranched polyglycerol core surrounded by double-layered shells consisting of C18-alkyl chain and of monomethoxy poly(ethylene glycol) [98]. Able to load lipophilic as well as hydrophilic agents and enhancing the delivery of dye particles [99-101], CMS nanoparticles appear to be free of cutaneous toxicity [102]. The highly adaptable structure allows a wide flexibility concerning the choice of a drug. The drug can be loaded to the monomers but also in between the spaces of the aggregated CMS polymers. Depending on the carrier concentration, unloaded methoxypoly(ethylene glycol) (mPEG)-based particles self-aggregate mainly to 5-8 nm hydrodynamic radii, but also larger aggregates up to 82 nm may occur [103]. Particle size changes after loading of the cargo and differs strongly depending on the cargo itself. Loading of the dye nile red results in particle sizes between 118 nm and 138 nm, while smaller particles (7-22 nm) are obtained by loading of methotrexate [103].

Focusing on the topical application of membrane active peptides, these particles may be able to enhance peptide delivery into the skin. The adaptable dendrimer structure allows interaction with the peptide's strong charged areas given by their high amount of basic amino acids. But also hydrophobic amino acids as present in CPPs and AMPs can interact with the lipophilic inner shell of the CMS nanotransporter.

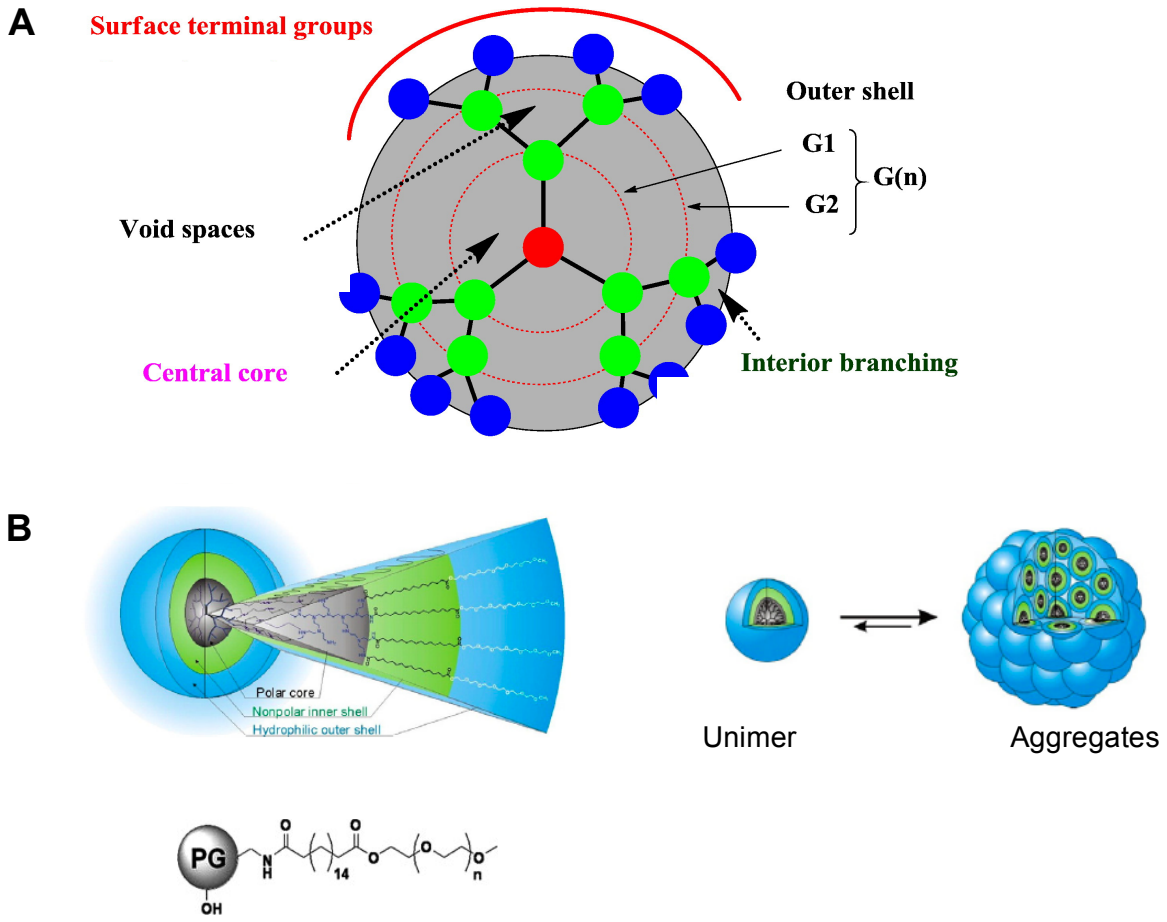

Figure 5: (A) Structure of dendrimer nanoparticles (modified after [95]). (B) Specific dendritic core-multishell nanotransporters with hyperbranched polymeric cores [98].

Solid lipid nanoparticles (SLNs) are aggregates of lipids, which are solid at room temperature and suitable for topical drug application. The advantages of drug loading to SLNs can be sustained release, enhancement of drug stability and skin penetration [104]. Various drugs such as tetracaine, etomidate or prednisolone have been loaded to SLNs with varying lipid matrixes. Prolonged drug release up to 5 weeks was observed by prednisolone particles while tetracaine and etomidate SLNs showed a burst drug release within 1 min [105]. These results underline the importance of the interaction between the lipid and the drug. Variations of lipid and emulsifiers influence the properties of SLNs and can modify drug penetration into the skin following topical application.

1.6. Aim of this work

Increased incidence of NMSC is a result from co-occurrence of careless sun exposure and the ageing society, while current therapeutic options are still limited. Beside lack in efficacy, severe adverse effects ask for novel treatment options.

The broad activity of membrane active peptides awakened the interest for new application areas in particular for skin diseases. The activity of AMPs on skin cancer has not been investigated due to the challenges of peptide penetration into the skin. Focused on the anticancer activity of cationic antimicrobial peptides, melittin, cecropin A, protegrin-1 and histatin 5 were selected and should be tested for their cytotoxic effects on SCC12 and SCC25 cell lines. SCC12 cells are derived from head and neck cancer and hence, most similar to cells found in lesions of AK and superficial SCC. Furthermore, the knowledge about AMP-toxicity on mammalian skin cells is still limited. As AK, noninvasive SCCs and superficial BCCs are located in the epidermis, AMP-toxicity on NHKs is of major interest.

In the treatment of cancer diseases, combination of drugs is often applied to reduce adverse effects and resistance establishment. Since 5-FU is the standard drug for the treatment of NMSC, synergistic effects of selected AMPs combined with 5-FU should be investigated.

The arrangement and composition of the human skin is extremely complex. Topical and transdermal drug delivery need to overcome stratum corneum and tight junction barriers for sufficient penetration to the target site without harming effects. Therefore, peptide penetration into human skin and their enzymatic cleavage following skin penetration is a major challenge. Nonetheless, CPPs seem to surmount the skin barrier and deliver linked cargoes. CPPs with antimicrobial activity as well as AMPs with enhanced translocation capacity have been reported [106]. Due to the strong physicochemical similarity between AMPs and CPPs [107,108], the penetration property and enzymatic cleavages of both peptide families should be compared. Focusing on penetration enhancement, additional loading of peptides to nanotransporter delivery systems should be investigated.

2. RESULTS

2.1 Cationic membrane-active peptides - anticancer and antifungal activity as well as penetration into human skin

The manuscript has been published in *Experimental Dermatology*:

Do N, Weindl G, Grohmann L, Salwiczek M, Koksch B, Korting HC, Schäfer-Korting M (2014) Cationic membrane-active peptides - anticancer and antifungal activity as well as penetration into human skin. *Exp Dermatol* 23: 326-331. http://dx.doi.org/10.1111/exd.12384

Amount performed by the author:

Design of experiments:	50 %
Practical, experimental part:	80 %
Data analysis:	70 %
Interpretation of results:	65 %
Writing:	50 %

2.2 Core-multishell nanotransporters enhance skin penetration of the cell penetrating peptide low molecular weight protamine

The manuscript has been published in *Polymers for Advanced Technologies*:

Do N, Weindl G, Fleige E, Salwiczek M, Koksch B, Haag R, Schäfer-Korting M (2014) Core-multishell nanotransporters enhance skin penetration of the cell penetrating peptide low molecular weight protamine. *Polym Adv Technol* 25: 1337-1341
http://dx.doi.org/10.1002/pat.3362

Amount performed by the author:

Design of experiments:	60 %
Practical, experimental part:	95 %
Data analysis:	60 %
Interpretation of results:	55 %
Writing:	45 %

2.2 Improving topical non-melanoma skin cancer treatment: In vitro efficacy of a novel guanosine-analog phosphonate

The manuscript has been published in *Skin Pharmacology and Physiology*:

Ali-von Laue C, Zoschke C, Do N, Lehnen D, Küchler S, Mehnert W, Blaschke T, Kramer J. Plendl KD, Weindl G, Korting HC, Hoeller Obrigkeit D, Merk HF, Schäfer-Korting M (2014) Improving Topical Non-Melanoma skin cancer treatment: In vitro efficacy of a novel guanosine-analog phosphonate. *Skin Pharmacol Physiol* 27: 173-180.
http://dx.doi.org/10.1159/000354118

Amount performed by the author:

Design of experiments:	5 %
Practical, experimental part:	20 %
Data analysis:	10 %
Interpretation of results:	10 %
Writing:	10 %

3. DISCUSSION

Regarding skin diseases topical treatment is preferred over systemic application to reduce side effects and enhance efficacy at the target site. Drug structure and formulation need to be carefully studied to guarantee an adequate healing without harming the skin function. Importantly, drugs and formulation must be tested for efficacy, safety and sufficient penetration to the target site.

Several AMPs have strong effects against various microorganisms and cancer cells *in vitro* and *in vivo* [40,50]. Due to the strong variety, modes of action and susceptibilities of AMPs against pathogens differ. Melittin's mode of action is controversially discussed and includes necrosis as well as apoptosis [54,109]. Here, necrotic cell death seems to be predominant since melittin induced toxicity occurs within 3 hours already. Especially, pore formation into membranes results in leakage of internal compounds and quick necrosis [110]. Among the investigated AMPs, melittin was most promising. It rapidly induced strong toxic effects to the cancer cell lines SCC12 and SCC25 (Figure 1a, Table S2 [111]), which is well in accordance with previous investigations [56]. While most of the studies did not include normal cells as control in the experimental design, the direct comparison within this work shows a clear toxicity of melittin to normal human keratinocytes and hence a lack of selectivity. However, melittin's cytotoxic effect exceeds 5-FU, the classical anticancer drug for the treatment of NMSC. Similarly, the DNA polymerase inhibitor aphidicolin, which is known for its potency to target the DNA polymerase but also for its toxicity on normal keratinocytes [37], was less active than melittin.

Whether and how AMPs exhibit selectivity is not clearly understood and strongly depends on the individual AMP. In general, increased expression of anionic-charged structures on membranes of cancer cells e.g. phosphatidylserin and O-glycosilated mucins can enhance AMP selectivity [112,113]. Moreover, a larger surface area, generated by the high amount of microvilli in cancer cells, may also contribute to an increase in selectivity [114] while, neutral charges of zwitterionic phospholipids and sterols can stabilize the membrane of normal mammalian cells [49,115]. Especially, cholesterol is important for membrane stability as its depletion increased cytotoxicity of melittin in Caco-2 and HT29 cell lines [109]. Nonetheless, transformed cells develop from normal cells and structural similarity may limit cell selectivity. This may

be true for the SCC12 and SCC25 cell lines and NHKs since melittin and protegrin-1 show strong cytotoxic effects on both cell lines but were also toxic on NHKs (Figure 1a, Table S2 [111]). Similarly, the human histatin 5 lacks in toxicity on NHKs and did not show anticancer effects on SCC12 and SCC25 cell lines, too (Figure 1a [111]).

In contrast, differences between cells of mammalian cells and microorganisms are more pronounced – the latter are protected by an additional cell wall, too. Higher amounts of anionic lipids e.g. phosphatidylglycerol, cardiolipin and phosphatidylserin build bacterial membranes while mammalian membranes compose of mainly neutral phospholipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin [49]. Enhanced electrostatic interaction between a negatively charged cell surface and cationic AMPs increases AMP-toxicity and selectivity. Accordingly, melittin and protegrin-1 show strong anti-*Candida* effects at non-toxic peptide concentrations on normal human keratinocytes as cell walls of *C. albicans* are coated with manosylated or phosphorylated glycophosphatidiylinositol, increasing the affinity to positively charged ions [116]. Notably, the standard antifungal amphotericin B was less potent than both peptides (Table S3 [111]). Histatin 5 and cecropin A did not show anti-*Candida* effects up to 5 µM. However, the well known anti-*Candida* activity of histatin 5 occurs at concentrations between 15-30 µM [117].

Combination of melittin or cecropin A with the anticancer drug 5-FU indicated strong synergistic effects on SCC12 and SCC25 cells. Most interestingly, this is accompanied by a reduced toxicity on NHKs (Table 1 [111]). This observation is well in accordance with the study by Hui *et al.*, where cecropin A showed synergistic effects in combination with 5-FU or cytarabine on leukemia cell lines [60]. Notably, cecropin A only was more toxic on NHKs than its combination with 5-FU. The mode of synergistic activity is not completely understood. AMP induced pore formation in the cell membrane may facilitate access of extracellular compounds such as anticancer agents into the cell resulting in enhanced effects by targeting two completely different structures. Interestingly, cecropin A at lower concentration (1 µM) antagonised 5-FU effects on SCC12 and SCC25 cells. Prior to channel formation AMPs attach to the surface of the cell membrane [38], which may impede 5-FU access to the cellular target site and may be the reason for the observed antagonistic activity by cecropin A.

Taken together, the results show that melittin is a promising candidate for dermal and in particular mucosal *Candida* infections and NMSC. Topical use is not considered up to now, because of the challenging skin penetration. Peptides and proteins cannot surmount the stratum corneum barrier, due to their high molecular weight and hydrophilicity. Superficial fungal infections, including tinea versicolor, piedra, and tinea nigra, are caused by pathogens restricted to the stratum corneum [118]. Here, treatment with topical antifungals, which do not penetrate into deeper tissues, can be advantageous to reduce adverse effects. In contrast, cutaneous cancer is more challenging. While clusters of actinic keratosis are located within the epidermis, these lesions can invade the dermis by becoming squamous cell carcinoma. Especially early treatment and a sufficient penetration to the target site are essential.

CPPs exhibit excellent membrane translocation capability, penetrate the viable skin and deliver linked cargoes across the skin *in vivo* and *in vitro* [41,119]. As AMPs and CPPs share similar physicochemical characteristics [107,108], AMPs might also be able to overcome the skin barrier. In fact, both CPPs, penetratin and LMWP, penetrated after 24 hours exposure into the viable layers of human skin *ex vivo* (Figure 2 [111]). This is well in accordance to the enhanced stability and absorption of salmon calcitonin into rat skin by co-incubation with tat peptide [120]. Here, the penetration of the CPP alone, without cargo, was investigated to determine the plain penetration ability of LMWP and penetratin. For penetration enhancement, a simple co-application allows access of the non-covalently bond model peptide P20 into the viable epidermis of porcine ear skin. This effect is additionally increased by covalent attachment to the CPP and a deeper access of the cargo into the skin is achieved [121]. Enhancement of skin penetration may be due to the interaction of CPPs with stratum corneum lipids resulting in destabilization of the stratum corneum barrier increasing permeability. Additionally, CPPs may affect tight junction proteins. Prevalently located in the stratum granulosum of the skin, TJ proteins have barrier function against the entrance of exogenous compounds from the environment but also inhibit the transepidermal water loss [122]. Interaction with CPPs may disturb structure and functionality of TJ proteins. Poly-L-arginine impairs the localization junctions occludin and ZO-1 between cells, promoting the paracellular permeability of fluorescent labeled dextran across rabbit nasal epithelium *in vitro* [123]. Cutaneous absorption of AMPs has been investigated only rarely. Yet, structural similarity

between AMPs and CPPs may result in similar penetration behavior. This was confirmed by similar penetration characteristics of melittin and both CPPs (Figure 2 [111]). Importantly, melittin clearly exceeds CPPs in molecular weight (M_r: 2846.5 melittin versus 1880.2 LMWP and 2246.7 AT) due to the higher number of amino acids (26 melittin versus 16 penetratin and 14 LMWP), respectively. Here, the cytotoxic and membrane disruptive effect of melittin may enhance skin penetration.

Another aspect to be considered is the biotransformation by skin enzymes. Although topically applied drugs are less affected by metabolism compared to oral administration, knowledge about biotransformation is crucial to guarantee sufficient efficacy and safety. Especially, peptides and proteins can be easily cleaved by various proteases, strongly depending on the peptide structure [124]. Nonetheless, there is a lack of knowledge so far about peptide penetration and peptide stability in the skin. Metabolic cleavage can result in loss of activity and quick clearance of the drug. Additionally, a change in penetration characteristics and altered toxic profile by biotransformation may be possible. Here, *in silico* analysis (PeptideCutter) was performed to predict possible cleavage sites of LMWP and penetratin by cutaneous enzymes. Accordingly, enzymatic degradation of LMWP was mainly directed by trypsin at 3 main cleavage sites. The combination of PMSF and phenanthroline inhibiting serine proteases as well as metalloenzymes in rat skin [125], was used for enzyme inhibition within this work allowing inhibition of LMWP cleavage in a trypsin solution and skin homogenate. Similar chromatographic pattern after exposure of LMWP to trypsin solution and skin homogenate confirmed the involvement of trypsin as key enzyme for LMWP cleavage (Figure S2 [111]). Here, RP-HPLC chromatography with fluorescence detection allowed visualization and quantification of the intact peptide. In addition, peptide fragments bond to the fluorescence dye can be detected. According to *in silico* analysis, penetratin cleavage may occur by several enzymes at various cleavage sites. This was visible by RP-HPLC after exposure of penetratin to trypsin or skin homogenate resulting in strong degradation of penetratin (Figure S2 [111]). During skin penetration experiments, cleavage of LMWP and penetratin was observed in skin with as well as without PMSF and phenanthroline pre-treatment (Figure 3 [111]). Hence, a complete inhibition of enzymes in human skin tissue was not possible probably due to the involvement of other yet unknown enzymes, insufficient penetration of the inhibitors into the skin

tissues or lack of stability of the inhibitors. Nonetheless, a significant higher amount of intact LMWP and penetratin was extracted 24 hours post-exposure from enzyme inhibited skin tissue and quantified by RP-HPLC, compared to untreated skin tissue.

Fluorescence microscopic evaluation of skin tissues visualizes the depth of penetration by the tagged fluorescence dye. Here, no discrimination between dye tagged intact peptide and dye tagged peptide fragments is possible. However, strong differences between the use of enzyme inhibited skin tissue and non-treated skin tissue during skin penetration experiments was visible. Peptide exposure to enzyme inhibited human skin resulted in no visible penetration into the viable skin as the fluorescence signal by microscopy was mainly detected in the stratum corneum even after 24 hours exposure with both peptides. In contrast, for untreated skin tissues fluorescence in deeper layers was observed, which most likely is derived from peptide fragments covalently linked to the dye since the smaller peptide fragments can surmount the stratum corneum more easily than the intact peptide.

If peptides are used for treatment of skin diseases, penetration of the intact peptide to the site of disease is essential. While lesions of actinic keratosis remain in the epidermis since their development starts at the basal layers and move upward to the stratum granulosum and stratum corneum, invasive SCCs also involve the dermis and deeper tissues [126]. Therefore, treatment of NMSC requires penetration of the drug across the stratum corneum to the viable epidermis and dermis, too. Maintenance of structure and hence activity e.g. by primary and secondary structures of peptides is crucial to achieve adequate effect and reduce resistance development and recurrence. Hence, peptide based drugs for topical application should always be tested for enzymatic degradation.

Up to now, CPPs have only been studied as carrier for the delivery of drug actives, proteins or nanoparticles across the skin [42]. Here, maintenance of peptide integrity was not in focus. For the use of melittin in NMSC, the good penetration characteristic, similar to CPPs, is most interesting for topical application. For further improvements of penetration capacity and stability, modification of peptide structure or loading to nanotransporter delivery systems may be options [127].

Loading of LMWP and penetratin to CMS nanoparticles aimed to improve penetration of the intact peptide into the viable skin. Unimolar CMS nanotransporter

with sizes about 8 nm tend to assemble to stable supramolecular aggregates (about 100 nm). Amphiphilic AMPs may interact with the hydrophobic inner shell of the monomer nanoparticle, the C18-alkyl chain, as well as with the outer, hydrophilic mPEG shell, and thus may incorporate between the aggregates of the single nanotransporter [103,128]. CPPs' high molecular size and hydrophilic nature appear to prevent entrance into the void spaces of the lipophilic inner shell. Small change in enthalpy as measured by ITC, confirms only a weak interaction between CPPs and CMS nanotransporter (Figure 1 [129]). In addition, formation of LMWP agglomerates (272 ± 3.2 nm) was observed by DLS measurements [129], which is a well-known property for CPPs due to their amphiphathic nature [130]. LMWP aggregates in water resulting in particle sizes in nanometer dimensions [129]. This is in accordance with the well-known characteristic of CPPs for aggregation [130] and also complies to ITC results: the titration of peptide in water (control) showed a small change in enthalpy already. Nevertheless enhanced skin penetration of (non-loaded) LMWP fragments but not of the intact peptide was observed in the presence of the CMS nanotransporter (Figure 3 [129]). CMS nanotransporters seem to disintegrate peptide aggregates and thus may contribute to an enhanced peptide penetration into the skin. Noticeable, CMS nanotransporters interact with the stratum corneum's lipids [131], which may contribute to the increased penetration of LMWP. Importantly, LWMP is mainly cleaved by trypsin, resulting in 3 main cleavage products [111]. These visible fragments are bond to the fluorescent dye lissamine rhodamine B and the conjugate is described by high molecular weight (lissamine rhodamine B tagged VSR, 938 Da; VSRRRRRR, 1502 Da; VSRRRRRRGGR, 1772 Da). Therefore, CMS nanotransporter may enhance the delivery of molecules up to 1772 Da across the skin by interaction with the skin surface. One possible approach to optimize drug encapsulation and delivery is the modification nanoparticle structure [103]. An increased interaction with cationic peptides may be achieved by anionic surfaces of the CMS outer shell, which promotes electrostatic interaction with the cationic net charge of the peptides. However, it needs to be assured that these modifications do not result in loss of peptide integrity, since the cationic charge is an essential property of membrane active peptides.

Another innovative approach for the treatment of NMSC is the inhibition of the polymerase alpha. Synthetic guanosine-analog phosphonates have been designed

by molecular modeling to optimally target this enzyme [34,35]. Focusing on topical treatment, the most promising candidate, OxBu, has been encapsulated into solid lipid nanoparticles to achieve sustained release as well as increased stability [132]. Solid lipid nanoparticles suspended in a hydrophilic gel formulation allowed prolonged release of OxBu for up to 48 hours, compared to OxBu embedded in hydrophilic gel matrix, aqueous solution, gel formulation and the aqueous SLN dispersion (Figure 3 [132]). As expected, the SLN dispersion of OxBu retarded drug release over control, OxBu solution. However, faster OxBu release from SLN dispersion than from the hydrogel suggests a weak binding of the drug to the lipid matrix. Here, the OxBu formulation with SLN embedded in hydrogel matrix demonstrated superior release, which is characteristic because of the reduced mobility of SLN by the gel matrix [132].

Topical therapy of NMSC requires sufficient skin penetration of the drug to the site of disease. AMPs and guanosine phosphonate analogues target two completely different structures – the former addresses the cell membrane and the latter the polymerase alpha. However, as cancer diseases and resistance continue to increase, both strategies are innovative and especially the combination of different modes of action is promising.

4. FUTURE PROSPECTS

From the selected AMPs, melittin has shown best activity against SCC12 and SCC25 cell lines but also toxicity on NHK. Therefore, increasing AMP selectivity should be focused on in future investigations. As melittin's activity seems to be driven by its interaction with the phospholipid membrane, differences between membranes, in particular cell surface proteins of SCC12 or SCC25 cell lines and NHKs should be evaluated. Then, structural modification of melittin may allow enhanced attraction to tumor specific surface proteins. This may allow reducing toxicity for NHKs. The combined effect of melittin or cecropin A with 5-FU resulted in reduced toxicity for NHK and enhanced activity on SCC12 and SCC25 cells. Here, additional investigations in synergistic effects should be performed. Especially combinations with other small molecules used for NMSC such as ingenol mebutate, diclofenac or imiquimod with AMPs seem to be an interesting approach. Furthermore, there is still a broad variety of AMPs, which should be tested for their activity against cancer cells. For example, the nontoxic magainin 2, which was isolated from the frog *Xenopus laevis,* is active against cancer cells *in vivo* and *in vitro [50]*. Interestingly, conjugation of magainin 2 to penetratin enhanced cytotoxic activity in tumor cell lines *in vitro* and reduced tumor growth of HeLa cells in BALB/c mice *in vivo* [133].

Penetration experiments of melittin, LMWP and penetration were performed in healthy human skin *ex vivo*. However, diseased skin may have altered barrier properties due to altered organization of the stratum corneum lipids or change in localization of tight junction proteins [134,135], which may influence skin penetration. Therefore, investigations of peptide penetration with and without nanocarrier systems on NMSC diseased skin or skin models would be of great interest.

5. SUMMARY

Innovative pharmacotherapy for non-melanoma skin cancer (NMSC) is still looked-for due to insufficient healing rates and frequent adverse effects (strong pain, scabbing and erythema) caused by current drugs. This work focused on the investigation of antimicrobial peptides (AMPs) as potential innovative option for the treatment of NMSC. The effect of several AMPs, including melittin, cecropin A, protegrin-1 and histatin 5, on viability and proliferation on SCC12 and SCC25 cell lines was compared to effects on normal human keratinocytes (NHKs). Especially melittin has shown strong and fast cytotoxic activity on SCC12 and SCC25 cancer cell lines. However, melittin does not exhibit selectivity and was toxic against NHKs. Interestingly, melittin efficacy was enhanced by the combination with 5-fluorouracil (5-FU) while NHK-toxicity was reduced. Similarly, cecropin A combined with 5-FU (the the gold standard for NMSC) was more potent on SCC12 and SCC25 cell lines and revealed less toxicity on NHKs than in monotreatment.

Regarding skin diseases, topical application is favored to reduce side effects and increase efficacy at the target site. In a time dependent manner, penetration of melittin into human skin *ex vivo* was compared with two nontoxic cell-penetrating peptides (CPPs), low molecular weight protamine (LMWP) and penetratin. Non-toxic CPPs serve as reference peptides since they have similar structures to AMPs. They allow detailed insight into the penetration of cationic, membranolytic peptides, without damaging effects. Penetration of the fluorescence-labeled peptides into viable layers of the skin was observed after 24 hours exposure. Peptide and peptide fragments, which are covalently bond to the fluorescent dye, can be detected by high pressure liquid chromatography (HPLC) with fluorescence detection. In order to determine influences of enzymatic cleavage during skin penetration, LMWP and penetratin were extracted and the amount of intact peptide was quantified by HPLC. Both CPPs were cleaved to a high extent by skin enzymes after 6 hours exposure, already. The inhibition of enzymes, which are responsible for LMWP cleavage, resulted in an enhanced recovery (compared to no-inhibition) of intact LMWP to 91.7 % (25.3 %) in trypsin, 91.9 % (39.4 %) in skin homogenate and up to 31.9 % (2.3 %) in skin tissue after 24 hours exposure, respectively. However, fluorescence microscopy showed

that the intact peptides remained to a high extent in the stratum corneum and only a small amount was detected in the viable skin.

In order to improve peptide penetration into the skin, loading onto dendritic core-multishell (CMS) nanotransporter systems was investigated. Although failed to be loaded, the penetration of LMWP into the skin was enhanced in the presence of nanoparticles. This observation indicates an influence of CMS nanotransporter on the skin barrier.

Another target for the treatment of NMSC is the inhibition of the polymerase alpha. Previous investigations have shown cytotoxic and antiproliferative effects of the guanosine phosphonate, OxBu, against various cancer cell lines. OxBu release from different dosage forms, including OxBu encapsulated in solid lipid nanoparticles (SLN), hydrophilic OxBu gel, OxBu-SLN embedded into hydrophilic gel matrix and aqueous OxBu solution, was part of this work. Embedment of OxBu-SLNs into hydrophilic gel allows strongest sustained release compared to the other dosage forms (SLN-Gel > Gel > SLN > solution).

Topical application is still a challenge for natural occurring as well as synthetic drugs. Detailed insights into release, penetration and metabolism of innovative agents within this work are the basis for future use in dermatotherapy.

6. ZUSAMMENFASSUNG

Neue Strategien für die Behandlung des hellen Hautkrebses sind aufgrund der bisher noch unzureichenden Heilung und häufig auftretender unerwünschter Wirkungen, wie starke Schmerzen, Juckreiz und Rötungen, Gegenstand aktueller Forschung. Der Fokus dieser Arbeit liegt auf der Erforschung antimikrobieller Peptide (AMPs) als potentielle neue Behandlungsoptionen für das Indikationsgebiet des hellen Hautkrebses. Dafür wurde die Wirkung von AMPs (Melittin, Cecropin A, Protegrin-1 und Histatin 5) auf die Viabilität und Proliferation von SCC12 und SCC25 Krebszelllinien im Vergleich zu normalen humanen Keratinozyten geprüft. Die Ergebnisse verdeutlichen, dass insbesondere Melittin sehr schnell und stark zytotoxisch auf SCC12 und SCC25 Zelllinien wirkt. Jedoch erwies sich Melittin als unselektiv, es wirkte ebenso toxisch auf normale Keratinozyten. Interessanterweise erhöht die Kombination von Melittin mit dem Goldstandard in der Behandlung des hellen Hautkrebses, 5-Fluorouracil (5-FU), die Selektivität der Wirkung. Ähnlich verhielt sich Cecropin A, dessen toxischer Effekt mit 5-FU gegenüber Krebszelllinien verstärkt und gegenüber Keratinozyten reduziert wird im Vergleich zu der Exposition des einzelnen Peptids.

Bei Hauterkrankungen wird die topische Applikation oftmals bevorzugt um unerwünschte Effekte zu reduzieren und eine optimale Wirkung am Ort der Erkrankung zu gewährleisten. Daher wurde zeitabhängig die Penetration von Melittin in Humanhaut *ex vivo* untersucht und vergleichend zu zwei zellpenetrierenden Peptiden (cell-penetrating peptides, CPPs), Penetratin und niedermolekulares Protamin (low molecular weight protamine, LMWP), getestet. Die Kontrollpeptide besitzen keine toxischen Wirkungen, sind den AMPs aber strukturell sehr ähnlich. Ohne Einfluss auf die Zellviabilität, erlauben CPPs die Penetration kationischer, membranolytischer Peptide näher zu untersuchen. Eine Penetration der fluoreszenzmarkierten Peptide in tiefe Hautschichten war nach 24 stündiger Exposition erkennbar. Peptide und Peptidfragmente, die kovalent am Fluoreszenzfarbstoff gebunden sind, wurden mit Hilfe einer HPLC-Methode mit Fluoreszenzdetektion erfasst. Um enzymatische Einflüsse während der Hautpenetration zu ermitteln, wurden LMWP und Penetratin aus der Haut extrahiert und die Menge des intakten Peptides mit HPLC und Fluoreszenzdetektion

quantifiziert. Beide CPPs unterlagen innerhalb von 6 Stunden einer ausgeprägten Biotransformation. Die Inhibition der am Abbau beteiligten Enzyme resultierte in einer erhöhten Wiederfindung (im Vergleich zu nicht inhibierten Enzymen) des intakten LMWPs von 91,7 % (25,3 %) in Trypsin, 91,9 % (39,4 %) im Hauthomogenisat und bis zu 31,9 % (2,3 %) in Humanhaut nach 24 stündiger Inkubation. Allerdings verweilte das intakte Peptid vorwiegend im Stratum corneum und wurde nur in geringen Mengen in der lebenden Epidermis und Dermis detektiert.

Um die Aufnahme von Peptiden in der Haut zu erhöhen, erfolgten Untersuchungen zum Einschluss von LMWP und Penetratin in Dendrimernanopartikel. Obwohl beide Peptide nicht eingeschlossen werden konnten, erhöhte allein die Anwesenheit der Nanopartikel die Hautpenetration von LMWP. Dendrimernanopartikel scheinen daher einen Einfluss auf die Hautbarriere zu haben.

Ein weiterer Angriffspunkt für die Behandlung des hellen Hautkrebses stellt die Inhibition des Enzyms Polymerase alpha dar. Frühere Studien zeigten zytotoxische und antiproliferative Wirkungen des Guanosinphosphonates OxBu auf verschiedene Krebszelllinien. Die Freisetzung von OxBu aus vier Formulierungen, OxBu-SLN, OxBu in einer hydrophilen Gelmatrix, OxBu-SLN eingebettet in einer hydrophilen Gelmatrix sowie eine wässrige OxBu-Lösung, war Gegenstand dieser Arbeit. Insbesondere die Einbettung von OxBu-SLNs in einer hydrophilen Gelmatrix erlaubt eine stärkere Retardierung im Vergleich zu den anderen Formulierungen (SLN-Gel > Gel > SLN > Lösung).
Topische Applikation stellt nach wie vor eine große Herausforderung sowohl für natürlich vorkommende als auch für synthetische Wirkstoffe dar. Die im Rahmen dieser Arbeit gewonnenen Erkenntnisse zur Freisetzung, Hautpenetration und Metabolisierung zur biologischen Wirkung neuartiger Wirkstoffe, legen den Grundstein für einen zukünftigen Einsatz in der Dermatotherapie.

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CURRICULUM VITAE

Due to data protection reasons, the CV has been removed.