Aus der Klinik für Innere Medizin I
Gastroenterologie, Infektologie und Rheumatologie
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DISSERTATION

Functional assessment of gastroenteropancreatic neuroendocrine tumor's (GEP-NET) sensitivity to bio- and chemotherapeutic drugs and the role of survivin and aurora kinase B expression in GEP-NET disease

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1. Introduction

1.1. Gastroenteropancreatic neuroendocrine tumors (GEP-NETs)

1.1.1. Classification and common features

Gastro**e**ntero**p**ancreatic **n**euro**e**ndocrine **t**umors (GEP-NETs) are poorly understood lesions that encompass a broad category of neoplasms derived from neuroendocrine cells of the gastrointestinal (GI) mucosa and the pancreas. The lesions are grouped based on the acceptance that they have a common cell lineage and produce similar secretory products: chromogranin A (CgA), synaptophysin and neuron-specific enolase (NSE).[1,2] The GEP-NET classification includes different entities such as carcinoids, insulinomas, and gastrinomas, tumors that secrete vasoactive intestinal peptide (VIPoma), glucagonomas, somatostatinomas and non-functional pancreatic neuroendocrine tumors (pNETs). Those tumors are rarer than adenocarcinomas, the estimated incidence of pNETs is 5 per 1,000,000 population, whereas carcinoids (bronchopulmonary and GI) is estimated 8.4 per 100.000 population.[3-5] Although they are categorized together, their clinical behavior is strikingly divergent in terms of both symptoms and outcome. Thus, the overall 5-year survival rate for all carcinoids is 67.2 %, whereas the overall 5-year survival rate for pNETs varies from 97 % (benign insulinomas) to 30 % in nonfunctional pNETs.[4,6-8] Completely different is the outcome of patients with undifferentiated neuroendocrine carcinomas (NEC). As our group recently reported, 95 % of the undifferentiated NECs presented with evidence of either regional lymph-node involvement (UICC stage III: 20 %) or distant metastases (UICC stage IV: 76 %) at the time of diagnosis.[9,10] Moreover, we proposed that in otherwise typical adenocarcinomas of the colon and rectum the partial neuroendocrine differentiation is an independent prognostic factor in stage III–IV cancers and correlates with a more aggressive course of the disease.[9]

In an attempt to define the neuroendocrine group of lesions, several classifications have been proposed in the last 5 decades. In 1963 Wiliams and Sandler presented a

classification based on the localization of the tumors: Foregut (bronchial, pancreas, duodenum), midgut (up to the Treitz' ligament) and hindgut.[11] The prognostic value of this classification has still to be confirmed. Kloppel et al. presented an updated World Health Organization (WHO) system for the classification of NETs of the GEP system. Three main categories were created: Well differentiated endocrine tumors (WDETs), well differentiated endocrine carcinomas (WDECs) and poorly differentiated PDECs with the objective of providing a prognostically relevant classification system that assessed tumors according to size, proliferative activity, angioinvasion, organ invasion, metastases, hormone activity, and clinical syndromes.[12]

Most recently the TNM classification was introduced to better classify GEP-NETs. This classification has the advantage of distinguishing between lymph node positive and negative tumors and incorporates the "grading" as a further parameter to discriminate tumors according to their tumor biology. G3 is defined as a proliferation beyond 20 % (as assessed by Ki67 expression). This classification is currently under clinical investigation.[13-15] The distinction between benign and malignant GEP-NETs is very important and has not yet been completely resolved. The present classification is based on histopathological criteria. Further predictive genetic and molecular markers have not been evaluated to date. Apart from tumor differentiation, angioinvasion and tumor site, no marker reliably predicts the course of the disease. Only Ki67 as a marker of spontaneous tumor growth has been proven to be of prognostic significance.[16] More than 90 % of GEP-NETs are sporadic. Little is known about the pathogenesis of sporadic GEP-NETs.

1.1.2. GEP-NET genetics

A large number of studies have shown that neither common oncogenes such as src, ras, myc, fos, jun, nor common tumor suppressor genes as p53 or retinoblastoma susceptibility gene are of general importance in the molecular genetics and pathogenesis of most GEP-NETs (for review see Grabowski et al.[115]). In recent years, other genes and genetic aberrations in the molecular pathogenesis of GEP-NETs including the MEN1

gene, DPC/SMAD4, p16/p14/p15 and a potential tumor suppressor gene at18q24[5,17-19] have been evaluated. Recently, we have shown that p27 is of prognostic significance in GEP-NET tumor disease.[116] Patients with the inherited MEN1 syndrome and up to 40 % of spontaneous NET tumors show a mutation in the MEN1 gene. This gene encodes a putative tumor suppressor protein menin, which was shown to bind to JunD and several other interaction partners. Wild-type menin repressed transcriptional activation mediated by JunD. More than 400 different MEN1 germ line mutations have now been identified in several independent national studies based on large series of MEN1 patients with mutations found to be spread over the entire coding and non-coding sequence of the MEN1 gene without significant clustering. Most patients with aggressive phenotypes share truncating mutations. However, no other genotype-phenotype correlations could be established to date. In the MEN2 syndrome, the RET proto-oncogene, a tyrosine kinase receptor, is activated by missense mutations occurring either in the extracellular dimerization domain or intracellular tyrosine kinase catalytic regions.[20,21]

1.1.3. GEP-NETs pathophysiology

One of the typical features of GEP-NETs is that due to their derivation from active endocrine cells these tumors can (over)produce and secrete a wide variety of hormones, the most prominent and clinically relevant substance being serotonin, which can be synthesized by the tumor cells in large quantities leading to a hypersecretion syndrome. In the tumor, tryptophan is metabolized to 5-hydroxytryptophan, which is then converted to serotonin by L-dopa decarboxylase and stored in the neurosecretory tumor granules or released in the systemic circulation. Whereas foregut and hindgut GEP-NETs usually have low serotonin content and rarely cause clinical symptoms, midgut tumors have high serotonin content and frequently cause a classical carcinoid syndrome. About half of all NETs are functional, i.e. producing excess amounts of peptide hormones and biogenic amines.[22]

The (over)expression of somatostatin receptors on the surface of GEP-NETs provides the structural molecular basis for the regulation by somatostatin and its stable analogues. In

recent years, a number of studies have been published defining the subtype distribution of these receptors in GEP-NETs. From all receptor subtypes presented in the tumor types, SSTR2 and SSTR5 are clearly the dominant receptor subtypes.

1.1.4. Principles for GEP-NETs' treatment

The principles of management of patients with GEP-NETs depend on a number of factors requiring a multidisciplinary approach. In cases of localized disease the surgical resection seems to be the best option. However, in cases of extended disease, as opposed to treatment decisions for other solid tumors of the digestive tract, 'wait-and-see' strategies can often be adopted in patients with GEP-NETs.[23] The recurrence rate of the tumor after surgery was 84 %, but 5-year survival was still 61 %.[6,24,25] The slow-growing nature of well-differentiated tumors means that chemotherapy and other treatment strategies should be reserved for patients with progressive disease. Because of the specific expression of receptors like SSTR 2 and 5 on the tumors, so called biotherapeutic agents like somatostatin analogues and more recently, multireceptor binding agents like SOM230 were synthesized with good results concerning the clinical syndromes, but uncertain effects in terms of inhibition of further tumor growth.[26,27] Indeed, to date interpretation of data on treatment of patients with GEP-NET tumors has been hampered by the lack of evidence for progressive disease in a number of studies. Documented progression should be based on accurate and comparable evaluation of clinical, biological and morphological data at least at 6-monthly intervals.

For WDETs, which are usually "benign-behaving", a classical chemotherapy is not evaluated and in most cases not indicated. Local endoscopic or surgical procedures seem to be sufficient to cure small tumors. In WDEC disease, foregut carcinomas can be effectively treated with a combination of 5-FU and streptozocin or doxorubicin, whereas a standard chemotherapy in midgut tumors is not established.[24,25,27,28] The poor prognosis of PDECs is little influenced by aggressive chemotherapeutic agents like etoposide and cisplatin.[13,29,30] Given that response to cytotoxic agents in patients with GEP-NETs may be short-lived, determining the correct moment to start treatment is often difficult. Early

treatment at the outset is, on the contrary, usually necessary for patients with aggressive well-differentiated carcinomas and for those with poorly differentiated lesions whose natural history and outcome is similar to that of small cell lung cancer. Another consideration in commencing treatment at the moment of diagnosis is the presence of bulky disease, especially the presence of extensive liver metastases. Here, local ablative strategies like RFTA, PEI, TACE or surgical resection are implemented. Another therapeutic option is the use of peptide radioreceptor therapy (PRRT) with ß-particle emitting molecules like Yttrium/Lutetium-octreotate.[6,21,22,31-35] In spite of all efforts, tumor control is often not satisfactory and future clinical trials are warranted, to evaluate the best therapeutic modality for the patient in this setting.

1.2. Survivin

1.2.1. Inhibitor of Apoptosis Proteins (IAPs)

The antiapoptotic proteins that counteract signaling through specific apoptosis pathways provide targets for possible drug discovery and new anticancer interventions. Two major pathways of apoptosis have been identified in mammalian cells. An extrinsic pathway is triggered by the binding of ligands to cell-surface trimeric membrane death receptors and leads to caspase-8 activation. An intrinsic pathway involves mitochondria, which respond to proapoptotic signals by releasing cytochrome *c*, which in turn binds and activates the apoptotic protease activating factor-1 (Apaf-1), causing assembly of a multiprotein caspase-activating complex (apoptosome) and leading to activation of caspase-9 and initiation of a protease cascade. The intrinsic and extrinsic pathways for apoptosis converge on downstream effector caspases. Some of these, such as caspase-3 and caspase-7, are targets of suppression by an endogenous family of antiapoptotic proteins, which also interfere with caspase-9 processing, the upstream initiation of the mitochondrial pathway of apoptosis.[36-38] The human genome encodes eight IAP family members including X-linked inhibitor of apoptosis (X-IAP), cIAP1, cIAP2, ML-IAP (Livin; KIAP), Naip, ILP2 (TS IAP), apollon/Bruce, and survivin.

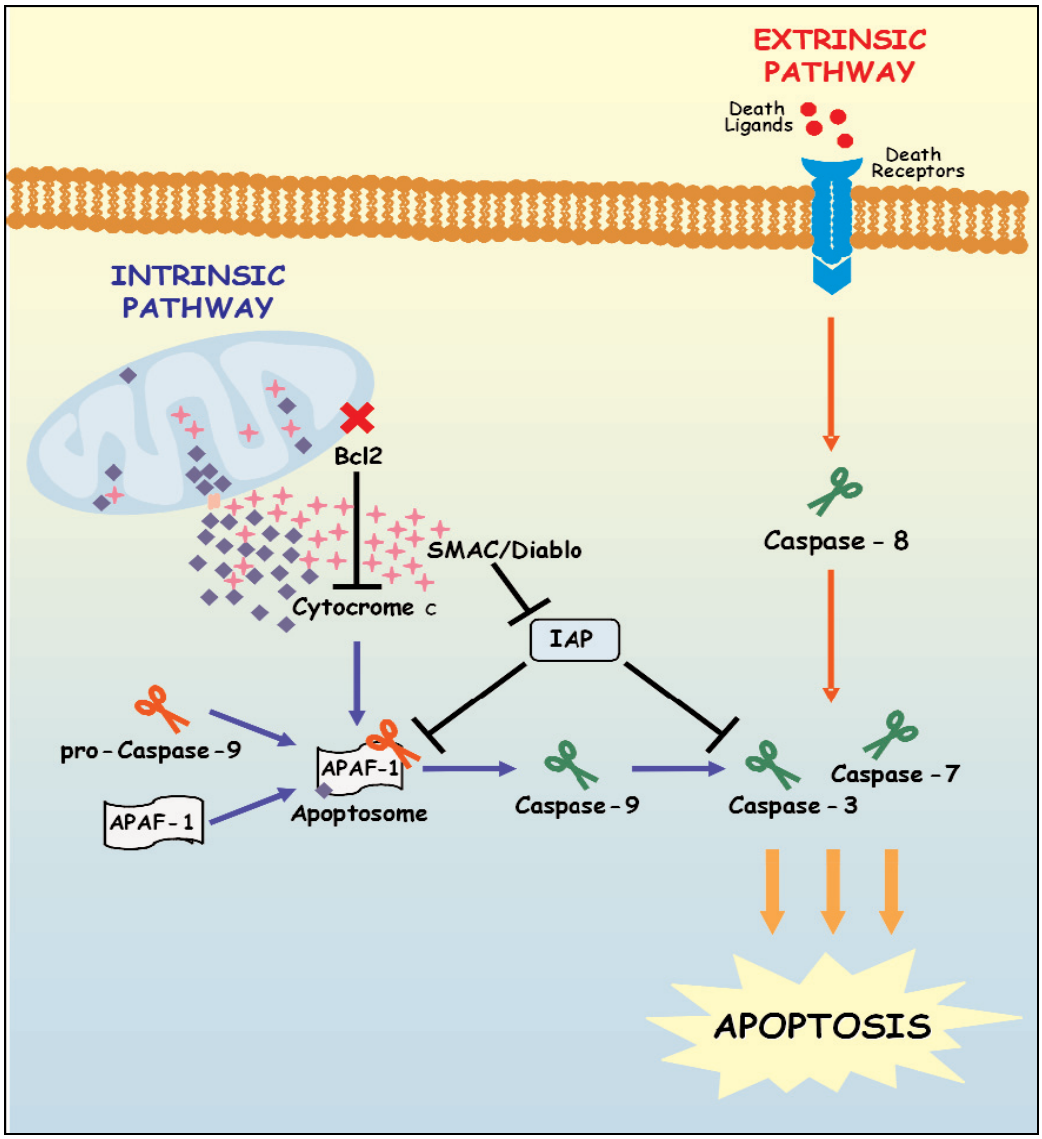


Fig. 1. Schematic representation of the two major apoptotic pathways in human cells: the "extrinsic" (the death receptor-mediated) and "intrinsic" (mitochondrial) programmed cell death (modified after Zaffaroni, 2006).

1.2.2. Structure and function of survivin

The human survivin gene spans 14.7 kb on the telomeric position of chromosome 17 and is transcribed from a TATA-less, GC-rich promoter to generate the wild-type transcript and four different splice variant mRNAs. Survivin is a 16.5 kD protein of 142 amino

acids and is composed of a single **B**aculovirus **I**AP **R**epeat (BIR) domain and an extended COOH-terminal α-helical coiled-coil domain; it does not contain the RING-finger domain found in other IAPs. Five functional splice variants of human survivin have been described, full-length survivin, survivin-2α, survivin-2B, survivin ΔEx3, and survivin-3B.[36,39-42] While survivin-3B is a truncated form of 120 amino acids, survivin-2α and survivin ΔEx3 both result from alternative splicing at the interface between exons 2 and 3. Survivin-2B has an additional exon of 23 amino acids, exon 2B, inserted in its BIR domain, which is predicted to alter its tertiary structure and thus to affect its anti-apoptotic function. Survivin ΔEx3 omits exon 3 causing a frame shift (Fig. 2). Little is known about the functions of the alternative splice forms of survivin – except their participation in mitosis. Preliminary data suggest that heterodimerization of wild-type survivin with survivin ΔEx3 is essential for the inhibition of mitochondrial-dependent apoptosis. Subcellular compartment localization of survivin in mitochondria or the nucleus seems to play a role in the antiapoptotic function of the protein and is a powerful prognostic marker.[43]

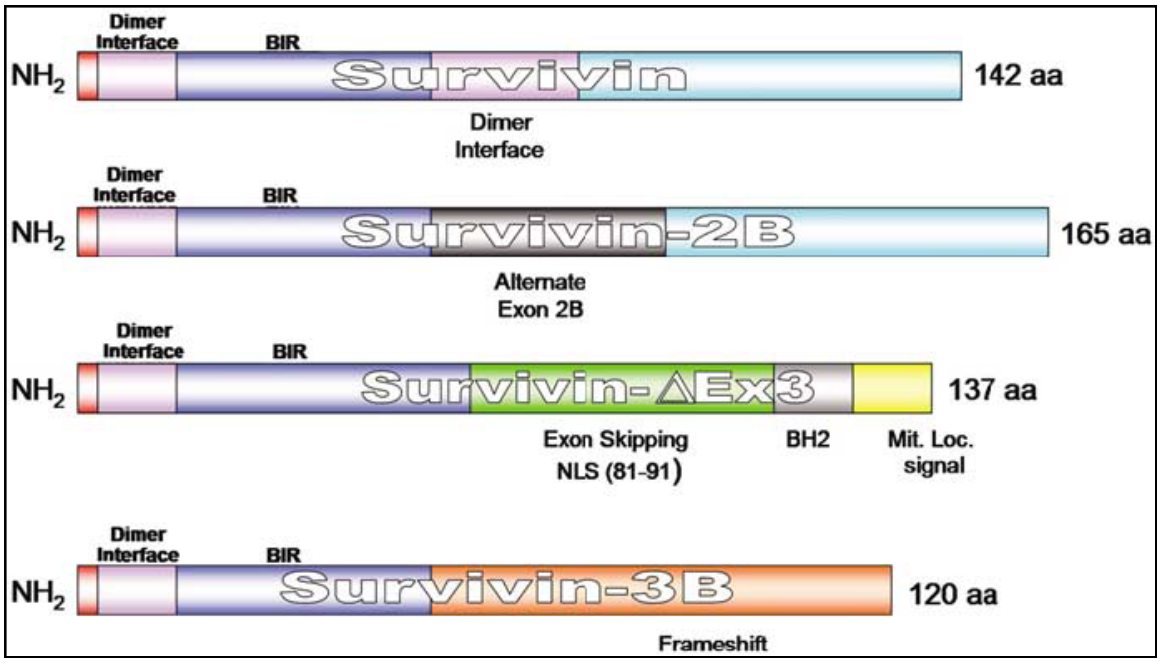


Fig. 2. Comparison of the protein structure of survivin's main splice variants. The survivin protein encoded by four exons consists of an N-terminal domain, a BIR domain (aa 38-87), and a C-terminal a-helix tail (modified after Caldas, 2006).

1.2.3. Survivin as a participant in the mitotic cycle

Survivin is a component of the chromosomal passenger complex. Expression of survivin peaks in G2/M phase of the cell cycle and it was proposed that its subcellular localization in the nucleus is characteristic of its function in the chromosomal passenger complex, where survivin interacts directly with INCENP and aurora kinase B [41,44-46] (Fig. 3).

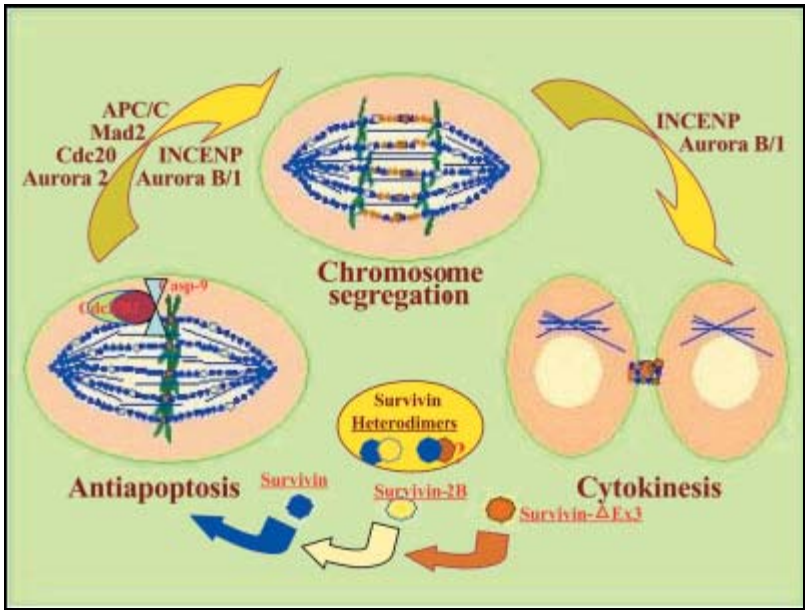


Fig. 3. A hypothetic model to unify the function and subcellular localization of survivin and survivin-splicing variants. In metaphase, survivin binds to the mitotic spindle fibers on which survivin is associated with and phosphorylated by CDC2/cyclin. The phosphorylated survivin offers survivin the ability to physically interact and functionally inhibit the caspase-9 activity. During prometaphase and metaphase, survivin and survivin ΔEx3 together with aurora B and INCENP bind to kinetochore. During anaphase the kinetochore-associated survivin and survivin ΔEx3 together with INCENP and aurora B will be transferred to spindle midzone (modified after F.Li, 2003).

1.2.4. Survivin expression in normal and tumor tissues

Survivin expression in normal tissues is developmentally regulated and the protein was found to be absent or low in most terminally differentiated tissues.[47] However, recent studies tend to attribute a role to survivin in regulating the function of normal adult cells including vascular endothelial cells, polymorphonuclear cells, T cells, erythroid cells, and hematopoietic progenitor cells.[48,49] However, although survivin is expressed in normal tissues characterized by self-renewal and proliferation, its expression is significantly lower than in transformed cells. In fact, several studies have demonstrated strong survivin expression in most human solid tumor types and hematological malignancies.[48,50-52] The upregulation of survivin at the transcriptional level in human tumors has been confirmed in genome-wide searches, which indicated survivin as the fourth top "transcriptome" in cancers of various histology.[53] Growing evidence suggests that survivin expression in cancer cells is associated with clinicopathologic variables of aggressive disease, resistance to radio- and chemotherapy and may represent an important prognostic marker for patient outcome.[51,52,54,55] In fact, several studies on different types of solid tumors and hematological malignancies showed that high levels of the protein were predictive of tumor progression in terms of either disease-free or overall survival.[16,36,42,43,56,57] As it is possible to immunohistochemically distinguish two intracellular pools of survivin, a nuclear and a cytosolic one, the prognostic significance of the nuclear survivin expression was proven in a subset of GEP-NET tumors by Grabowski and co-workers.[43]

1.2.5. Molecular mechanisms for inhibition of the survivin expression

In recent years considerable efforts have been made by researchers to develop strategies for modulating apoptosis in cancer and other human diseases. In this context, approaches to counteract survivin in tumor cells have been proposed with the dual aim to inhibit tumor growth through an increase in spontaneous apoptosis, and to enhance tumor cell response to apoptosis-inducing agents.[58] Different kinds of survivin molecular antagonists, including antisense oligonucleotides, ribozymes, small interfering RNAs

(siRNAs), dominant-negative mutants and cyclin-dependent kinase inhibitors as well as small inhibitory molecules have been used.[59,60]

Methods	Mechanism	Effects	Result
Antisense-Oligonucleotide[56,61]	Specific Inhibition	Inhibition of proliferation Induction of apoptosis Cell cycle dysregulation	Increased chemosensitivity Increased radiosensitivity Increased sensitivity towards Immunotherapy
siRNA[60,62]	Inhibition of the survivin gene expression	Inhibition of proliferation Induction of apoptosis Cell cycle dysregulation	Increased chemosensitivity Increased radiosensitivity
Hammerhead-Ribozyme[54,62-64]	Degradation of RNA target sequences	Caspase 9 - dependant apoptosis	Increased chemosensitivity
Dominant-neg. mutants[64]	Competition with the wild type survivin	Inhibition of proliferation	Increased chemosensitivity
Cyclin-dependent kinase-Inhibitors[64]	Inhibition of mitosis Phosphorylation	Induction of apoptosis Inhibition of proliferation	Increased chemosensitivity

Table 1. Strategies to downregulate the survivin expression level in the preclinical setting. In a number of studies it was demonstrated that survivin downregulation induces apoptosis and increased sensitivity towards chemotherapy and/or radiotherapy.

1.2.6. The potential role of survivin in chemo- and radioresistance

Growing evidence has indicated that survivin expression plays an essential role in drug resistance, and that genetic or pharmacological modulation of survivin expression affects drug effectiveness in apoptosis induction. Recently it was reported that expression of survivin antisense RNA significantly reversed multiple drug resistance and sensitized various tumor cells to TRAIL or chemotherapeutic drug induced apoptosis, but had no effect on normal human fibroblasts.[40,56,60,64]

Evidence has also revealed an essential role for survivin in cancer cell resistance to radiation therapy. Downregulation of survivin by siRNA increased apoptosis in colorectal cancer cells. One potential mechanism to explain the antagonistic effects of the drug/radiation combination treatment could be the induction of survivin expression by means of drug and radiotherapy.[54,65]

1.2.7. Preclinical studies

Recent extensive research on survivin has revealed that a number of existing anticancer drugs show survivin suppressing activity through different cell signaling pathways, for example: hedamycin, the ErbB2 inhibitor lapatinib, the janus-activated kinase-2 inhibitor AG490, the heat shock protein-90 antagonist 17-N-allylamino-17-demethoxygeldanamycin 17-AAG) and the histone deacetylase inhibitor LAQ824.[60] When exerting their antitumor activities, however, these drugs are also thought to act on other normal cell signaling pathways, which often results in severe systemic toxicity.[59,60,66] YM155 is a new first-in-class synthesized compound that suppresses selectively survivin and shows potent antitumor activities in vitro and in vivo.[66] A phase II, open-label study of YM155 with 7-day continuous i.v. infusion in patients with different hematologic and non-hematologic malignancies is currently under way (preliminary results with this compound were presented at EORTC/NCI/AACR Meeting on “Molecular targets and cancer therapeutics, 2007”, abstract B 278).

1.3. Aurora kinases

Among the network of regulatory proteins of cellular division, aurora kinases play a crucial role in cellular division by controlling chromatid segregation. Defects in chromatid segregation cause genetic instability, a condition associated with tumorigenesis. There are three aurora kinases: A, B and C.

1.3.1. Structure of aurora kinases

Human aurora kinases (Fig. 4) range in size from 309 to 403 amino acid residues, which exhibit a relatively high sequence divergence between species.

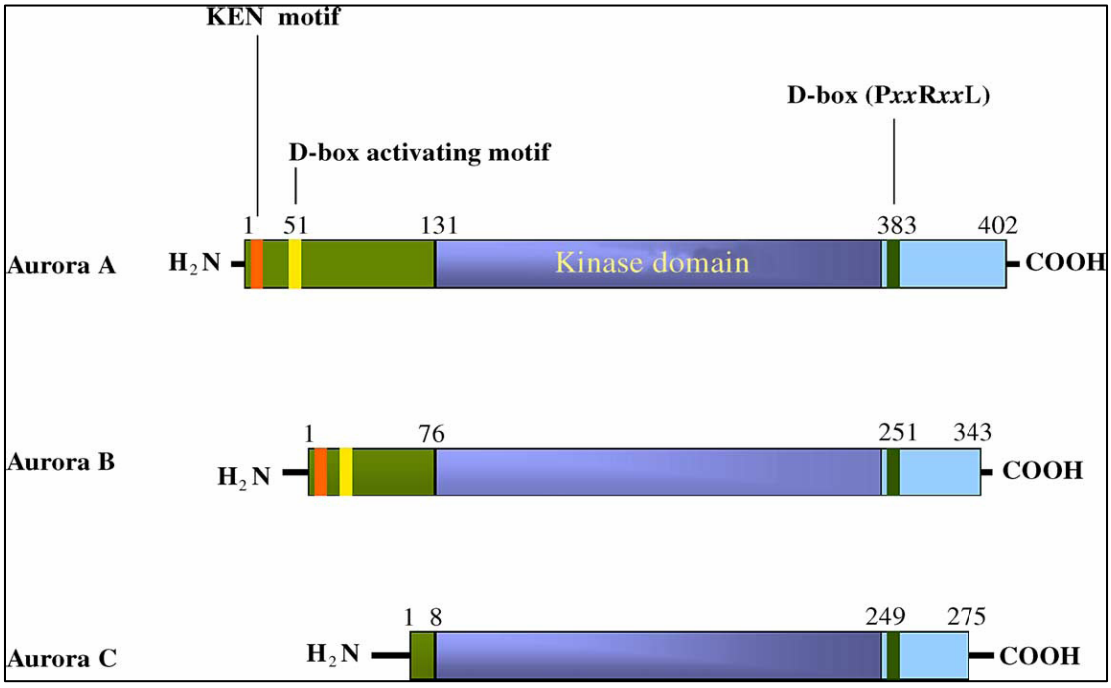


Fig. 4. Domain organisation of aurora kinases A, B and C. As shown here, aurora kinases present three domains: the N-terminal and the C-terminal domains contain most of the aurora's regulatory motifs, while the central region contains the catalytic domain. In addition to the kinase activity, this central domain also presents regulatory motifs (modified after Bolanos-Garcia, 2007).

Aurora kinases A–C present a similar domain organization: an N-terminal domain of 39 – 129 residues in length, a protein kinase domain and a short C-terminal domain of 15 – 20 residues. The N-terminal domain of aurora kinases shares low sequence conservation, which determines selectivity during protein – protein interactions [67-70] (Fig. 4). The alignment of auroras A and B allows the identification of one distantly conserved 'KEN' motif, spanning 11–18 residues. The C-terminal domain of human aurora B shares 53 % and 73 % sequence similarity to human auroras A and C, respectively.

1.3.2. Biological function

1.3.2.1. Aurora A

Aurora A overexpression contributes to genetic instability and tumorigenesis by disrupting the proper assembly of the mitotic checkpoint complex.[71] Its overexpression also causes resistance to apoptosis induced by taxol in human cancer cell lines.[72] Moreover, this kinase is a key regulatory component of the p53 pathway as its overexpression leads to increased p53 degradation, which facilitates oncogenic transformation.[63,73]

1.3.2.2. Aurora B

Aurora B also plays an essential role in chromosome segregation and cytokinesis and its kinase activity is required for bipolar chromosome orientation and condensation.[46] Aurora B kinases are 'chromosomal passenger' proteins, which are found in cells in a complex with inner centromere protein (INCENP) and survivin. This complex first localizes along the chromosome during prophase, becomes concentrated at the inner centromere region during prometaphase and leave the chromosome and localize to the central spindle in anaphase. The overexpression of an aurora B kinase-dead mutant (K-R) causes multiple defects in the mitotic machinery, including the loss of kinetochore attachment to microtubules and the exit from mitosis without anaphase or cytokinesis.[63,74,75] It seems that aurora kinase B could be a regulator in the p53 pathway.[73]

1.3.2.3. Aurora C

Very little is known of the function and regulation of this kinase. Aurora C is a chromosomal passenger protein localizing first to centromeres and then to the midzone of mitotic cells that cooperates with aurora B to regulate mitotic chromosome segregation and cytokinesis in mammalian cells and like aurora B, interacts with the inner centromere protein (INCENP).[70]

1.3.3. Possible medical applications

Aurora A has been recognized as a good marker of tumor progression and prognosis. Previous studies found that aurora A and B were aberrantly expressed in a variety of solid tumors including prostate, testicular[76-78], colon[78], pancreas[79], breast[80], and thyroid cancers.[81] In addition, increased levels of aurora kinases correlated with advanced clinical stage in individuals with prostate cancer, as well as those with head and neck squamous cell carcinoma.[76,82] It has been recently shown that hematological malignant cells including those from acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML) aberrantly expressed aurora A and B kinases.[83,84] Moreover, the activation loop region of aurora A exhibits unique conformations, features that can be exploited for drug design. Certainly, the analysis of ATP-binding domains has opened a promising new front in the search of novel anti-oncogenic drugs that target aurora kinases.

1.3.4. Preclinical studies

Several aurora inhibitors have been synthesized to date: hesperadin and AZD1152[85], inhibitors of aurora B; the quinazoline ZM447439 and the pyrazole PHA-739358[86], inhibitors of auroras A and B and more recently, the phenylamide VX-680, a selective inhibitor of the three auroras. Most of these selectively block the aurora kinases in the nM range and other kinases in the μM and mM range, have a profound effect on tumor growth and have potential for efficacy in multiple tumor types.[87] Parental administration

of AZD1152 and ZM447439 in tumor xenograft models resulted in profound tumor growth-sustaining effects accompanied by low or moderate, reversible side effects. Currently, phase I trials are ongoing with ZM447439 and AZD1152[85], PHA-680632[87,88] in patients with hematological malignancies and solid tumors.

2. Aim

In this study, we investigated the functional role of survivin in a subset of gastrointestinal neuroendocrine tumor cell lines representing the heterogeneous growth pattern of this tumor entity. We used survivin downregulating strategies with specific survivin siRNA to elucidate the cytotoxic effects.

Furthermore, we evaluated the antiproliferative and pro-apoptotic effects of a novel highly selective aurora kinase inhibitor ZM447439, alone and in combination with bio- and chemotherapeutic agents and its preclinical profile, as a candidate for anticancer therapy in gastroenteropancreatic neuroendocrine tumor diseases.

3. Materials and Methods

3.1. Materials

3.1.1. Common solutions and chemicals.

Reagents	Manufacturer
Aqua redistilata	DeltaSelect, Dreieich
Bromophenol blue	Sigma, Deisenhofen
DMSO	Sigma, Deisenhofen
Ethanol	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
PBS Dulbecco w/o Ca2+ and Mg2+	PAA, Berlin
Propidium Iodide	Fluka, Switzerland
Rnase A	Qiagen, Hilden
L-Glutamine	PAA, Berlin
Penicillin/Streptomycin	PAA, Berlin
Nonidet P-40 (Igepal C-630)	Sigma, Deisenhofen
NaCl	Merck, Darmstadt
KCl	Fluka, Switzerland
Tris Base	Amersham, Sweden
K2HPO4	Merck, Darmstadt
Na2CO3	Merck, Darmstadt
HCl	Merck, Darmstadt
KH2PO4	Merck, Darmstadt
Na2HPO4 x 2H2O	Merck, Darmstadt
Lauryl Sulfate (SDS)	Sigma, Deisenhofen
NaDeoxycholic Acid	Sigma, Deisenhofen
Hepes Puffer	PAA, Berlin
Biocoll Separating solution	Biochrom, Berlin
FCS Gold	PAA, Berlin

3.1.2. Laboratory materials

Material	Manufacturer
Culture flasks	NUNC, Wiesbaden
Cell scrapers	Sarstedt, Nuembrecht
6-well- multidishes	NUNC, Wiesbaden
12-well-multidishes	NUNC, Wiesbaden
24-well-multidishes	NUNC, Wiesbaden
96-well-multidishes	NUNC, Wiesbaden
FACS tubes	BDFalcon, Heidelberg
Falcon tubes (15 ml and 50 ml)	BDFalcon, Heidelberg
Neubauer chamber	Sigma, Deisenhofen
Serological pipettes	BDFalcon, Heidelberg
Petri plates	Eppendorf, Hamburg
Eppendorf tubes 1.5 ml	Eppendorf, Hamburg
ElecTips	BD Falcon, Heidelberg

3.1.3. Laboratory inventory

Product	Name	Manufacturer
Agarose gel chamber	Horizon 58	GibcoBRL, Karlsruhe
Camera	Polaroid	Polaroid, Offenbach
Centrifuge	Eppendorf 5424	Eppendorf, Hamburg
Electrical pipette	Pipetus-accu	Hirschmann, Hamburg
Flow cytometer	FACScan	Beckton Dickenson, Heidelberg
Incubator	APT line CB	Binder, Tuttlingen
Microscope	Zeiss	Zeiss, Goettingen
Pipettes	Eppendorf	Eppendorf, Hamburg
Quevette	Ultrospect	Pharmacia Biotech, UK
Safety bench	Clean air	Woerden, Holland

Vortexer	REAX 2000	Heidolph, Kelheim
ELISA Reader	GEMINI	Bio- RAD, München
Multi-pipette	Transferpette-12	Brand, Germany
Thermomixer comfort	Eppendorf	Eppendorf, Hamburg
Scale	Universal	Sartorius, Goettingen
Labor pH/mV-Meter	pH-Meter	WTW, Weilheim
Power PAC 3000	Electromagnetic emission apparatus	BIO- RAD, München
Shaker	MTS 2	IKA, Staufen
Western Blot's chamber	Criterion Cell	BIO- RAD, München
Magnetic shaker	KMO2	IKA, Staufen
Fluorometer	VersaFluor Fluorometer System	BIO- RAD, München

3.1.4. Bio- and chemotherapeutic agents

Name	**Cat. N.**	**Manufacturer**
5-FU	F-6627	Sigma-Aldrich Chemie, Steinheim
Etoposid	E1383	Sigma-Aldrich Chemie, Steinheim
Irinotecan (SN-38)	I1406	Sigma-Aldrich Chemie, Steinheim
Doxorubicin	D1515	Sigma-Aldrich Chemie, Steinheim
Streptozocin	S0130	Sigma-Aldrich Chemie, Steinheim
Docetaxel	1885	Sigma-Aldrich Chemie, Steinheim
Oxaliplatin	9512	Sigma-Aldrich Chemie, Steinheim
Cisplatin	P4394	Sigma-Aldrich Chemie, Steinheim
ZM447439	1A/74564	Tocris, USA
SOM230	1A/74564	Kindly provided by Novartis, Switzerland
Octreotide	O1014	Sigma-Aldrich Chemie, Steinheim

5-FU is a commonly used pyrimidine antagonist. Like the other pyrimidine antagonists, 5-FU is similar in structure to the normal pyrimidine molecule. It inhibits DNA synthesis both by blocking the formation of normal pyrimidine nucleotides via enzyme inhibition

and by interfering with DNA synthesis after incorporation into a growing DNA molecule. In oncology, 5-FU is used in the adjuvant as well as in the palliative setting, to treat patients with gastrointestinal, pancreatic, urinary bladder and prostate cancer.

Doxorubicin is a DNA-interacting drug widely used in chemotherapy. It is an anthracycline antibiotic, which intercalates with DNA. It is commonly used in the treatment of a wide range of cancers.

Cisplatin is the first platinum-based drug used to treat various types of cancers. It binds to DNA and DNA interacting proteins and inhibits the activity of the cell telomerase.

Oxaliplatin is a platinum-based chemotherapeutic drug of the same family as cisplatin and carboplatin. Compared to cisplatin the two amine groups are replaced by cyclohexyldiamine for improved antitumor activity and lesser side effects. The chlorine ligands are replaced by the oxalate bidentate derived from oxalic acid in order to improve water solubility.

Irinotecan is a chemotherapeutic agent that is a topoisomerase 1 inhibitor. Chemically, it is a semisynthetic analogue of the natural alkaloid camptothecin. SN-38 is the active metabolite and leads to inhibition of both DNA replication and transcription.

Etoposide phosphate is an inhibitor of the enzyme topoisomerase II. It is used as a form of chemotherapy for various types, but especially SCLC and PDEC.

Docetaxel is a tubulin-binding chemotherapeutic agent, which acts mainly on M-phase cells. Docetaxel is a clinically well-established anti-mitotic drug used mainly for the treatment of breast, ovarian and non-small cell lung cancer.

Streptozocin is a type of antibiotic that is only used for chemotherapy. It slows or stops the growth of cancer cells and is used mainly in WDEC tumors.

Octreotide is an octapeptide that mimics natural somatostatin pharmacologically (somatostatin agonist), though it is a more potent inhibitor of growth hormone, glucagon, and insulin than the natural hormone.

SOM230 is another Somatostatin-mimicking analogue. However, it is acting on all five somatostatin receptors.

3.1.5. Cell lines

Cell line	Origin	Provider
BON	Pancreas NET	kindly provided by Prof. Townsend CM Jr, University of Texas Medical Branch
DU-145	Prostate NEC	ATCC, USA
HT29	Colon adenocarcinoma	ATCC, USA
MIP-101	undifferentiated NEC	kindly provided by Prof. Tai, University of British Columbia, Vancouver
CM	Insulinoma	ATCC, USA
LCC-18	Colon adenocarcinoma	ATCC, USA
NCI-H716	Colon adenocarcinoma	ATCC, USA
HT29	Colon adenocarcinoma	ATCC, USA
Colo320	Colon adenocarcinoma	ATCC, USA
NCI-H747	Colon adenocarcinoma	ATCC, USA
Colo205	Colon adenocarcinoma	ATCC, USA
LS-174-T	Colon adenocarcinoma	ATCC, USA
Caco2	Colon adenocarcinoma	ATCC, USA
QGP-1	Pancreatic NEC	kindly provided by Prof. Wiedenmann, Charite- CVK, Berlin

3.1.6. Cell cultures and mediums

Medium	Contents	Manufacturer
DMEM (HAM'S F-12)		Biochrom, Berlin
	10 % FCS	
	1 % Penicillin/Streptomycin	
	1 % Glutamine	
	w stable glutamine	
	LE (low Endotoxin)	
RPMI 1640		Biochrom, Berlin
	10 % FCS	
	1 % Penicillin/Streptomycin	
	1 % Glutamine	
	w 2,0 g/l NaHCO3	
	w stable glutamine	
	LE (low Endotoxin)	
D-MEM		PAA, Berlin
	10 % FCS	
	4500 mg/l Glucose	
	L-Glutamine	
	w/o Pyruvate	
Opti-MEM		Invitrogen, USA

3.1.7. Reagents used in the crystal violet growth assay

Reagents	Manufacturer
Glutaraldehide Solution	Sigma, Deisenhofen
Cristal Violet	Sigma, Deisenhofen
Triton X-100	Sigma, Deisenhofen

10 x PBS	Biochrom, Berlin
2 gr KCL	Sigma, Deisenhofen
14,4 gr Na2HPO4	Sigma, Deisenhofen
2,4 gr KH2PO4	Sigma, Deisenhofen
80 gr NaCl	Sigma, Deisenhofen
1:10 dilute, pH 7,4	

3.1.8. Common materials and reagents used for Western Blot

Reagents	**Manufacturer**
SeeBlue Plus2 Pre-stained standard	Invitrogen, Karlsruhe
XT MOPS, Running Buffer, 2 0x	BIO-RAD, München
Criterion XT Precast Gel, 12 %Bis-Tris	BIO-RAD, München
ECL Western Blotting Analysis System	Amersham Biosciences, UK
ECL Plus Western Blotting Detection	Amersham Biosciences, UK
XT Reducing agent, 20 x	BIO-RAD, München
XT Sample Buffer, 4 x	BIO-RAD, München
Blotting Grade Blocker Non-fat Dry Milk	BIO-RAD, München
10 x LOS (WEB)	
30 gr Tris Base	Sigma, Deisenhofen
	144 gr Glycine
10 x TBS	
	60,61 gr Tris Base
	80 gr NaCl
	2 gr KCl
	HCL ad pH 8,0
Tween 20 for electrophoresis	Sigma, Deisenhofen
Glogos II Autorad Markers	Strategene, USA

3.1.9. Antibodies and kits

Name	Manufacturer
Anti-Survivin-antibody polyclonal	Biozol, Eching
Survivin ΔEx3 antibody	Biozol, Eching
Survivin-2B antibody	Biozol, Eching
AIM-1	BD Biosciences, USA
Monoclonal anti-ß-Actin	Sigma, Saint Louis, Missouri, USA
Monoclonal anti-ß-Tubulin	Sigma, Saint Louis, Missouri, USA
Anti-Synaptophysin	BioGenex, USA
SNAP25 (mAB)	Synaptic Systems, Göttingen
Anti-Synaptobrevin-VAMP2 (mAB)	Synaptic Systems, Göttingen
Anti-Syntaxin	Synaptic Systems, Göttingen
Mouse anti-Human Chromogranin A	Linaris, Wertheim
ECL Anti Maus IgG HRP-linked antibody(from sheep)	Amersham, UK
ECL Anti- rabbit IgG HRP-linked antibody(from donkey)	Amersham, UK
Fix und Perm, Cell permeabilization kit	Catalag, Austria
Alexa fluor 488 goat anti-mouse IgG	Molecular Probes, USA
Negative Control Mouse IgG1	DakoCytomation, Denmark
Apo-ONE Homogeneous Caspase-3/7 Assay	Promega, USA
Apoptotic DNA Ladder Kit	Roche Diagnostics, Penzberg
TiterZymeEIA human Total Survivin Kit	Assay designs, USA

3.1.10. Reagents and kits used for protein isolation, separation and determination

Reagent	Manufacturer
BCA Protein Assay Reagent A	Pierce, USA
BCA Protein Assay Reagent B	Pierce, USA

Albumin Standard 2 mg/ml		Pierce, USA
Protease Inh. "complete Mini"		Roche, Mannheim
1 x TEN-Buffer		
	15 ml 1 M NaCl	
	200 µl 0,5 M EDTA-Sol pH 8	
	4 ml 1 M Tris-HCl-Sol pH 7,5	
	ad 100 ml aqua destillata	
RIPA-buffer		
	2 ml Igepal C-650	
	1 ml 20% SDS	
	1gr NaDeoxycholic Acid	
	0,1 M PMSF in ETOH	
	Protease Inh. "complete Mini"	
	ad 100 ml aqua destillata	

3.1.11. Cell transfection material, reagents and siRNA oligonucleotides

Reagents	**Manufacturer**
Lipofectamine 2000	Invitrogen, Karlsruhe
Negative Control siRNA	Qiagen, Hilden
siRNA survivin	kindly provided from Prof. Zaffaroni, Milano, Italy

3.2. Patients

The human tumor material was used according to the standards set by the Ethics Committee of the Charité University Hospital. Blood from the patients was obtained after receiving an informed consent. Blood samples were obtained from patients with GEP-

NET cancer (n = 20) and healthy volunteers; hematological malignancies and aggressive solid tumors (n = 20) and healthy volunteers (n = 20) by venipuncture. The exact patient characteristics are described in Table 3 (Chapter 4.3.3).

3.3. Methods

3.3.1. Cultivation of tumor cells

Only sterile mediums, medium additives, cell scrapers and serological pipettes were used in the cultivation of tumor cell lines. Medium change and all other procedures with cell culture flasks were carried out under a clean air bench. Frozen cells were thawed in water bath at 37°C and dissolved in warm medium. Then the cells were centrifuged for 5 minutes at 1200 rpm speed at room temperature and washed twice in a medium to remove DMSO (final concentration < 0.01 %) and sowed in flasks at the recommended by the provider density. Every three days the cell count was checked and cells repetitively repopulated at the recommended density. The cells were split as follows: first the "old" medium was removed and cells were washed with 10 ml pre-warmed PBS, then 1000 µl Trypsin-EDTA was added and finally cells were left for 5 minutes in a cell incubator at 37°C, 5 % CO2 till they were detached. Then the desired volume of cells was transferred with 15 ml fresh medium to a new flask.

All cell lines were cultured in doubles in flasks at 37°C, 5 % CO2 and 95 % humidified sterile air. QGP-1 and DU-145 cell lines were cultured in RPMI 1640 medium supplemented with 10 % FCS, 1 % L- Glutamine, 1 % penicillin/streptomycin. Colo320, NCI-H716, NCI-H747 cell lines were also cultured with RPMI 1640 medium but supplemented only with 10 % FCS. BON cell line was cultured in DMEM (HAM'S F-12) supplemented with 10 % FCS, 1 % L- Glutamine and 1 % penicillin/streptomycin. MIP-101, LCC-18, Colo 205 cell lines were cultured in D-MEM supplemented only with 10 % FCS and LS-174-T cell line was also cultured in D-MEM but supplemented not only with 10% FCS but also 1 % L-Glutamine. CaCo 2 cell line was cultured with MEM

medium supplemented with 10 % FCS. HT29 cell line was used as negative control and cultured in RPMI 1640 medium, containing 10 % fetal bovine serum.

3.3.2. Freezing of cell samples

The medium, in which the cell samples were frozen, was prepared from 20 % FCS, 10 % DMSO and RPMI 1640 medium ad 50 ml. After harvesting the cells, 20 ml fresh medium was added to the cells, which were then centrifuged for 5 min, at 1600 rpm speed. 1.5 ml freezing medium was added to the pellet and the cells were stored first at - 80°C and then transferred in liquid nitrogen.

3.3.3. Fluorescence based methods: Fluorescent flow cytometry (FACS)

Flow cytometry is a method by which more than 5000 particles per second can simultaneously be evaluated for up to five parameters in a liquid flow. This is realized as follows: diluted cells are adsorbed from a FACS tube and by the means of hydrodynamic forces focused on an argon-ionic laser. The scattered light is measured by five detectors. The forward scatter (FSC) detects waves scattered along the primer beam and in that way gives information about the cell size. The side scatter (SSC) detects waves scattered perpendicularly to the primer source of light and depicts cell granularity. In this study three fluorescent dyes were used - FITC, detected at fluorescent channel 1 with emission maximum at 520 nm; phycoerythrin (PE), detected at fluorescent channel 2 with emission maximum at 580 nm and propidium iodide (PI), detected at fluorescent channel 3 with emission maximum at 650 nm. Each dye emits light with a definite spectrum after being excited by an argon laser. However, the spectrums overlap each other and thus have to be compensated. At each measurement at least 10000 events were counted.

3.3.3.1. Intracellular staining

The following protocol was used when staining intracellular GEP-NET markers as: synaptophysin, synaptobrevin, chromogranin A, syntaxin and SNAP25.

After harvest, the cells were centrifuged for 5 min at 14000 rpm. Then the cells were washed once in PBS, the supernatant was removed and 100 µl Fix & Perm Medium A was added. After 15 min incubation, the cells were washed with 1 ml 1 x PBS and centrifuged 40 sec at 14000 rpm. The supernatant was removed, the cells were washed a second time with 1 ml 1 x PBS and then centrifuged again 40 sec at 14000 rpm. The supernatant was removed again and the first antibody was added in appropriate concentration. The incubation with the first antibody is about 50-60 min at room temperature. Afterwards, the cells were twice washed in 1 ml 1 x PBS and centrifuged for 40 sec at 14000 rpm. Then the second FITC-conjugated antibody was added in dilution 1:500 and the cells were incubated with it at about 1 hour in a dark place on ice. After the incubation cells were washed twice with 1 ml 1 x PBS and centrifuged for 40 sec at 14000 rpm. Finally, 300 µl 1 x PBS was added to the cells, they were transferred into FACS tubes and fluorescence was measured at FACS.

3.3.3.2. Fluorescent microscopy

The cell samples already prepared for flow cytometry were also used for immunofluorescence. The stained cells were simply transferred and pelleted onto coverslips using spin down tubes after centrifugation in a swinging bucket rotor at 10000 rpm for 10 min and then examined in a Leica fluorescent microscope. At least three measurements were performed.

3.3.3.3. DNA content analysis/cell cycle analysis

Cell cycle analysis was done as previously described.[89] After harvesting using cold PBS, 1x 10^6 cells per sample were incubated for 10 min on ice to obtain a single cell suspension and then centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the pellet was resuspended in 1 ml ice-cold PBS and again centrifuged. The pellet was resuspended in 1 ml ice cold 70 % ethanol in PBS (kept at - 20°C). Cells were incubated for at least 2 hours at – 20°C (cells in ethanol could be stored at - 20°C for

several months) and centrifuged and then resuspended in 1 ml PBS (room temperature). After a final washing the pellet was resuspended in 1 ml PI/ Triton – X 100 staining solution with RNase and incubated for 30 min at room temperature in a dark place before being measured at FACS.

3.3.4. Western Blotting

Western Blotting is a technique which identifies proteins that have been separated from one another according to their size by gel electrophoresis, using specific antibodies. The blot is a membrane, almost always of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins in the gel to move to the membrane where they adhere. The membrane is then a replica of the gel's protein pattern, and is subsequently stained with an antibody. Western Blotting includes: sample preparation, gel electrophoresis, transfer from gel to membrane, and immunostain of the blot. Depending on the needs of the experiment two different basic techniques are used in the protein extraction – total and cytoplasmic/nuclear protein extraction.

3.3.4.1. Total protein extraction

The medium was first removed from the 10 cm^2 flask or Petri dish and 1000 µl of TEN buffer was added. The cells were harvested with cell scraper and transferred in 1.5 ml Eppendorf tubes. The cells were centrifuged for 1 min at 14000 rpm and 4°C. The supernatant was removed and the pellet was resuspended in 50-100 ml RIPA-buffer by pipetting many times up and down for homogenization. After incubation on ice for 15 min the cells were pelleted by centrifugation for 15 min at 15000 rpm and 4°C. The supernatant was transferred to new 1.5 ml Eppendorf tube, aliquoted and frozen at -80°C.

3.3.4.2. Cytoplasm and nuclear protein extraction

The volumes of solutions and buffers indicated in the protocol were optimized for a 10 cm^2 culture dish. The culture medium was first removed from confluent cultures. The cells were washed by pipetting sufficient PBS to cover them and then were gently swirled and the PBS was decanted. Pre-warmed trypsin/EDTA solution was added to the culture enough to cover adhering cell layer. Bottom of plate was tapped on the countertop to dislodge cells. Culture was checked with an inverted microscope to be sure that cells were rounded up and detached from the surface, then 10 ml PBS was added. Cell suspension was drawn and cell layer was rinsed two or three times to dissociate cells and to dislodge any remaining adherent cells.

All following steps were performed on ice. The cells were pelleted by centrifuging 5 min at 1200 rpm at 4°C. PBS was removed and the cells were washed again with 10 ml PBS. All supernatant was removed, 300 ml buffer A and 3 µl 12.5 % Nonidet P-40 were added. After 5 min incubation on ice the nuclei were collected by centrifuging 10 min at 4000 rpm at 4°C. The supernatant was collected (cytoplasmic and membrane proteins) in a separate tube and stored at - 80°C. Then the nuclei were resuspended in 80 µl buffer C by rapid mixing and nuclear proteins were collected by centrifugation for 10 min at 10000 rpm at 4°C. The supernatant was placed in a separate tube or aliquoted into tubes if desired and rapidly frozen by submerging in liquid nitrogen. The extracts were stored at -80°C. If all of the extract was not going to be used at one time, the extract was divided into aliquots before freezing to avoid unnecessary freezing and thawing. Thawing, freezing was avoided more than five cycles, because the stability of specific proteins may differ. The frozen extracts were always thawed on ice.

3.3.4.3. Determination of protein concentration

The experiment was performed in a 96-well plate. The Buffer A and Buffer B (commercially available from Pierce) were mixed in ratio 50:1. Protein standard or the evaluated probes (cell lysates), 20 µl of each, were added to each well in triplicate and 100 µl from the pre-diluted Buffer A: Buffer B 50:1 was added. After 30 min incubation

at 37°C the color reaction was measured by an ELISA Reader. The protein concentration was obtained by means of a standard regression curve fitting analysis using 2000, 1500, 1000, 750, 500, 250 and 125 µg/ml of BSA (bovine serum albumin) as standard concentration.

	Final concentration (µg/ml)	**BSA-Buffer (µl)**	**Water (µl)**
A	2000	500 (stock)	
B	1500	564 (stock)	188
C	1000	475 (stock)	475
D	750	250 (B)	250
E	500	450 (C)	450
F	250	400 (E)	400
G	125	300 (F)	300
H	0		500

3.3.4.4. Running, transfer, immunoblotting and detection of cytoplasm and nuclear protein

Whole cell extracts were prepared by harvesting and lysing the cells with lysis buffer (0.1 SDS, 0.5 % sodium deoxycholic acid, 1 % Nonidet P-40, 0.1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A). The protein content of the lysate was determined using the BCA protein assay kit (Pierce, Rockford, USA). The cell lysate was mixed with gel loading buffer (62.5 mM Tris-HCl, 10 % glycerol, 1 % SDS, 2.5 % beta-mercaptoethanol). After boiling for 10 minutes, the lysates were subjected to SDS/polyacrylamide gel electrophoresis (30 µg of protein per lane; gel: 12 % polyacrylamide, 0.1 % SDS, 25 mM Tris-HCl; running buffer: 25 mM Tris-base, 50 mM glycine, 0.1 % SDS). Proteins were transferred to PVDF membranes by electroblotting using transfer buffer (25 mM Tris-base, 50 mM glycine, 20% methanol). Blots were

blocked in 5 % BSA and then incubated at 4°C overnight with the following antibodies: polyclonal survivin antibody (1:500), polyclonal Survivin ΔEx3 antibody (1:250), polyclonal Survivin-2B antibody (1:500), AIM-1 (1:250), ß-actin (1:5000) and monoclonal anti-ß-tubulin antibody (1:5000). After washing with PBS containing 0.1 % Tween20 and incubating with horseradish peroxidase-coupled anti-IgG antibody (1:10000) at room temperature for 1 hour, the blot was washed extensively and developed using enhanced chemiluminescent detection (Amersham, Uppsala, Sweden). Blots were exposed to Hyperfilm ECL film (Amersham, Uppsala, Sweden) for 1-30 minutes and analyzed densitometrically using TINA software (Raytest Isotopenmessgeräte, Straubenhardt, Germany). As a loading control, ß-actin (mouse-anti-human ß-actin antibody from Sigma, Deisenhofen, Germany) was detected on each blot.

3.3.5. Drug combination studies

To test for possible additive or even super-additive antiproliferative effects, co-incubation of ZM447439 and conventional bio- and cytotoxic drugs (5-FU, cisplatin for MIP-101 cell line; streptozocin, doxorubicin for QGP-1 cell line and octreotide, SOM230 for BON cell line) was carried out. Increasing concentrations of the drugs were combined with sub-IC50 value of ZM447439. The cytotoxic activities of the combinations were compared to those of each drug alone. Concentration ranges and effectiveness of each agent was determined in prior experiments using a crystal violet assay (Chapter 3.3.6). In all experiments the cell growth was evaluated by crystal violet staining, as further described (Chapter 3.3.6).

3.3.6. Determination of cell growth

Cell growth was evaluated using the crystal violet staining procedure.[90] In brief, cells were sown in 96-well plates at the desired density and incubated for 24 hours in a cell incubator and incubated with the evaluated substance. The concentration used for 5-FU, doxorubicin, SN-38, docetaxel and cisplatin was similar to that mentioned before[89,91,92]

and modified in our protocol. At the desired time point the medium was removed from the 96-well plate and the cells were washed with 200 µl per well 1 x PBS and fixed for 15 min with 1 % glutaraldehyde in Hanks' Balanced Salt Solution (HBSS) and kept hydrated with 1 ml of HBSS until staining.

Samples were stained for 30 min with 1 ml of 0.1 % crystal violet solution in deionized water. After that the plates were submerged in deionized water and destained for 30 min with a continuous, slow (0.5 liter per minute) stream of deionized water. The plates were then air-dried and the cells were incubated with 0.2 % Triton X-100. The absorbance of the solution was measured at 590 nm using a Bausch and Lomb Spectronic spectrophotometer. Absorbance readings were analyzed by using an in-house Pascal program. This program averages data and normalizes data to the control, and provides statistical evaluation. In our experience, the standard deviation within a column (four identically treated samples) had never exceeded 10 % of the median value.

3.3.7. Caspase -3/7 activity assay

The Apo-ONE Homogeneous Caspase -3/7 Assay was done according to the manufacturer's instructions in 96-well plates. First, 100 µl of Apo-ONE Caspase 3/7 Reagent was added to each well of a 96-well plate containing 100 µl of blank, control or cells in culture in triplicates. The content was gently mixed using a plate shaker at 300 - 500 rpm for 30 sec. Incubation at room temperature followed - from 30 min to 18 hours depending upon expected level of apoptosis in the cell type analyzed. The optimal incubation period was determined empirically. Then the fluorescence of each well was measured using a fluorescence reader with optimal excitation wavelength detection of 499 nm.

3.3.8. Measurement of serum survivin levels

Blood samples were obtained from patients with well-differentiated GEP-NET disease (n = 20), hematological malignancies (n = 20) and healthy volunteers (n = 22) by venipuncture, and were clotted at room temperature. The sera were collected following

centrifugation, and frozen immediately at – 20°C until analysis. The human total survivin enzyme immunometric assay (EIA) kit (TiterZyme EIA, Assay Design, Inc., Ann Arbor, MI, USA) was used for the quantitative determination of survivin in serum. Measurements were carried out following the manufacturer's instructions. In brief, a monoclonal antibody to survivin immobilized on a microtiter plate was used in the kit to bind survivin in the standards or samples. After a short incubation, the excess sample or standard was washed out and a rabbit polyclonal antibody to survivin was added. This antibody binds to the survivin captured on the plate. After a short incubation, the excess antibody was washed out and goat anti-rabbit immunoglobulin G (IgG) conjugated to HRP was added, which binds to the polyclonal surviving antibody. Excess conjugate was washed out and substrate was added. Following a short incubation, the enzyme reaction was stopped and the color generated was read at 450 nm. The measured optical density was directly proportional to the number of cells surviving in either standards or samples.

3.3.9. Gene silencing

RNA interference is the biological mechanism by which a double-stranded RNA molecule induces gene silencing by targeting for degradation complementary mRNA. In many non-mammalian systems, introducing or expressing long double-stranded RNA (dsRNA) triggers the RNAi pathway (Figure 5, pathway 1). In mammalian cultured cells, a long DNA or RNA molecule would trigger an interferon-based defense mechanism, that is why RNA inhibition is typically induced by either transfecting cells with short siRNAs (typically 21 bp RNA molecules with 3' dinucleotide overhangs) or by using DNA-based vectors to express short hairpin RNAs, (shRNAs), that are processed by Dicer into siRNA molecules (Fig. 5, pathways 2 and 3, respectively).

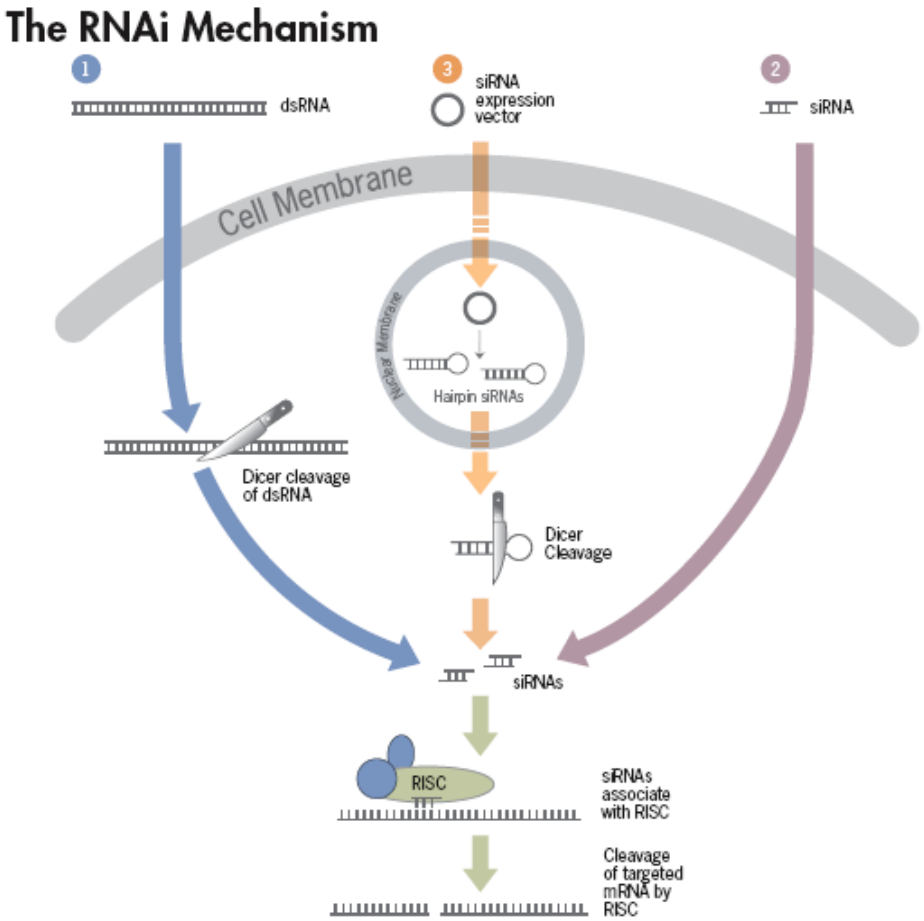


Fig. 5. Three ways to trigger the RNAi pathway. (1) dsRNA in non-mammalian systems. In mammalian systems - siRNA (2) or by DNA based expression vectors designed to express short hairpin RNA (shRNA) molecules (3) (modified after Ambion Inc.).

3.3.9.1. Protocol for survivin siRNA transfection assays

All oligonucleotides were used as a stock in a 100 x higher concentration than the final concentration in the wells. The oligonucleotides were diluted in OptiMEM and the siRNA surviving stock was usually 10 mM.

The lipid stock (Lipofectamine2000; Invitrogen) used was 1 mg/ml and the final concentration in this protocol was 5 µg/ml.

First, transfection using fluorescine-labeled siRNA was carried out to establish to most appropriate cell density, siRNA concentration and siRNA/Lipofectamine ratio for each

cell line. For the purpose cell samples from DU-145, QGP-1, MIP-101 and BON cell lines were incubated in 12-well plates in a different cell density – ranging from 5 x 10^5 to 4 x 10^6. Then they were transfected with 50, 100 and 200 nM siRNA, each with three different volumes of Lipofectamine2000 – 2.5, 4 and 7 µl. The transfection efficiency was measured at FACS, using Channel 1. The most appropriate siRNA and Lipofectamine2000 quantities for all cell lines were 100 pmol siRNA and 4 µL Lipofectamine2000.

One day before transfection, 5 x 10^5 cells (DU-145 and MIP-101 cell lines) or 1.5 x10^6 (QGP-1 and BON cell lines) were seeded in 12-well plates in complete growth medium (1 ml medium/ well). The confluence of the cell monolayer at the time of transfection was 60 - 70 % (DU-145 and MIP-101 cell lines) or 80 - 95 % (QGP-1 and BON cell lines). Cells were transfected either with siRNA oligos against survivin, All-Star negative control siRNA or fluorescine-labeled All-Star negative control siRNA (100 nM final concentrations) using Lipofectamine2000 reagent. Cells were also treated with Lipofectamine2000 alone as a mock control. The cell samples were harvested 24, 48 and 72 hours post-transfection and proteins were then eluted.

In brief:

1. Cells were first washed with OptiMEM (0.5 ml).
2. 0.4 ml of lipid mix (A) was added onto the cells in 12 well-plates.
3. Incubation for 10- 15 min at room temperature.
4. 0.1 ml of oligonucleotides solution (B) or mock solution (C) was added to the wells with cells containing media and lipid.
5. Incubation for 4 hours.
6. Cells were washed twice with OptiMEM (0.5 ml)
7. 0.5 ml culture medium was added and the cells were incubated at 37°C and 5 % CO2 for 24 - 72 hours.

A. Lipid mix (5 µg/ml) 1x: 2.5 µl Lipofectamine2000
397.5 µl OptiMEM

B. Oligos solution 1x: 5 µl Oligonucleotides
5 µl OptiMEM

C. Mock solution 1x: 5 µl H_2O
95 µl OptiMem

3.4. Statistical analysis

The statistical analysis was performed using SPSS Version 14.0 software. Descriptive statistics were obtained and a value for every studied parameter was achieved. The normality of distribution was evaluated using the Kolmogorov-Smirnov test with the Lilliefors significance correction. The nonparametric Wilcoxon's matched pairs signed test was used to calculate the significance if not mentioned otherwise.

4. Results

4.1. Expression of neuroendocrine markers in colorectal, pancreatic and prostate cancer cell lines

As a first step in this study we searched for cancer cell lines possessing neuroendocrine characteristics using flow cytometry. In order to prove neuroendocrine differentiation of the cell lines we used chromogranin A (marker of „large dense-core vesicles“) and synaptophysin (marker of „small dense core vesicles“) as well as SNAP25 and syntaxin (as components of the SNARE-Complex). The hormone-resistant prostate cancer cell line DU-145 was previously reported as a typical neuroendocrine cell line and was used as a positive control in this study. BON and QGP-1 cell lines were reported to express chromogranin A, synaptophysin and syntaxin[92], which was not only confirmed, but expanded with reproducible expression of SNAP25 and synaptobrevin in the course of this study (Table 2). The BON cell line represented the slow-growing well-differentiated GEP-NET of the foregut[93], as the QGP-1 cell line the well- and moderate-differentiated GEP-NET carcinomas of the foregut.[92,94] In a number of experiments we tried to establish a cell line as a model for poorly differentiated tumors (PDEC) of the hindgut. The LCC-18 cell line showed some neuroendocrine characteristics, although it was repetitively negative for syntaxin, SNAP25 and synaptobrevin. Caco2 and NCI-H716 cell lines also demonstrated some neuroendocrine characteristics, but did not cause a high proliferation rate as typical for a PDEC tumor, so those cell lines were not used in this study. Instead, we report here for the first time that the poorly differentiated colorectal cell line MIP-101[95] also expresses neuroendocrine markers (Fig. 6 and Table 2) and was used as a model of the poorly differentiated colorectal (hindgut) NEC in our study. The cell lines already mentioned represent the different growth pattern of neuroendocrine tumors.

Cell lines	Origin	Reference	Synaptophysin	Cg A	SNAP25	Synaptobrevin	Syntaxin
HT29	Colon carcinoma	Fogh et al., 1977	Negative	negative	negative	negative	negative
DU-145	Prostate carcinoma	Abdul et al., 1994	Positive	negative	negative	low positive	positive
BON	Pancreas NET	Evers et al., 1991	Positive	positive	low positive	low positive	positive
Caco2	Colon carcinoma	Jumarie et al., 1991	Negative	negative	negative	low positive	negative
Colo205	Colon carcinoma	Bjork et al., 1993	Negative	negative	negative	negative	negative
Colo320	Colon carcinoma	Quiunn et al., 1979	Negative	positive	negative	negative	negative
LCC-18	Colon carcinoma	Lundquist et al., 1991	Positive	positive	negative	negative	negative
LS-174-T	Colon carcinoma	Tom et al., 1976	Negative	negative	negative	negative	negative
MIP-101	Colon carcinoma	Wagner et al., 1990	Positive	negative	negative	low positive	positive
NCI-H716	Colon carcinoma	Park et al., 1987	Positive	low positive	negative	low positive	positive
NCI-H747	Colon carcinoma	Park et al., 1987	Negative	negative	negative	negative	negative
QGP-1	Pancreas NEC	Iguchi et al., 1990	Positive	positive	low positive	negative	positive

Table 2. Cells from 11 GEP-NET and one prostate carcinoma cell lines (1 x 10^5) were washed, fixed, incubated with 1st and 2nd antibody and mean fluorescence was calculated at FACS against the isotype control. The BON and DU-145 cell lines were used as a positive control for neuroendocrine differentiation, the HT29 colon cancer cell line, as a negative control. Here we show representative results of at least 3 independent experiments, made in duplicates. CgA – chromogranin A; NET – neuroendocrine tumor; NEC – neuroendocrine carcinoma.

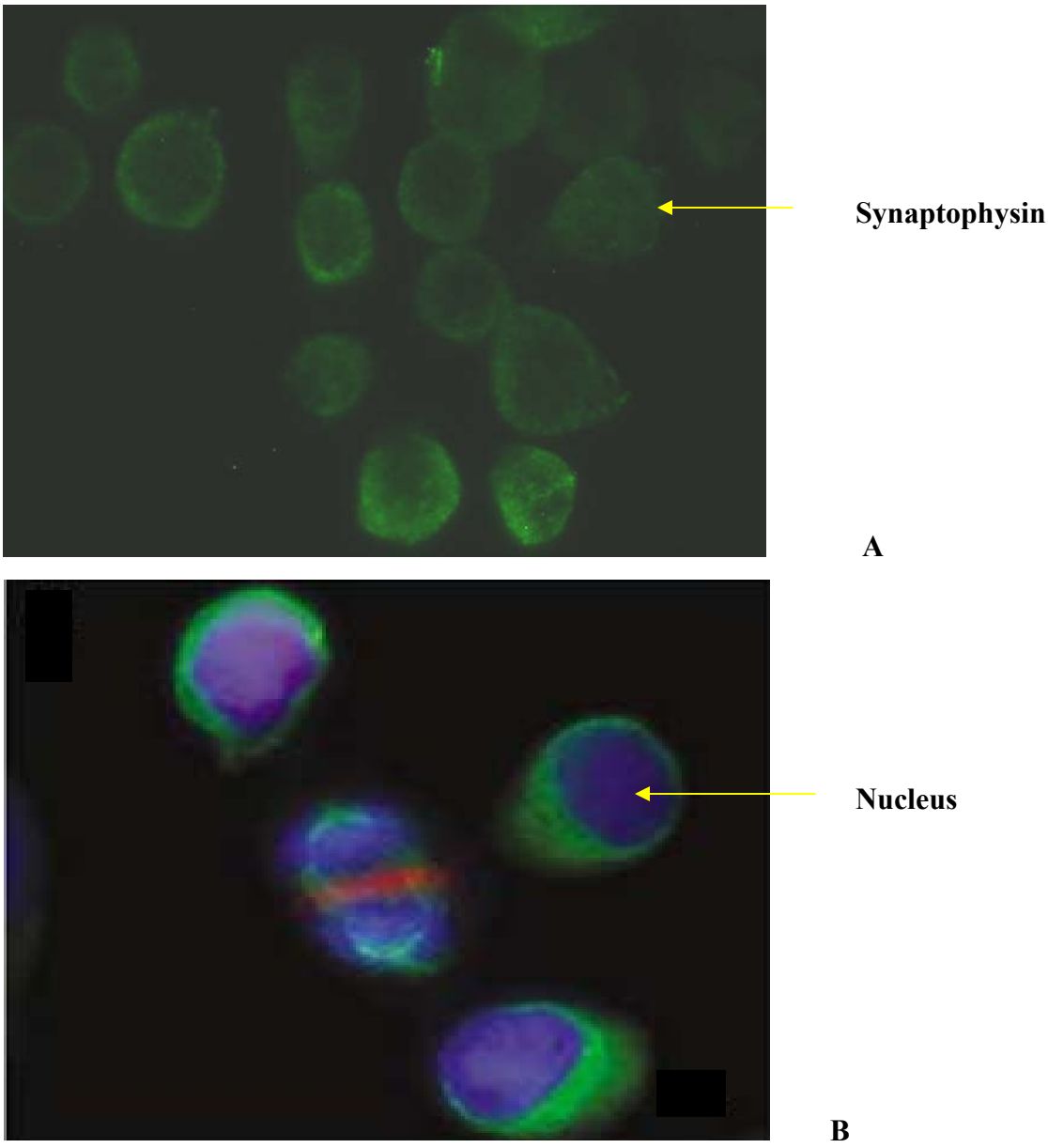


Fig. 6. Expression of synaptophysin in the cytoplasm of MIP-101 cells, marked either with only FITC-labeled antibody (A) or with consecutive staining of the nucleus (B), using propidium iodide. The fluorescent antibody-labeled cells were spun down onto coverslips using spin down tubes and examined with a Leica fluorescent microscope. Here we show a representative photo of at least 3 independent staining procedures.

4.2. Effects of bio- /chemotherapeutic drugs on the cell growth, viability and cell cycle transition in GEP-NET cells

Since the sensitivity of a therapeutic agent could depend on the growth phase of the tumor cells and NETs exhibit a wide spectrum of growth patterns ranging from very slow to very fast growing aggressive types, we performed our studies on cell lines which represent the different growth patterns. As a model of moderate to fast-growing GEP-NET cells, we chose QGP-1 cell line, which although a well-differentiated line has a doubling time about 20 - 24 hours while slower growing cells were represented by the pancreatic neuroendocrine BON cell line with a doubling time of 36 – 40 hours. As a positive control we chose the DU-145 neuroendocrine prostate cancer cell line. Additionally, we proved for the first time that the fast-growing MIP-101 cell line has neuroendocrine characteristics and we propose that this line might be used as a model for poorly differentiated NECs.

4.2.1. Effects of bio- /chemotherapeutic drugs on the cell growth of GEP-NET cells

Choosing chemotherapeutic agents for the treatment of the different cancer cell lines, we used clinical experience gathered over 20 years with various cytotoxic drugs in order to provide a situation which allows transferring our results to the clinical setting later on. For example biotherapeutic agents like octreotide were only used in the WDET-representing BON cell line, whereas cisplatin was only applied for the PDEC-representing cell line MIP-101.

4.2.1.1. Growth inhibitory effects of 5-FU on MIP-101, QGP-1 and BON cell lines.

Growth inhibition by 5-FU and all other cytotoxic drugs was studied by measuring cell proliferation using crystal violet assay. 5-FU time- and dose-dependently inhibited proliferation of MIP-101, QGP-1 and BON cell lines. In the same cell lines time- and

dose-dependent growth inhibition appeared following incubation with 5-FU above 5 – 10 µM (Fig. 7A and B). The IC50 value of 5-FU, determined after 96 hours of incubation, was 7.5 µM for QGP-1 cell line, 5.5 µM for MIP-101 cell line and 7 µM for BON cell line. After 96 hours of 5-FU treatment, the QGP-1 cell line displayed a remarkable growth inhibition of up to 100 %, interestingly after the same period of incubation with 5-FU at the same concentration, the BON cell line also demonstrated a growth inhibition rate of up to 100 %. In MIP-101 cell line, after 96 hours incubation with 5-FU, the observed growth inhibition was up to 80 %.

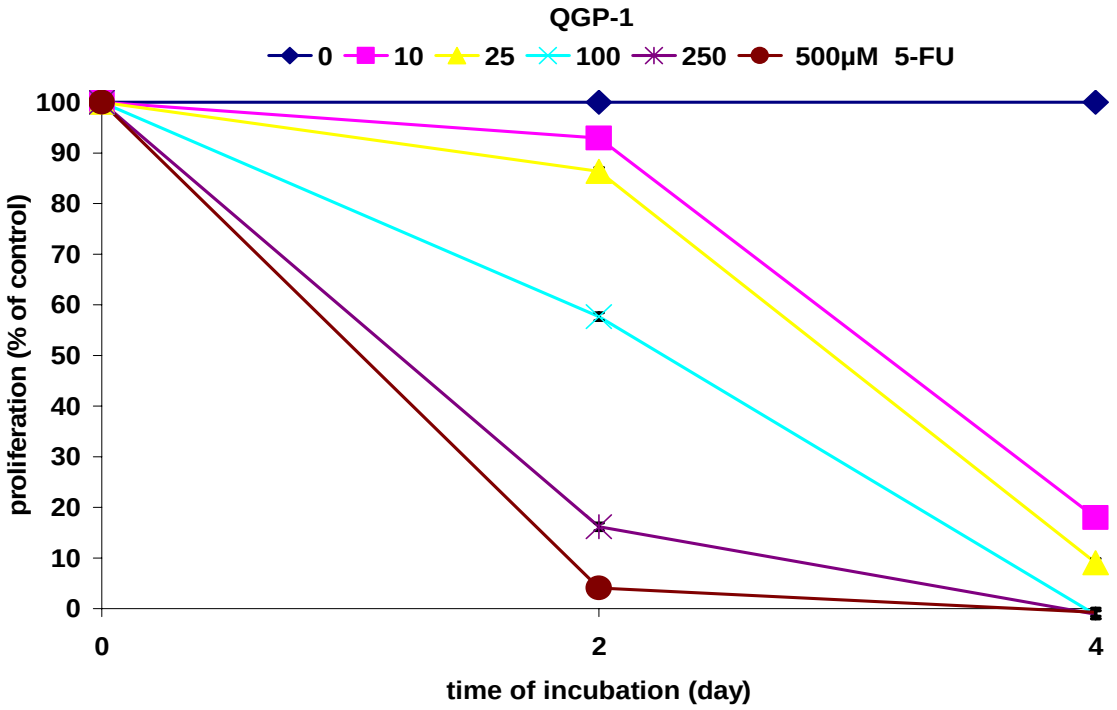


Fig. 7A. 5-FU-induced growth inhibition in QGP-1 neuroendocrine cells. Representative results of at least three independent experiments as measured by crystal violet assay (error bars - S.E.M.). Data are given as percentage of untreated controls.

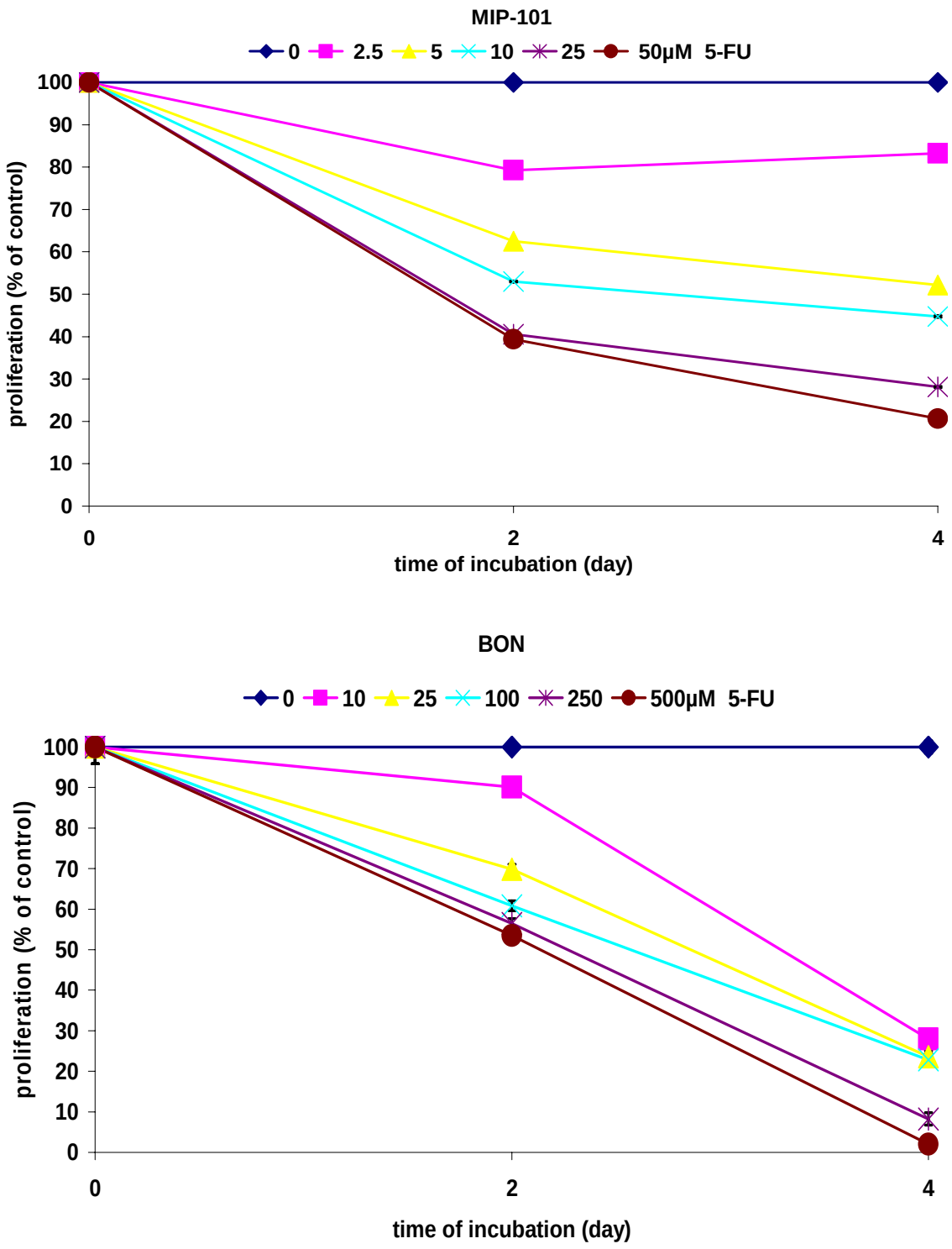


Fig. 7B. 5-FU-induced growth inhibition in MIP-101 and BON neuroendocrine cells. Representative results of at least three independent experiments as measured by crystal violet assay (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.2. Growth inhibitory effects of doxorubicin on MIP-101 and QGP-1 cell lines

Doxorubicin time- and dose- dependently inhibited the growth of the MIP-101 and QGP-1 cell lines studied. The IC50 values for doxorubicin determined after 96 hours were 12 nM and 4 µM for QGP-1 and MIP-101 cell lines, respectively (Fig. 8A and B). After 96 hours treatment with doxorubicin, QGP-1 cell line displayed an remarkable growth inhibition in the nM range, up to 100 %, meanwhile MIP-101 cell line demonstrated almost the same growth inhibition but in the µM range.

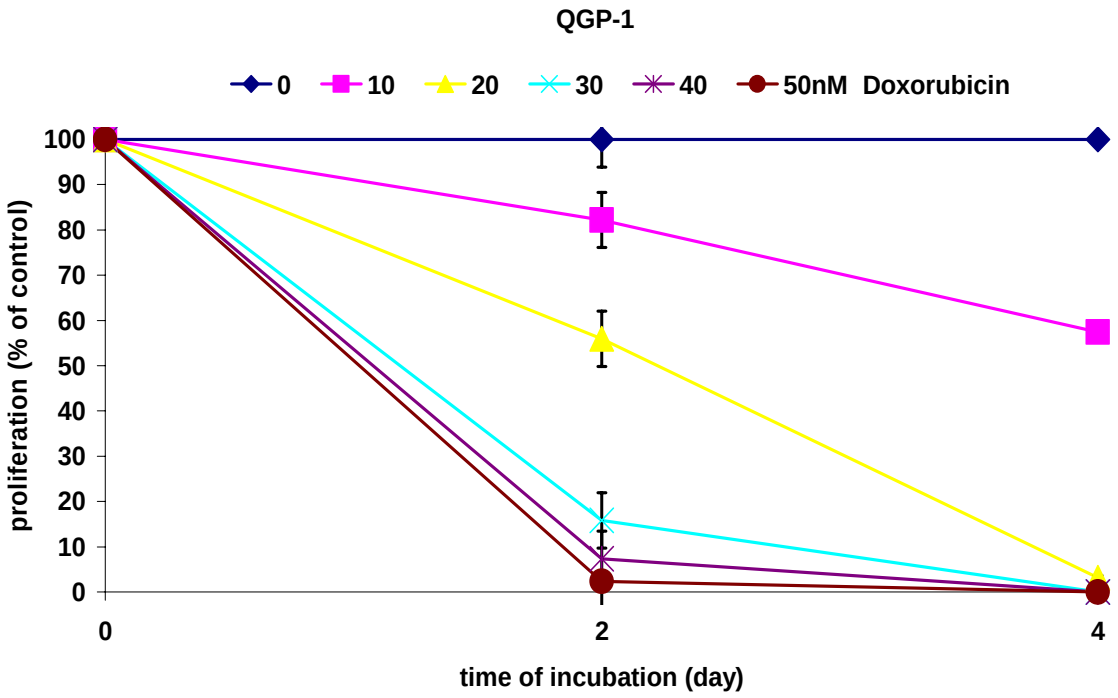


Fig. 8A. Doxorubicin-induced growth inhibition in QGP-1 pancreatic neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

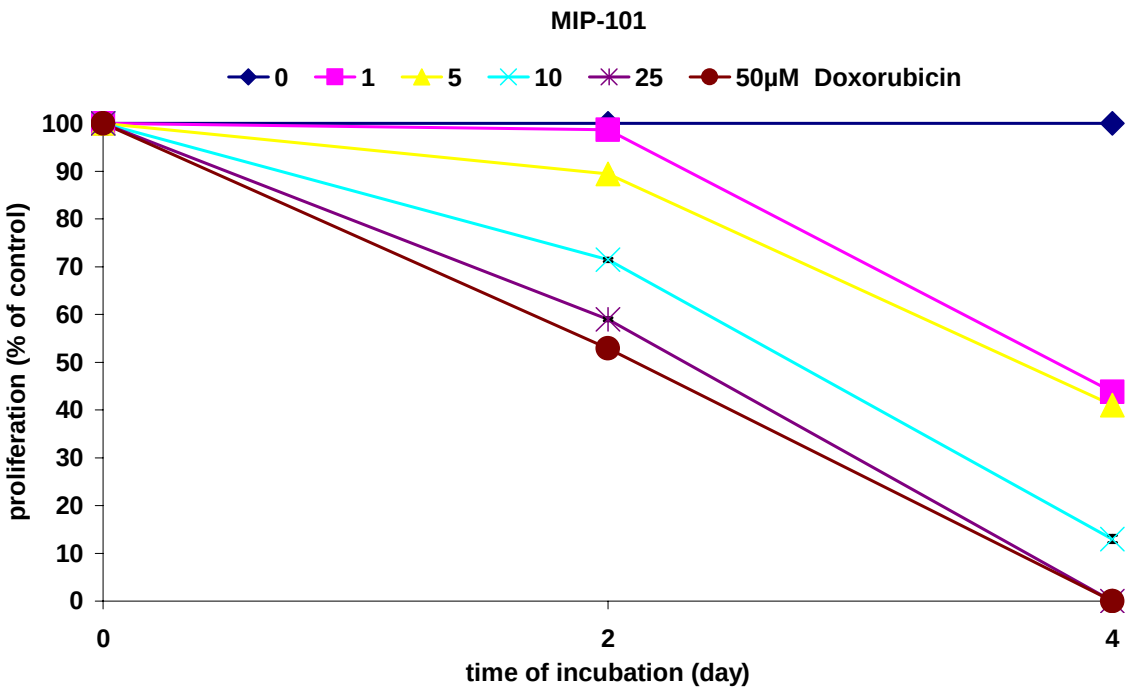


Fig. 8B. Doxorubicin-induced growth inhibition in MIP-101 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.3. Growth inhibitory effects of oxaliplatin on MIP-101 and QGP-1 cell lines

In rising concentrations oxaliplatin time- and dose-dependently inhibited the growth of QGP-1 and MIP-101 neuroendocrine cell lines. The IC50 values for oxaliplatin determined after 96 hours amounted to 4.4 µM and 4.7 µM in QGP-1 and MIP-101 cell lines, respectively (Fig. 9). The observed growth inhibition by both cell lines was evaluated in µM range and was up to 95 %.

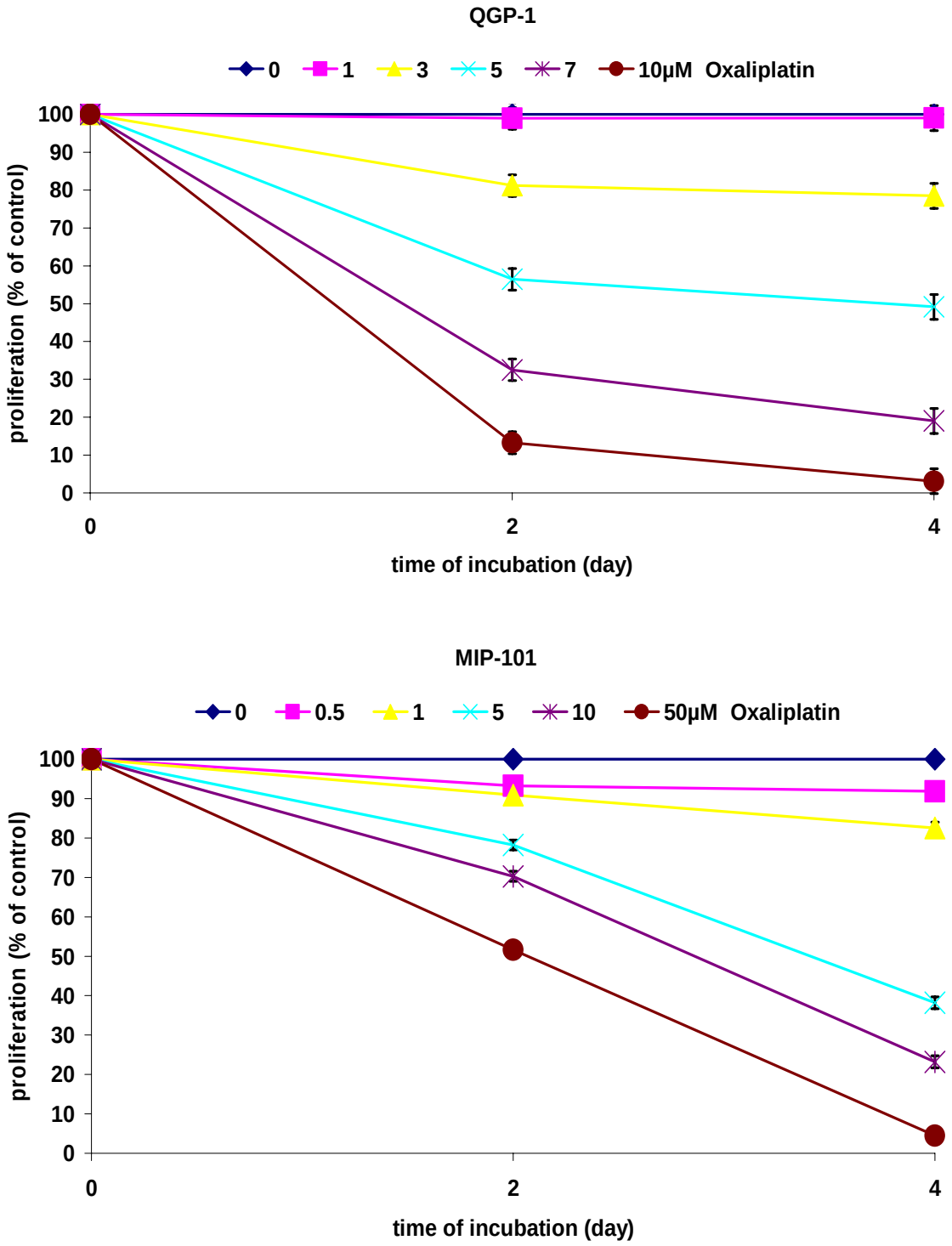


Fig. 9. Oxaliplatin-induced growth inhibition in QGP-1 and MIP-101 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.4. Growth inhibitory effects of SN-38 on MIP-101, QGP-1 and BON cell lines

Very low concentration of SN-38 1-10 nM led to fast time- and dose-dependent growth inhibition in QGP-1 cell line with IC50 1.3 nM after 96 hours. The growth of MIP-101 and BON cells was inhibited at higher concentrations, but also in nM range, with IC50 42.6 nM and 37.2 nM, respectively (Fig. 10A and B). The growth inhibition in QGP-1, MIP-101 and BON cells after 96 hours of treatment with SN-38 was up to 100 % and 70 %, 99 %, respectively.

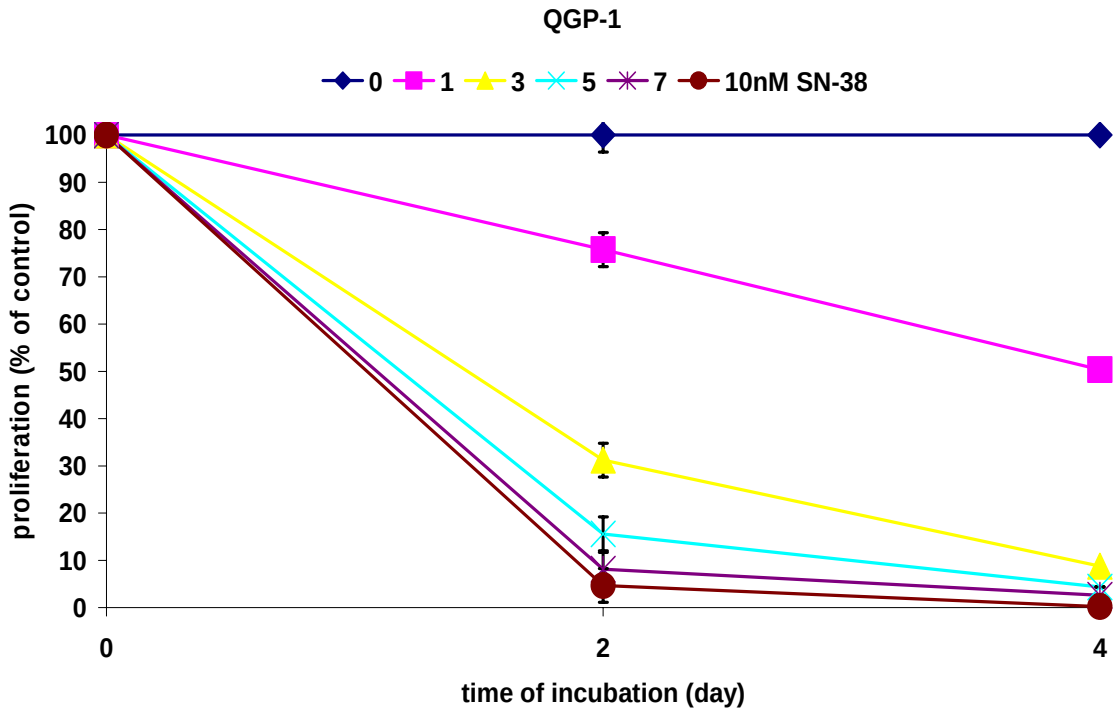


Fig. 10A. SN-38-induced growth inhibition in QGP-1 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

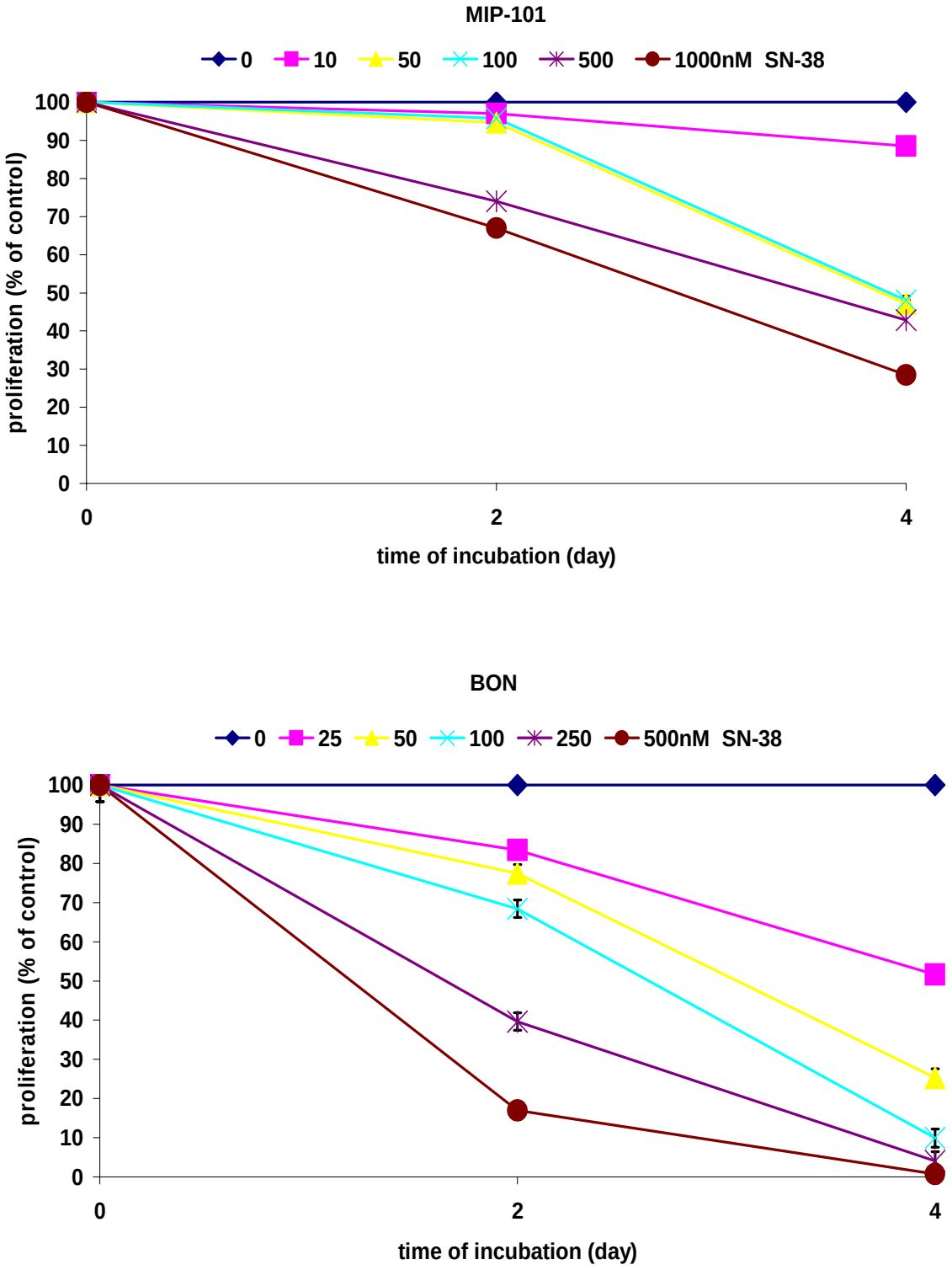


Fig. 10B. SN-38-induced growth inhibition in MIP-101 and BON neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.5. Growth inhibitory effects of etoposide on MIP-101 and QGP-1 cell lines

Changes in cell number caused by etoposide showed a time- and dose-dependent growth inhibition up to 70 - 100 % in QGP-1 and MIP-101 cell lines (Fig. 11A and B). IC50 values after 96 hours of incubation were 376 nM and 163.8 nM for QGP-1 and MIP-101 cells, respectively.

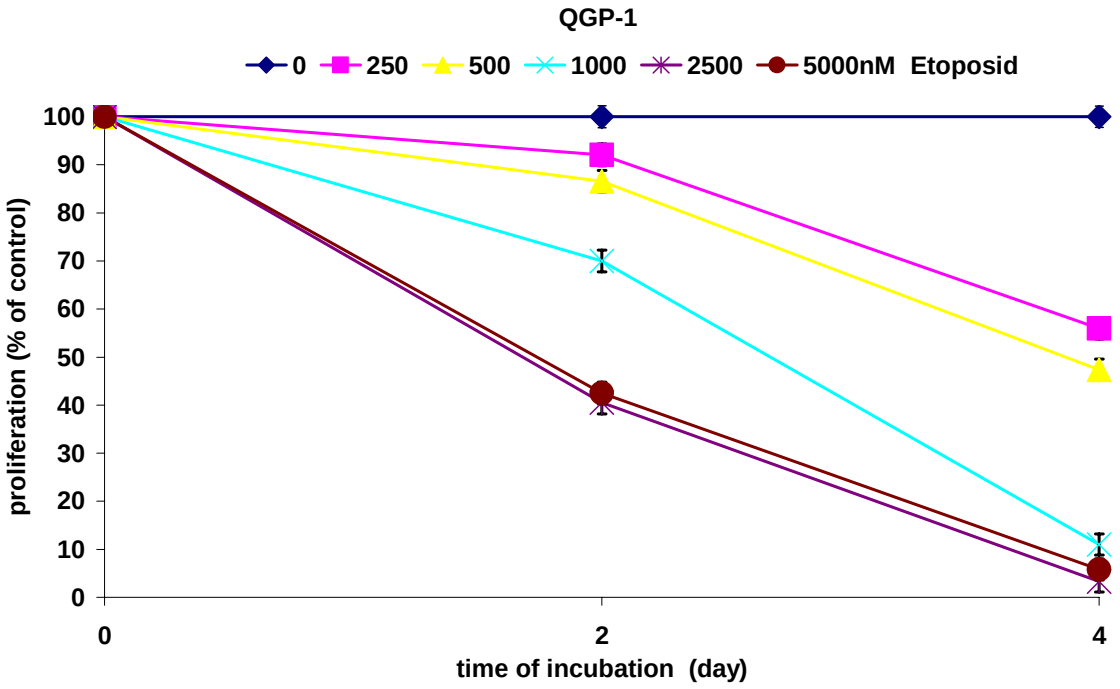


Fig. 11A. Etoposid-induced growth inhibition in QGP-1 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

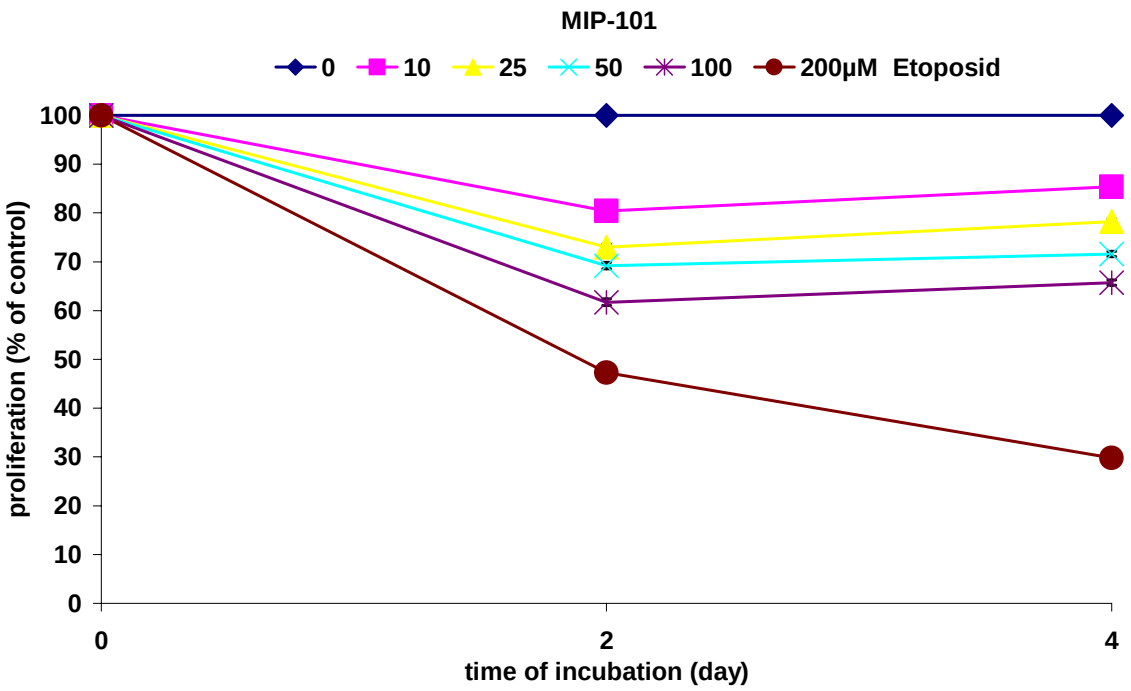


Fig. 11B. Etoposid-induced growth inhibition in MIP-101 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.6. Growth inhibitory effects of cisplatin on MIP-101 cell line

In standard rising concentration (1 - 50 µM) we observed time- and dose-dependent growth inhibition up to 95 %. IC50 values for MIP-101 cell line after 96 hours of incubation with cisplatin were 21.1 µM (Fig. 12)

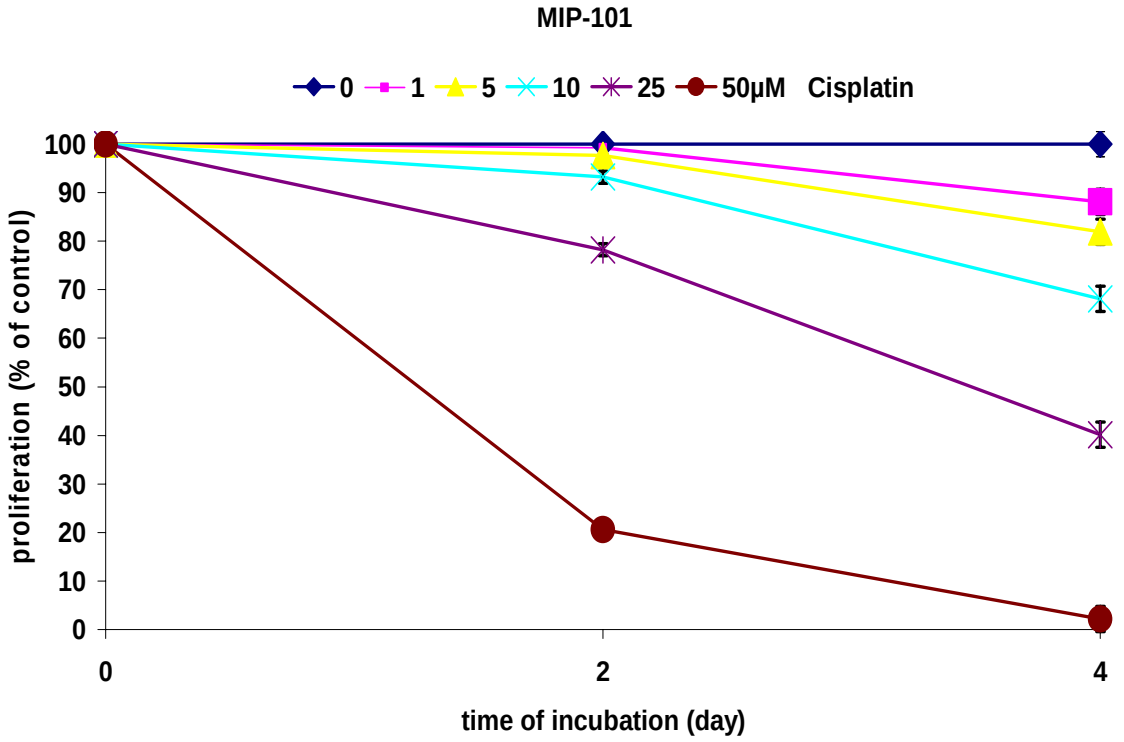


Fig. 12. Cisplatin-induced growth inhibition in MIP-101 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.7. Growth inhibitory effects of docetaxel on DU-145 cell line

The prostate neuroendocrine cancer cell line DU-145, was used in this study as a positive control for neuroendocrine differentiation. However, as we know that the prostate extensive disease is often treated with docetaxel, we tried to establish if DU-145 could be sensitive to docetaxel treatment. We proved the growth inhibitory effect of docetaxel on DU-145 prostate carcinoma cell line in extremely low concentrations (1 - 50 nM). The IC50 value for DU-145 after 96 hours of incubation with docetaxel was 3.4 nM and the observed growth inhibition was up to 90 % (Fig. 13).

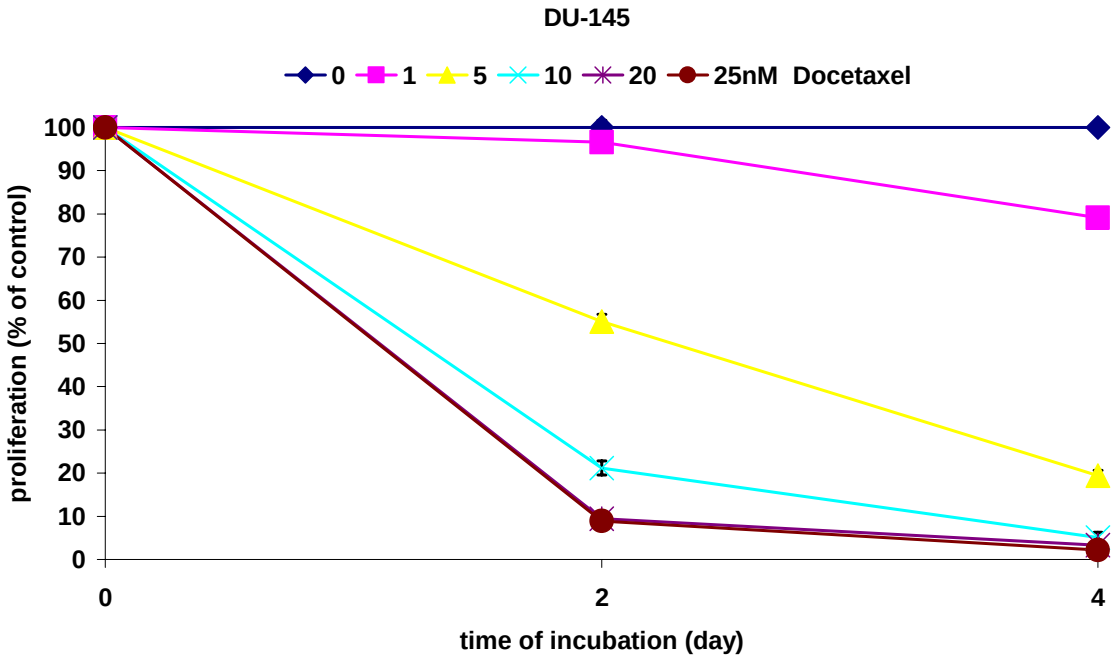


Fig. 13. Docetaxel-induced growth inhibition in DU-145 neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.8. Growth inhibitory effects of streptozocin

In QGP-1 cells, streptozocin time- and dose-dependently inhibited proliferation up to 90 % when used in concentrations 1 – 100 µM. However, DU-145, MIP-101 and BON cell lines were not responsive to streptozocin even in the mM range. The IC50 value for QGP-1 cells after 96 hours of incubation with streptozocin was 19.3 µM (Fig. 14).

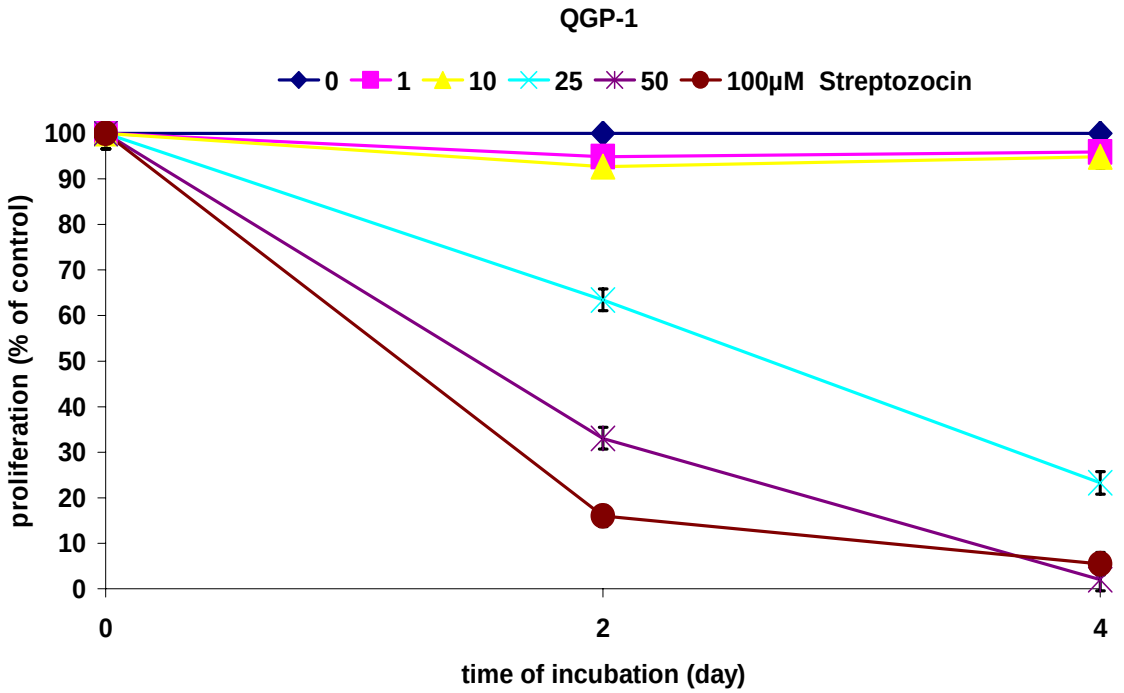


Fig. 14. Streptozocin-induced growth inhibition in QGP-1 neuroendocrine cell lines. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.2.1.9. Growth inhibitory effects of octreotide and SOM230 in BON cell line.

In the BON cell line octreotide time- and dose-dependently showed an albeit small, but significant and reproducible inhibition of proliferation though at very high nM concentrations (Fig. 15). The IC50 values, however, have not been reached. A similar, but quite less significant effect was observed 24 hours after incubation with SOM230. However, after 96 hours no significant difference was observed in any of the 3 independent experiments (Fig. 16).

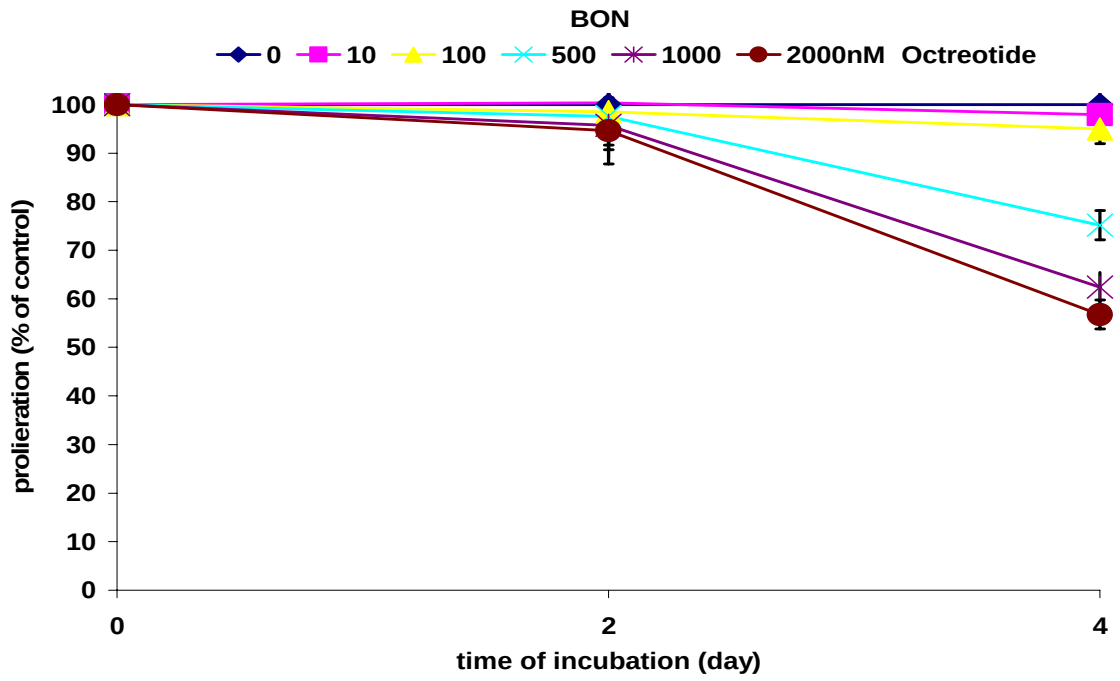


Fig. 15. Octreotide-induced growth inhibition in BON neuroendocrine cells. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

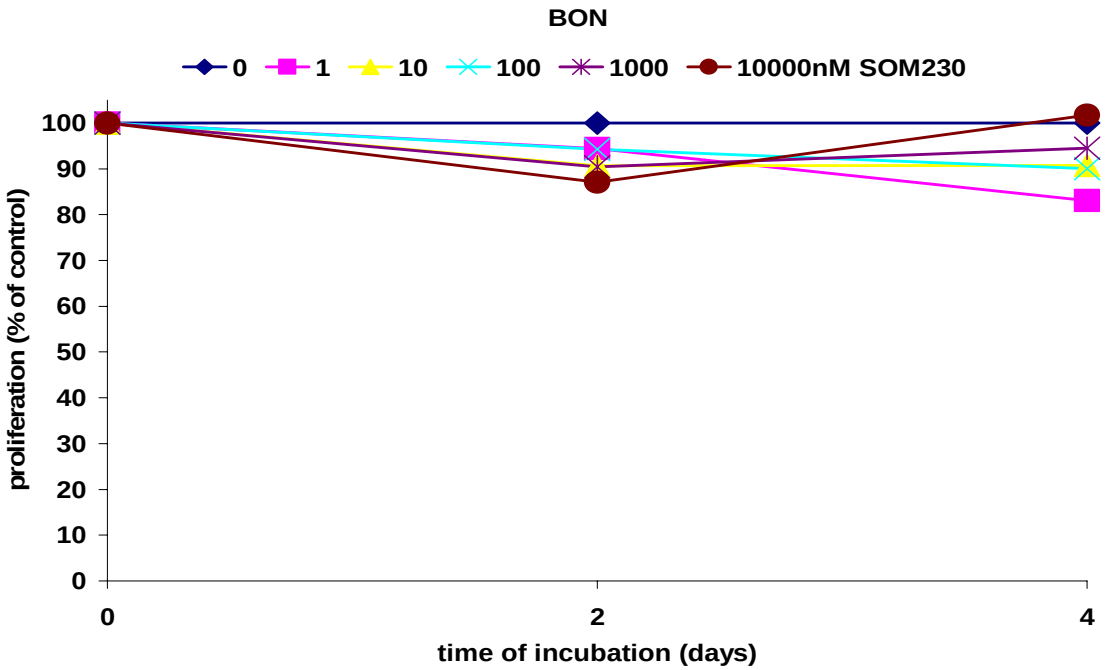


Fig. 16. SOM230 did not induce growth inhibition in BON neuroendocrine cells after 96 hours of incubation. Representative results of three independent experiments as measured by crystal violet staining. Data are given as percentage of untreated controls.

4.2.2. Induction of apoptosis by bio- and chemotherapeutic drugs

To study the potency of bio- and chemotherapeutic drugs to induce apoptosis in GEP-NET cells, we investigated activation of caspases-3 and -7, key enzymes in the apoptotic pathway. Activation of caspases-3 and -7 was evaluated in QGP-1, MIP-101 and BON cell lines 24 and 48 hours following incubation with cytotoxic drugs. The concentrations used corresponded to the respective IC50 value previously established by the crystal violet assays. Additionally, we carried out experiments with concentrations 100 - 1000 times exceeding the IC50 values. In QGP-1 cells, the treatment with 1 µM doxorubicin and SN-38 resulted in a significant increase of caspases-3 and -7 enzyme activities. Similar effects using streptozocin and 5-FU were only obtained when they were used in concentrations 1000 times higher than the respective IC50 value established by crystal violet (Fig. 17).

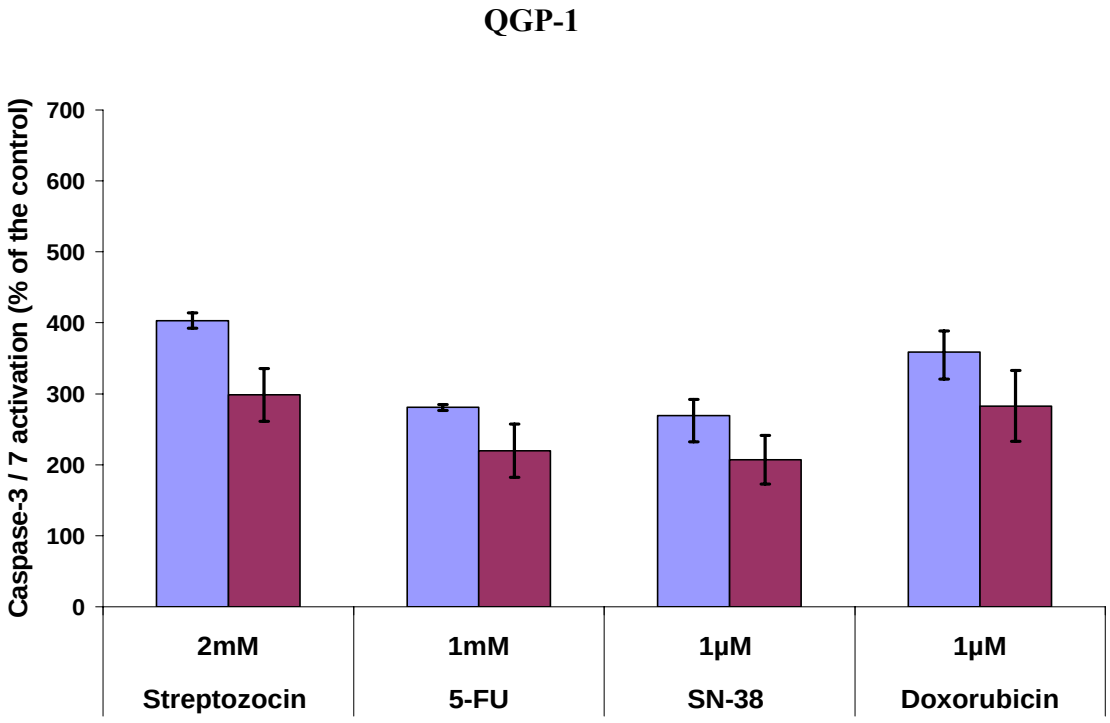


Fig. 17. Apoptosis-specific caspases-3 and -7 activity increase in QGP-1 neuroendocrine cells following 24 hours (blue columns) or 48 hours (red columns) of incubation with cytotoxic drugs (mean ± SEM, $n > 3$ independent experiments, $P < 0.05$ vs. controls).

In MIP-101 cell line the incubation with relatively low concentrations of cisplatin and SN-38 resulted in a significant increase of caspases-3 and -7 enzyme activities (Fig. 18). The incubation with 5-FU, oxaliplatin and etoposide led to caspases-3 and -7 activation, though in higher concentrations.

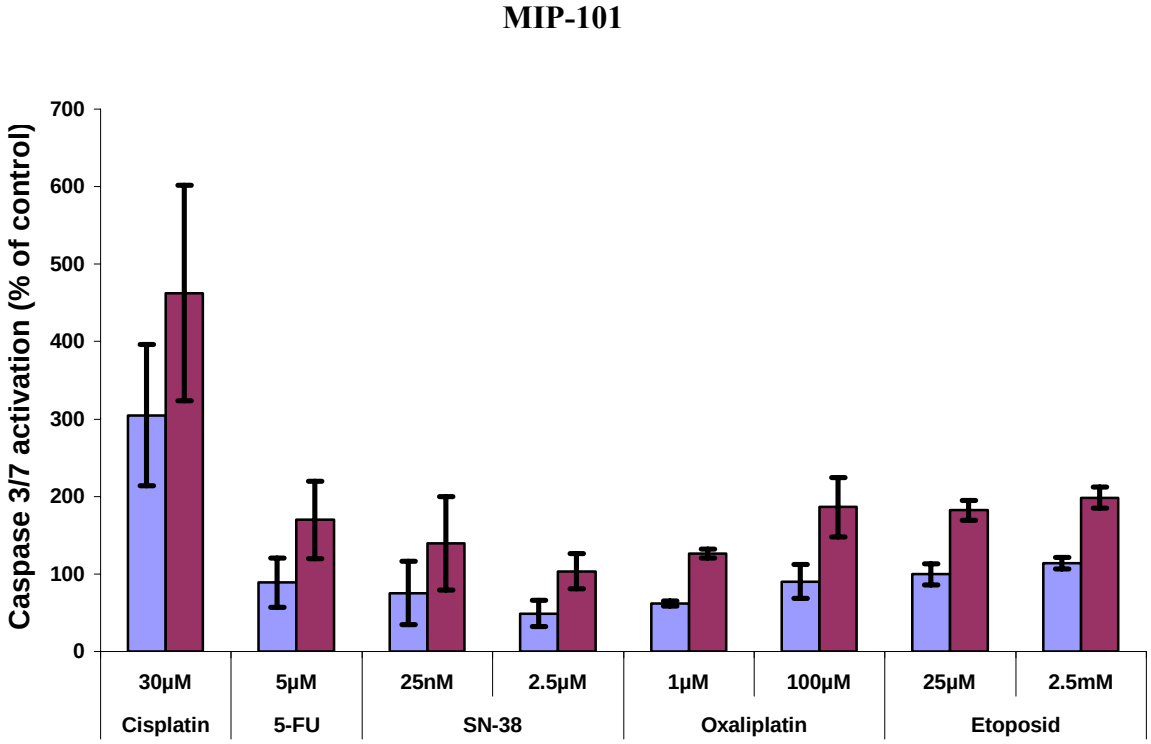


Fig. 18. Apoptosis-specific caspases-3 and -7 activity increase in MIP-101 neuroendocrine cells following 24 hours (blue columns) or 48 hours (red columns) of incubation with cytotoxic drugs (mean ± SEM, $n > 3$ independent experiments, $P < 0.05$ vs. controls).

In BON cells, no significant activation of caspases-3 and -7 was observed when SN-38, 5-FU and octreotide were applied in concentrations up to 100 times higher than the respective IC50 established by the crystal violet assay. Incubation with SOM230 did not induce caspases-3 and -7 activation even in the mM range.

4.2.3. DNA fragmentation analysis

To further substantiate induction of apoptosis from cytotoxic drugs, we determined DNA fragmentation as a biochemical hallmark of apoptosis. DNA fragmentation was evaluated in QGP-1, MIP-101 and BON cell lines 24 and 48 hours following incubation with cytotoxic drugs. The concentrations used corresponded to the respective IC50 value previously established by the crystal violet assays. Additionally, experiments with concentrations exceeding 100 times the IC50 values were carried out.

In MIP-101 cell line DNA fragmentation was observed following incubation with SN-38, 5-FU, oxaliplatin and etoposide, but not cisplatin (Fig. 19), which did not fully corresponded with the results obtained by the caspase-3 and 7 activation assay.

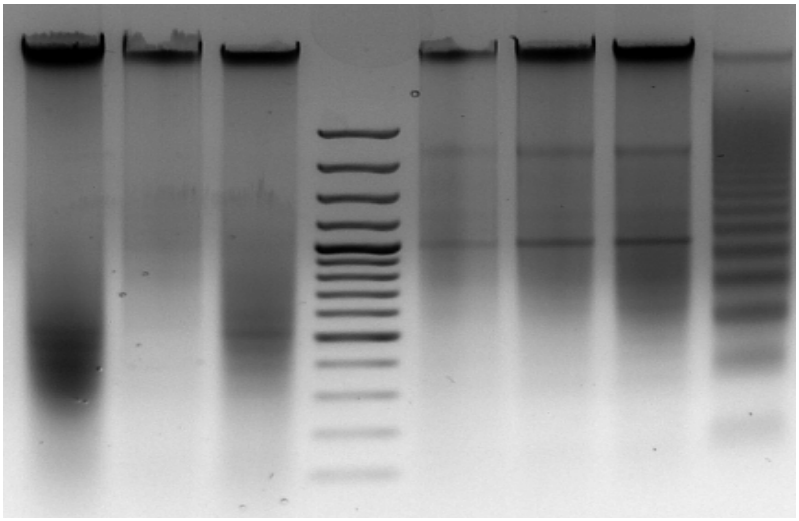


Fig. 19. Cytotoxic drugs-induced DNA fragmentation in MIP-101 neuroendocrine tumor cells. DNA isolated from 2 x 10^5 cells, treated for 48 hours with increasing concentrations of cytotoxic drugs. After elution DNA was subjected to agarose gel electrophoresis. Representative tracings of three independent experiments are shown. C - untreated control, M - molecular weight marker (100 bp), P - positive control.

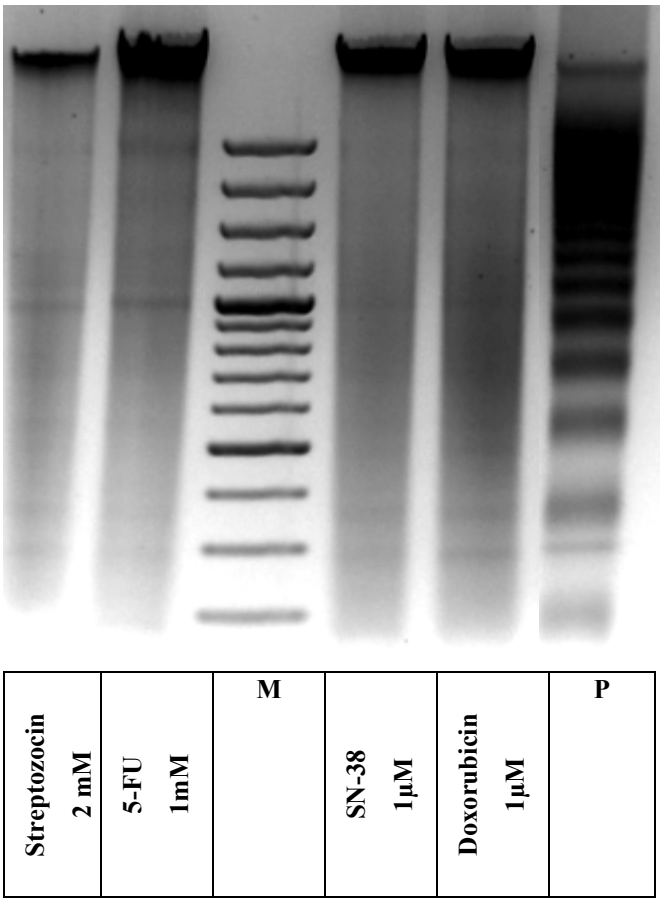


Fig. 20. Cytotoxic drugs-induced DNA fragmentation in QGP-1 neuroendocrine tumor cells. DNA isolated from 2 x 10^5 cells, treated for 48 hours with rising concentrations of cytotoxic drugs. After elution DNA was subjected to agarose gel electrophoresis. Representative tracings of three independent experiments are shown. C - untreated control, M - molecular weight marker (100 bp), P - positive control.

DNA fragmentation was observed in QGP-1 cell line 48 hours only after incubation with streptozocin and 5-FU. However, the concentrations which were used exceeded 100 times the respective IC50 value established previously by crystal violet. DNA fragmentation though weak, was observed after incubation with SN-38 and doxorubicin (Fig. 20). DNA fragmentation was not observed in BON cell line following incubation with octreotide, SOM230, 5-FU or doxorubicin (data not shown).

4.2.4. Effects of bio- /chemotherapeutic drugs on the cell cycle in GEP-NET cell lines

To test whether induction of cell cycle arrest contributed to the antiproliferative potency of bio- and cytotoxic drugs in our cell lines, we performed flow cytometric cell cycle analyses. Incubating MIP-101 cells with escalating doses of etoposide and SN-38 for 48 hours resulted in a cell cycle arrest in the G2/M phase, thereby decreasing the proportion of cells in the G0/G1 phase. Interestingly, when cisplatin and 5-FU were used, there was a statistically significant increase of the proportion of cells in S phase, but not of the G2/M phase. Additionally, cisplatin, etoposide and SN-38 induced an apoptosis-specific increase in the proportion of sub-G1 cells (Fig. 21).

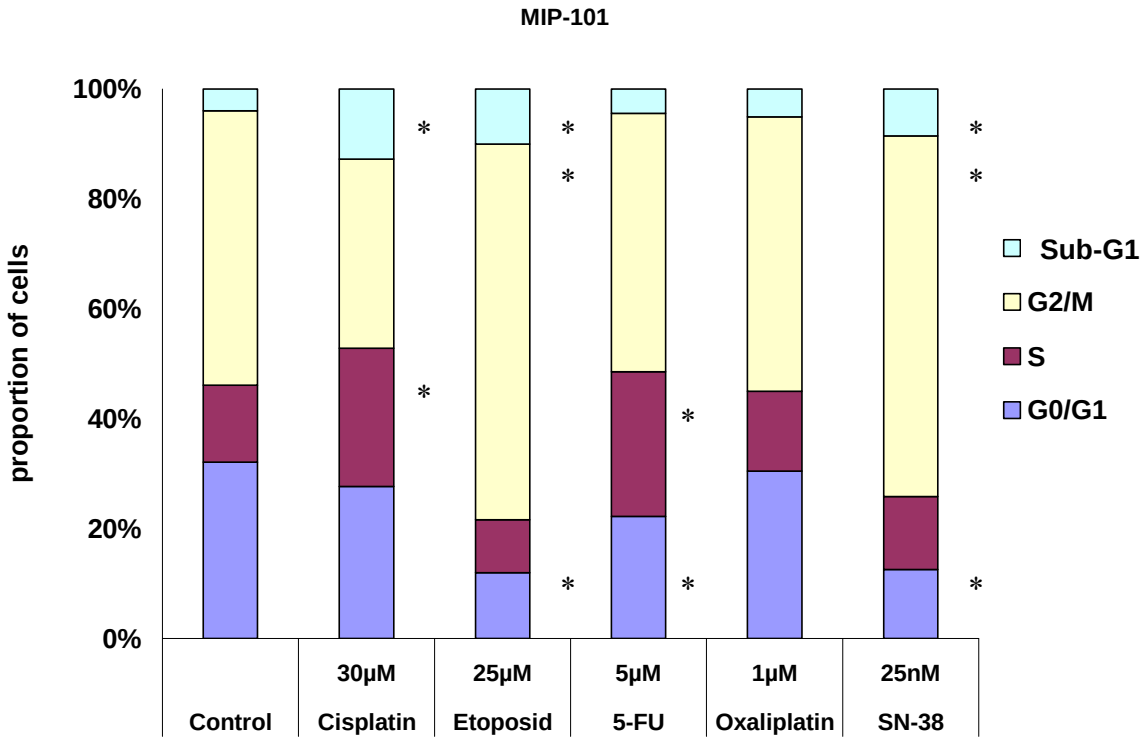


Fig. 21. Induction of cell cycle specific effects in MIP-101 cell line 48 hours after incubation with cytotoxic drugs. The difference of the proportion of cells in a particular phase of the cell cycle versus control was significant for cisplatin, etoposide, 5-FU and SN-38. *, statistical significance - $P < 0.05$.

Incubating QGP-1 cells with escalating doses of 5-FU, streptozocin and SN-38 for 24 hours decreased the proportion of cells in the G0/G1 phase with increase of the cells in S phase. Streptozocin and to lesser extent SN-38 and 5-FU (typical G2/M blocking agents) incubation resulted in a statistically significant arrest of the cell cycle in the G2/M phase. Additionally, 5-FU and streptozocin induced an enormous apoptosis-specific increase in the proportion of sub-G1 cells.

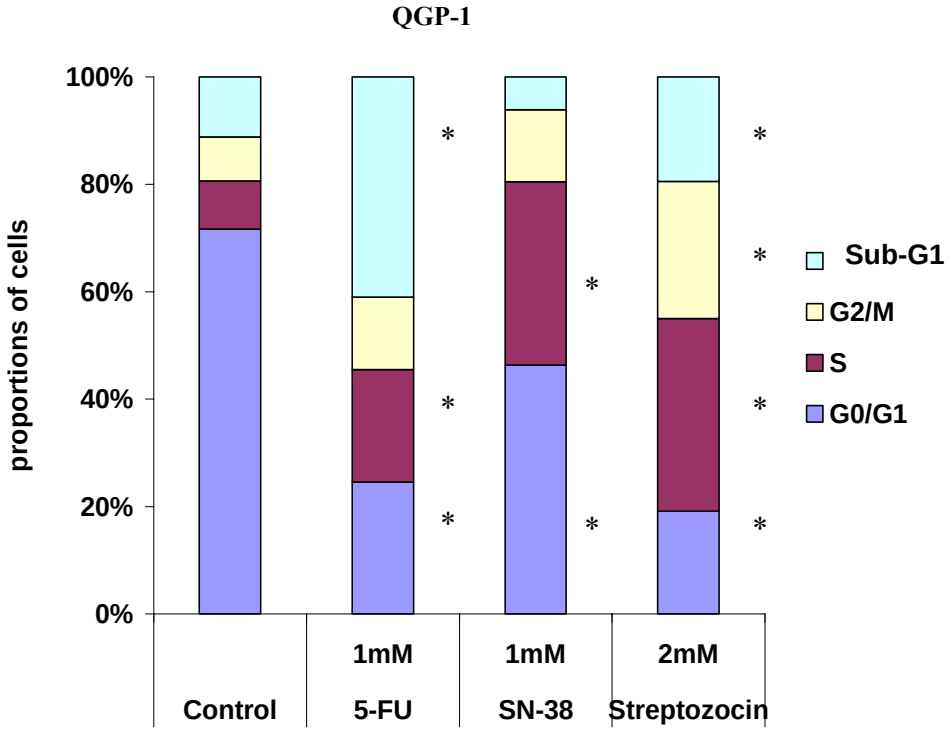


Fig. 22. Induction of cell cycle specific effects in QGP-1 cell line 24 hours after incubation with cytotoxic drugs. The difference of the proportion of cells in a particular phase of the cell cycle versus control was significant for streptozocin, 5-FU and SN-38. *, statistical significance - $P < 0.05$.

In BON cells no cell cycle dysregulation was observed following incubation with the biotherapeutic agents (octreotide and SOM230).

4.3. The functional role of survivin in GEP-NETs

4.3.1. Expression of survivin and survivin splice variants on protein level in DU-145 (prostate neuroendocrine carcinoma cell line) and BON, QGP-1 and MIP-101 (GEP-NET cell lines)

To our knowledge this is the first study of the expression of survivin splice variants on protein level in GEP-NET tumors and the DU-145 prostate carcinoma cell line, used here as a positive control. The results of the Western Blot analysis showed a relatively low survivin expression in the well-differentiated BON cell line (Fig. 23), which in this study is used as a model for WDET disease. Here the low survivin expression was predominantly due to low cytoplasmic expression and to a lesser extent to nuclear survivin expression (Fig. 24). Higher survivin expression was detected in QGP-1, a cell model for WDEC disease, due to a high nuclear survivin expression (Fig. 24). The highest survivin levels were observed in the poorly-differentiated MIP-101 cell line, which in this study was introduced as a cell model for PDEC disease. Moreover, this cell line had the highest nuclear survivin expression (Fig. 24). The positive control DU-145 cell line had survivin levels comparable with other well-differentiated carcinoma cell lines in this study (Fig. 23; Fig. 24).

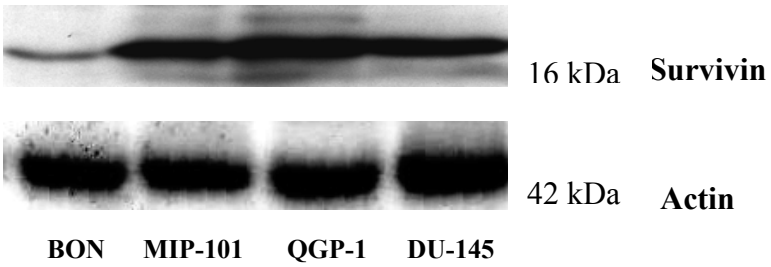


Fig. 23. Survivin expression in NET cell lines. Survivin expression was detected by Western Blotting in total cell lysate. DU-145 cell line was used as a positive control for NET cell line with strong expression of survivin.

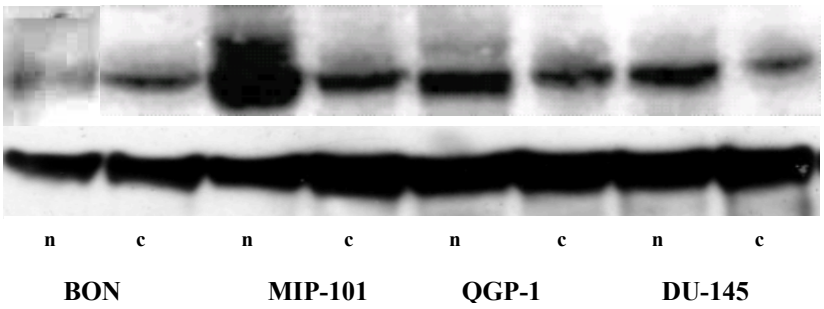


Fig. 24. Survivin expression in nucleus (N) and cytoplasm (C) in neuroendocrine tumor cell lines proved by Western Blot. DU-145 cell line was used as a positive control with known strong expression of survivin.[62]

In order to characterize the isoform expression of survivin in GEP-NET cell lines we subjected nuclear and cytoplasmatic extracts from GEP-NET cell lines and DU-145 cell line to immunoblotting with polyclonal antibodies to survivin-2B and survivin ΔEx3. Protein bands corresponding to the correct size of survivin-2B were detected only in the nucleus of the well-differentiated cell line BON and the QGP-1 cell line (Fig. 25). Survivin-2B expression was not detected in MIP-101 and DU-145 cell lines.

Even more intriguing were the results with survivin ΔEx3. This splice variant was detected as expected with a specific 17 kDa band, but in the cytoplasm of DU-145, MIP-101 and QGP-1 cell lines. No protein was found in the well-differentiated, benignly-behaving BON cell line (Fig. 25). However, in the nucleus of DU-145, BON and especially MIP-101 cells we observed with an additional 37 kDa protein in the nucleus. Additionally, in the nucleus of the poor-differentiated MIP-101 cell line and to a much lesser extend in the cytoplasm of QGP-1 cell line another protein with the weight of 32-33 kDa was seen (Fig. 25).

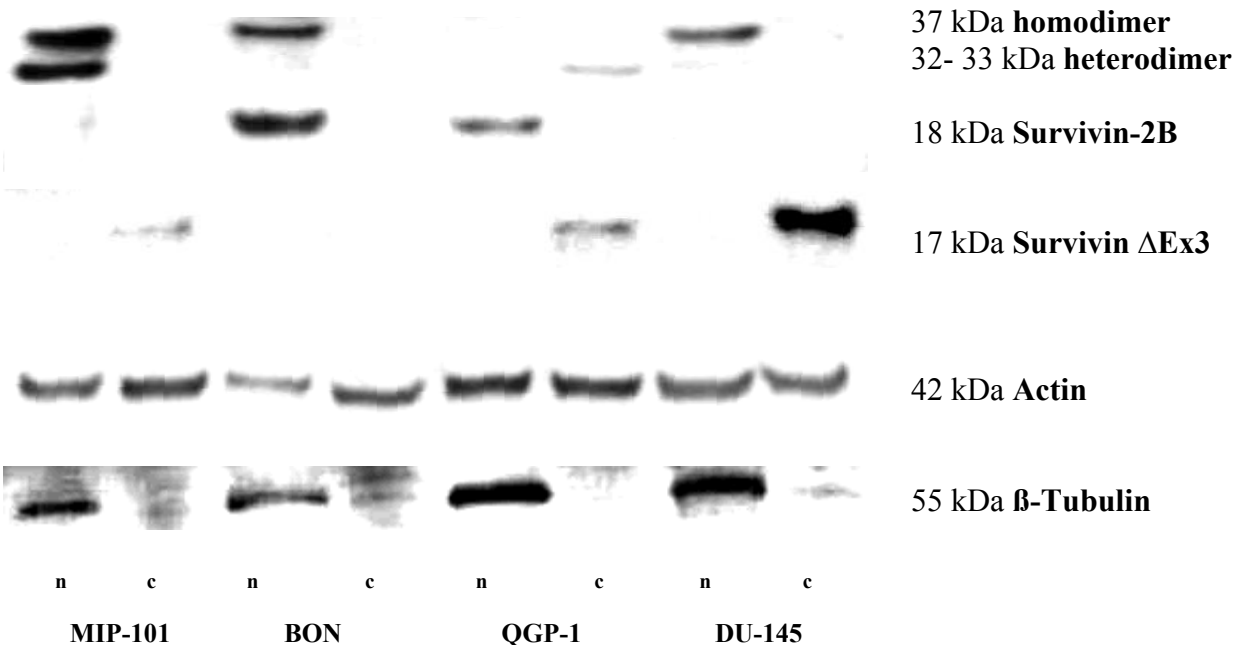


Fig. 25. Differential expression of survivin splice variants in neuroendocrine tumor cell lines. Western Blot analysis detects different levels of protein expression of the splice variants survivin-2B and delta Exon3 (ΔEx3), as well their homo-/heterodimers in the nucleus (n) in the cytoplasm (c) of DU-145 prostate carcinoma cell line and GEP-NET cell lines. ß-tubulin was used as a control for nuclear protein separation.

4.3.2. Survivin knock-down model in NET cell lines

In this study, we established a model for transient down regulation of the survivin expression using siRNA in four GEP-NET cell lines - QGP-1, MIP -101, BON and DU-145. We tested the effectiveness of a 21-mer siRNA targeting portion within exon 1 of survivin mRNA to silence survivin gene expression in the DU-145 cell line. Western Blot experiments carried out in QGP-1, MIP -101, BON and DU-145 cells, using protein lysate collected at different intervals after the transfection with 100 nmol/l of survivin-specific siRNA showed a reduction of survivin protein expression compared with Lipofectamine2000 alone and All-Star Control siRNA/Lipofectamine2000 – exposed

cells (Fig. 26). Such inhibition ranged from 39 % to 60 % in DU-145 cell line depending on the time-point. The transfection efficiency was proven to be > 95 % in all cell lines, as measured by FITC-labeled siRNA by FACS.

In the DU-145 cell line the effect of survivin down-regulation was observed on protein level (detected by Western Blot) 24 hours after siRNA transfection and lasted up to 72 hours after transfection. In MIP-101 cells the survivin downregulation appeared first 72 hours after siRNA transfection. In QGP-1 cells an albeit small, but significant survivin downregulation was observed first 72 hours post-transfection. However, as we suppose that there is a transient siRNA transfection we did not observe effects after 72 hours of siRNA transfection. In BON cell line no effect on the survivin protein level could be obtained, even though the transfection protocol was well established (data not shown).

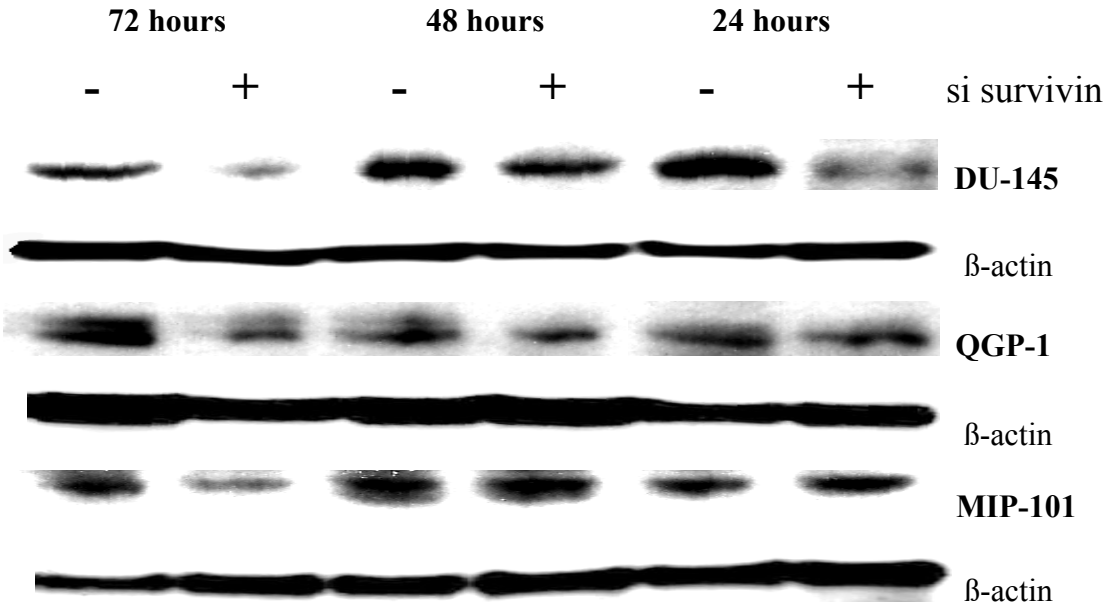


Fig. 26. Representative Western Blot experiments illustrating survivin protein expression in DU-145, QGP-1 and MIP-101 cell lines after transfection with siRNA against survivin in three different endpoints. ß-actin was used as loading controls.

4.3.3. Survivin as a predictive value (tumor marker) in patients with hematological malignancies, GEP-NETs and other solid tumors

The next step of the study included the establishment of a method by which the level of survivin could be used as a predictive value in the treatment outcome by patients with GEP-NET disease. Blood samples were obtained from patients with GEP-NET cancer (n = 20), hematological malignancies (HM) and solid cancers (n = 20) and healthy volunteers (n = 22) by venipuncture (Table 3), and were clotted at room temperature. The patients of the GEP-NET group had either stable disease with asymptomatic metastasis, predominantly liver (n = 16). Others were progressive and/or had symptomatic distant metastases at the time of venipuncture (n = 4). We used an EIA-based method for determination of survivin in human serum from patient before and during treatment for GEP-NET tumors. In order to establish if survivin plasma levels may have significance in other cancer patients we included in the present study also patients with other solid tumors and also with hematological diseases.

There was a statistically significant increase of the survivin protein levels in patients with HM and solid cancers compared to the healthy controls (Fig. 27). However, there was a subgroup of patients within the HM/solid cancer group (marked as A) in whom the survivin level was comparable to that in the healthy controls. Most of these patients had solid cancer with low tumor burden or had HM, for which they had recently received a cytoreductive therapy. However, there was a breast cancer patient who had very high survivin plasma levels. In the course of subsequent examinations multiple bone metastases were encountered in this patient.

There was an increase of the survivin protein level in the group of patients with GEP-NET tumors compared to the healthy controls. However, patients with stable GEP-NET disease had low survivin levels, except those who experienced a progression of the disease. Nevertheless, there were also patients who had relatively high serum survivin level, but no signs of progression. Follow-up is ongoing in order to establish if survivin levels correlate with the time to progression and the overall survival in those patients. However, the difference in survivin plasma levels between healthy controls and patients with GEP-NETs did not reach statistical significance. Interestingly, we observed that

healthy controls who had recently suffered a virus infectious disease (marked as B and D) and a pregnant healthy woman (marked as C) had considerably elevated levels of survivin (Fig. 27), as patients with stable GEPNET disease had a low or undetectable survivin level (marked as E).

Group	Subtype	N	male : female ratio	mean age (years)	age range (years)
Non-GEP-NET	All	20	14 : 6	59.5	30 - 82
Hematological Malignancies	NHL M. Hodgkin CLL AML	7 2 2 2			
Solid Tumors	Breast cancer NSCLC SCLC Ovarial cancer Melanoma malignum	1 2 1 1 2			
Healthy Controls	All	20	9 : 11	29.9	25 - 39
(GEP-) NET	All	20	9 : 11	64.3	43 - 81
	Esophagus Stomach /Duodenum Pancreas Small intestine Colorectal Renal	2 1 2 1 13 1			
Healthy Controls	All	20	13 : 7	29.5	24 - 39

Table 3. Characteristics of patients (and healthy controls) taking part in the plasma survivin measurement using the TiterZymeEIA Human Total Survivin Kit. One patient, who was first diagnosed with NSCLC, was confirmed later to suffer from a SCLC (a type of cancer, which usually demonstrates neuroendocrine differentiation).

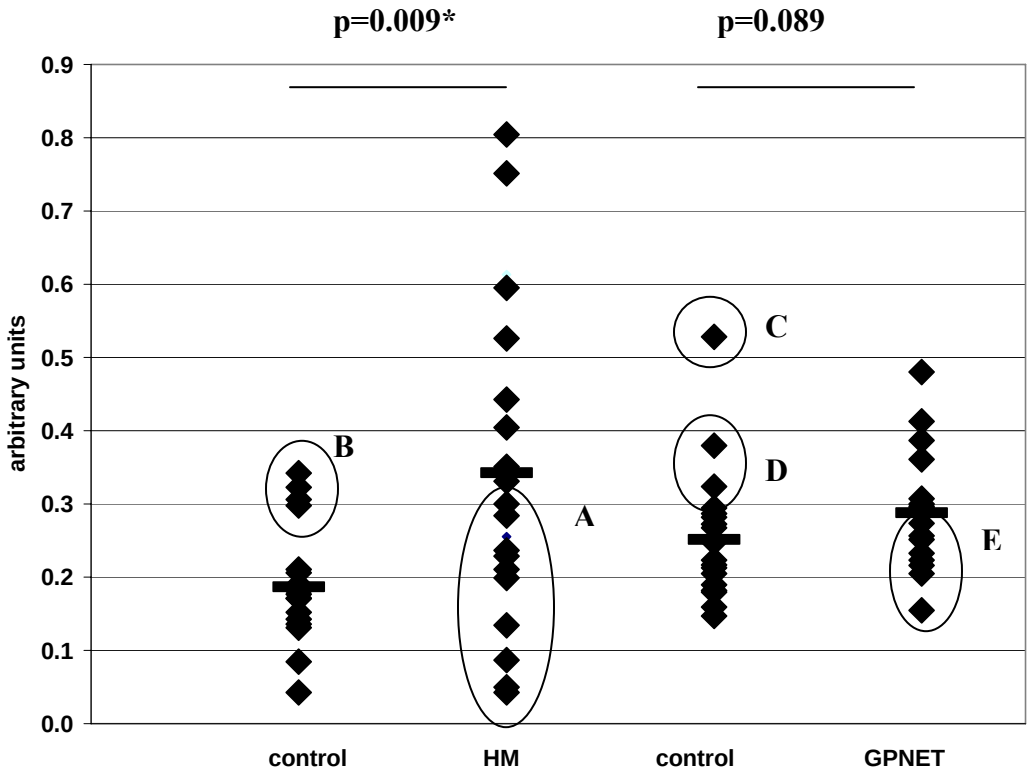


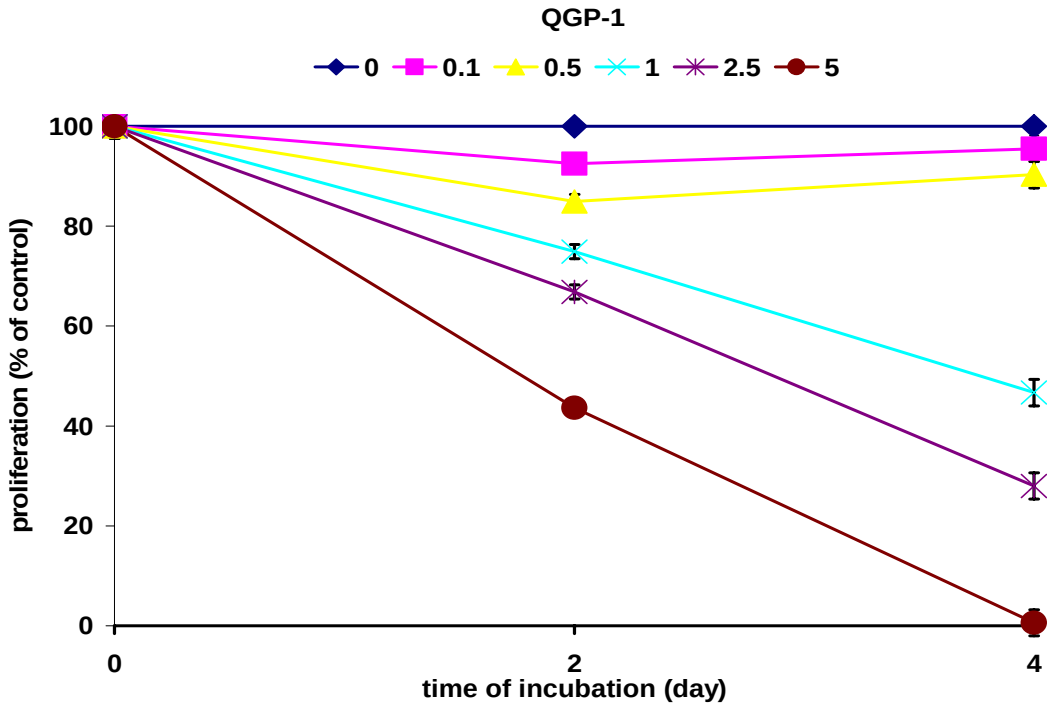
Fig. 27. Survivin expression in serum from patients with GEP-NET tumors and hematological malignancies (HM) vs. healthy controls. Two different control groups were used due to lack of serum from two patients. The results are obtained using EIA Survivin Kit and depicted as arbitrary units. The median is marked as a bar. * - level of significance $P < 0.01$.

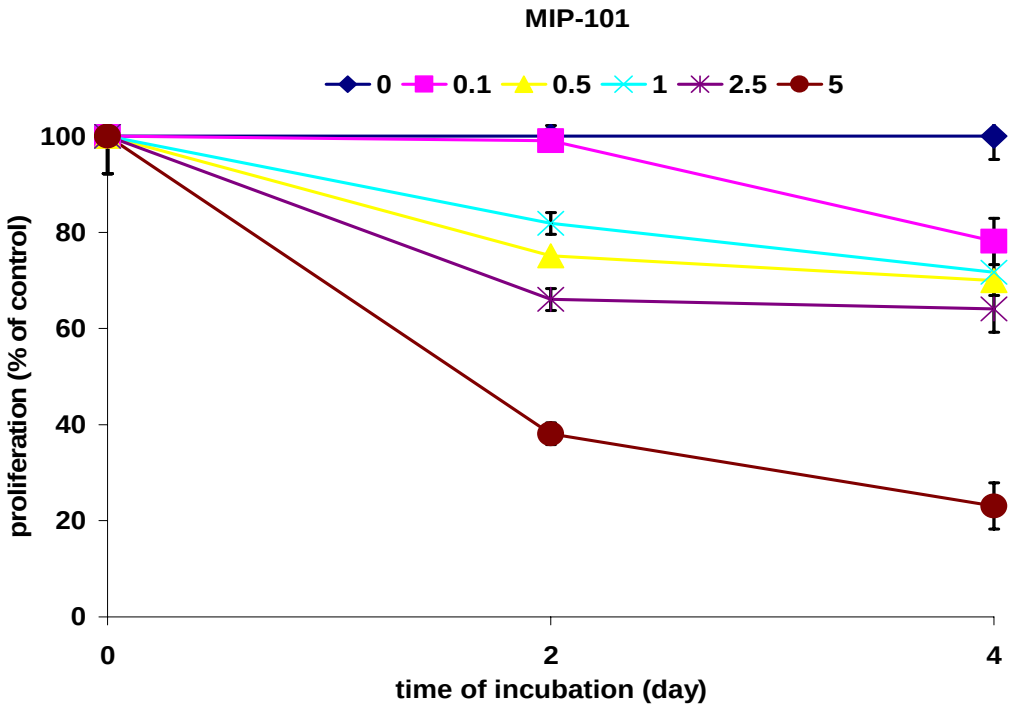
4.4. The role of aurora kinases A and B in GEP-NET disease

4.4.1. Effects of ZM447439 on the cell growth in GEP-NET cell lines and DU-145 prostate neuroendocrine carcinoma

The effects of ZM447439 on the growth of GEP-NET cell lines and DU-145 prostate carcinoma cell line were studied using crystal violet staining as described earlier (Chapter 2.2.6). Aurora kinases A and B inhibition using ZM447439 time- and dose-dependently inhibited the growth of all four studied cell lines (Fig. 28). After 96 hours of incubation

with rising concentrations of ZM447439 (0-5 µM) a significant decrease in cell number was observed. The IC50 values of ZM447439 determined after 72 h of continuous exposure to the respective drug were 3 µM (BON cell line), 0.9 µM (QGP-1 cell line), 3 µM (MIP-101cell line) and 2.1 µM (DU-145 cell line).




MIP-101
0
0.1
0.5
1
2.5
5
100
80
60
40
20
0
proliferation (% of control)
0
2
4
time of incubation (day)

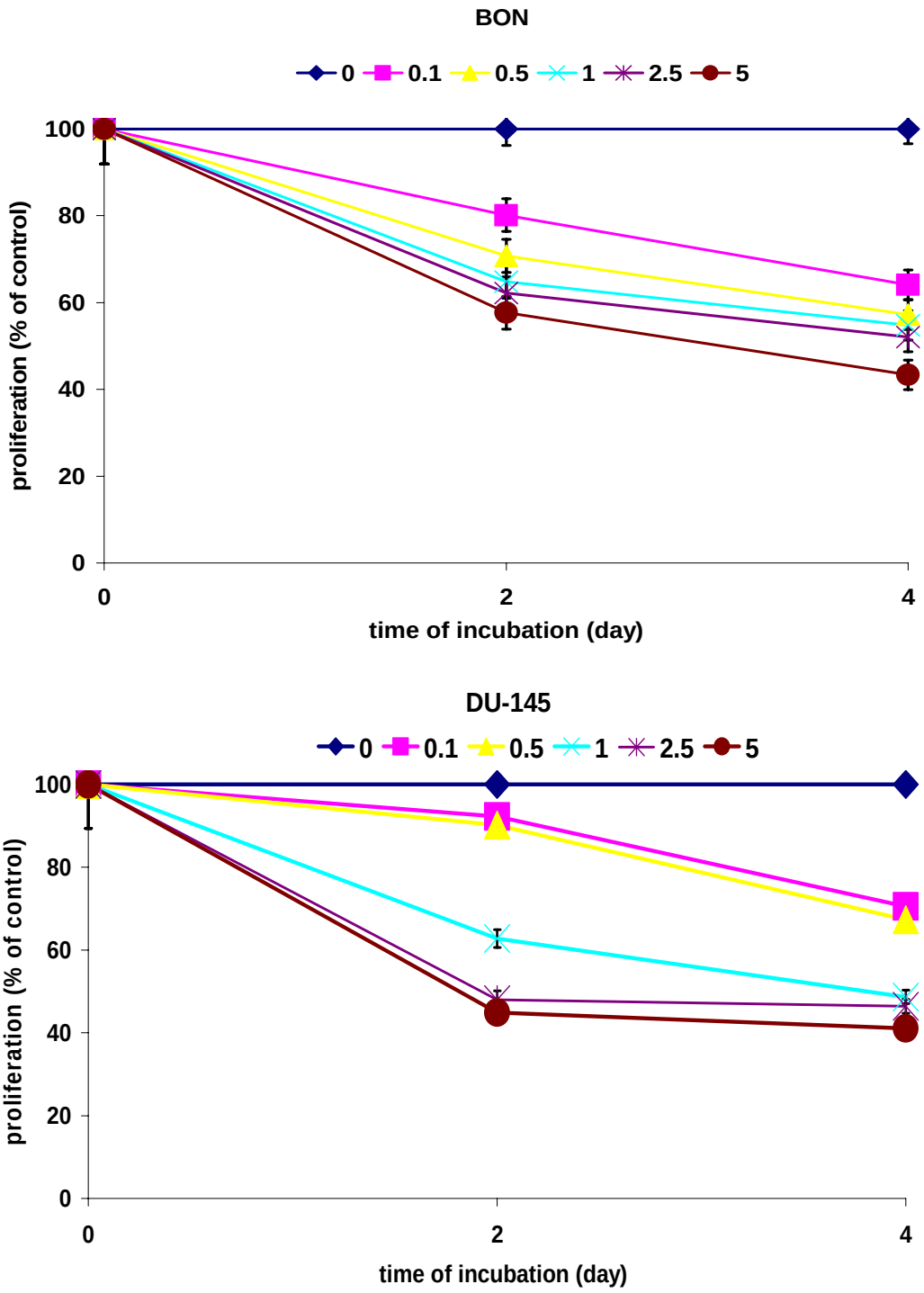


Fig. 28. ZM447439-induced growth inhibition in QGP-1, MIP-101, BON and DU-145 neuroendocrine cell lines. Representative results of three independent experiments as measured by crystal violet staining (error bars - S.E.M.). Data are given as percentage of untreated controls.

4.4.2. Expression of aurora kinases A and B in GEP-NET tumors before and after treatment with ZM447439

Aurora kinase B and survivin co-expression was proven in all cell lines included in this study by Western Blot analysis (Fig. 29). As previously mentioned, the experimental aurora kinase inhibitor ZM447439 produced a dose-dependent inhibition of aurora kinase B function when used in sub-IC50 concentrations. However, blocking the function of aurora kinase B led to its compensatory overexpression in MIP-101 and DU-145 cell lines which was followed by survivin overexpression in DU-145, but not in MIP-101 cell line (Fig. 29). In BON and QGP-1 cell lines the aurora kinase B inhibition led to dose-dependent aurora kinase B protein degradation followed by survivin down-regulation in both cases.

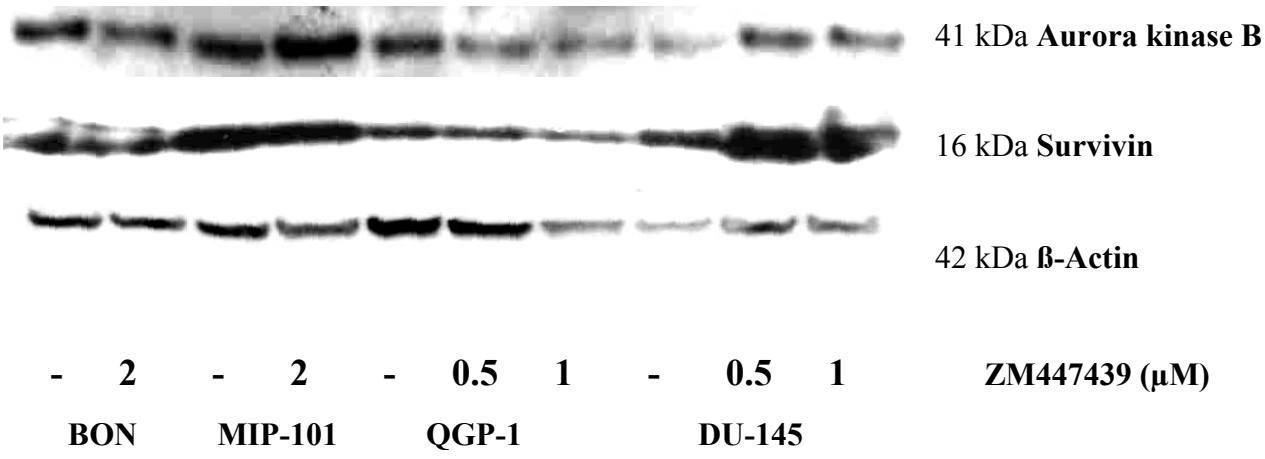


Fig. 29. Effects of ZM447439 on the aurora kinase B and survivin expression. BON, MIP-101, QGP-1 and DU-145 cells were treated with increasing concentrations of ZM447439 (0 – 5 µM) for 48 hours. The expression of aurora kinase B and survivin were analyzed by Western Blotting. Representative results from three independent experiments are shown.

4.4.3. Effects of ZM447439 on the cell cycle transition

To test whether induction of cell cycle arrest contributed to the antiproliferative potency of ZM447439 in GEP-NET cancer cells, we performed flow cytometric cell cycle analysis. Forty-eight hours of incubation with increasing concentrations of ZM447439 induced a dose-dependent cell cycle arrest in G1/G0 and G2/M phase, thereby decreasing the proportion of cells in the S-phase (Fig. 30). Additionally, in BON and DU-145 cell lines ZM447439 induced an apoptosis-specific increase in the proportion of sub-G1 cells.

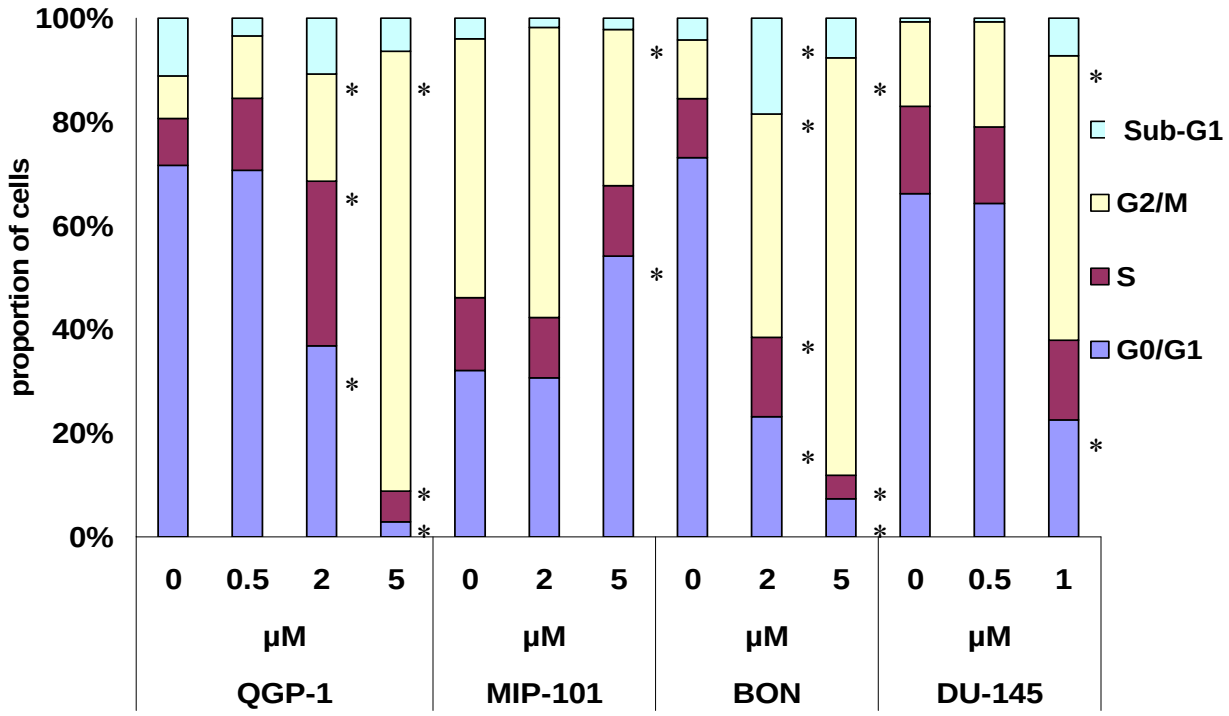


Fig. 30. Induction of cell cycle specific effects in QGP-1, MIP-101, BON and DU-145 cell lines 48 hours after of incubation with ZM447439. The difference in the proportion of cells in a particular phase of the cell cycle versus control was significant for all cell lines studied. *, statistical significance - $p < 0.05$.

4.4.4. Effects of ZM447439 on the induction of apoptosis in GEP-NET cells

4.4.4.1. Caspases- 3 and -7 activation as a hallmark of apoptosis

To determine how ZM447439 affects caspase dependent apoptosis levels in GEP-NET cells, we evaluated the activation of caspase-3 and -7, key enzymes in the apoptotic signaling cascade.

Cells were treated with increasing concentrations of ZM447439 and caspase-3 and -7 activities were determined after 6, 12, 24 and 48 hours (Fig. 31).

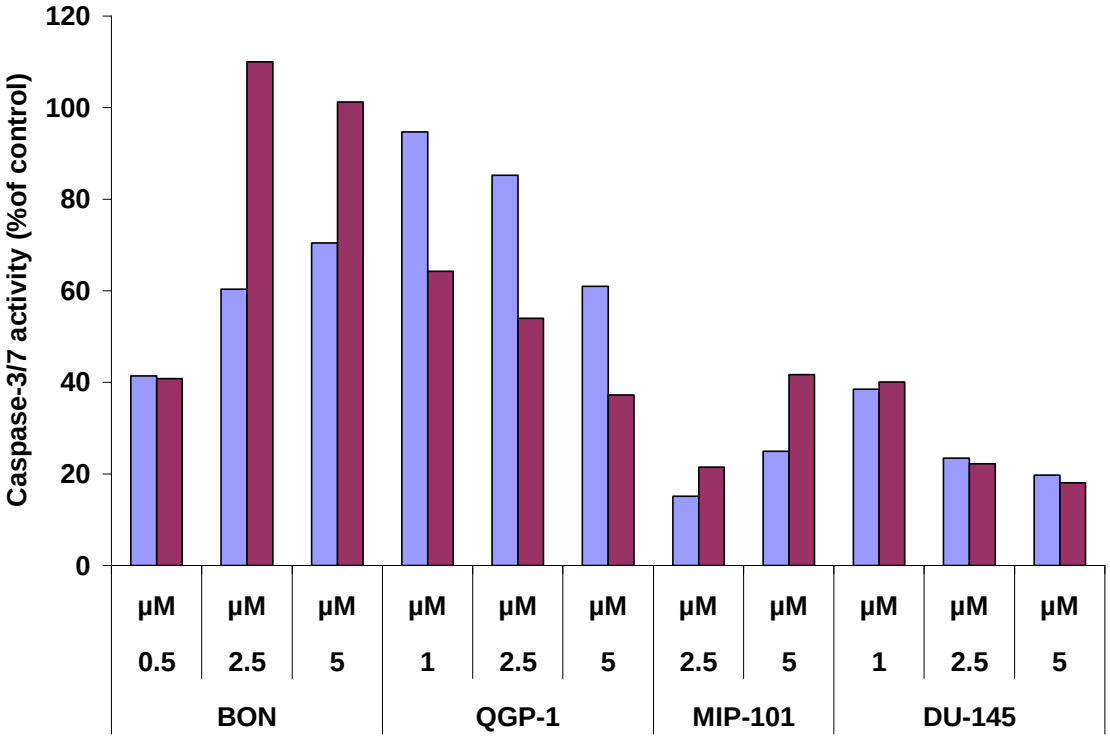


Fig. 31. Apoptosis-specific caspase-3 and -7 activity increase in neuroendocrine cell lines following 24 hours (blue columns) or 48 hours (red columns) of incubation with ZM447439 (n = 3 independent experiments, $P < 0.05$ vs. controls, only in BON and QGP-1 cell lines in concentrations above 1µM).

We observed that the highest levels of caspases-3 and -7 activation occurred after 24 or 48 hours depending on the cell type context. In BON and QGP-1 cells, ZM447439 induced significantly caspases-3 and -7 up to 110 % versus the control. In MIP-101 and DU-145 cell lines, however, there was only a low increase (25 - 50 %) in caspases-3 and 7 activity 24 - 48 hours after incubation (Fig. 31).

To further substantiate the anti-apoptotic effect of ZM447439 in GEP-NETs, we studied the DNA fragmentation as a biochemical hallmark of apoptosis. DNA fragmentation was observed in MIP-101, QGP-1 and DU-145 cell lines 48 hours after incubation with ZM447439 (Fig. 32). No induction of apoptosis was observed 24 hours after incubation with ZM447439. Moreover, no signs of DNA fragmentation were observed in BON cell line in either time endpoint.

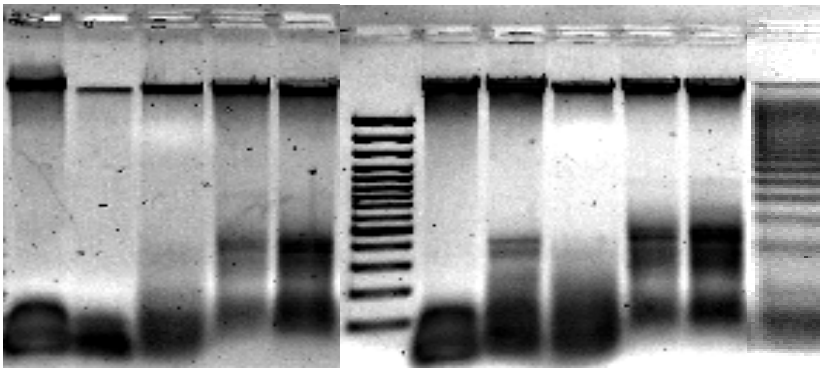
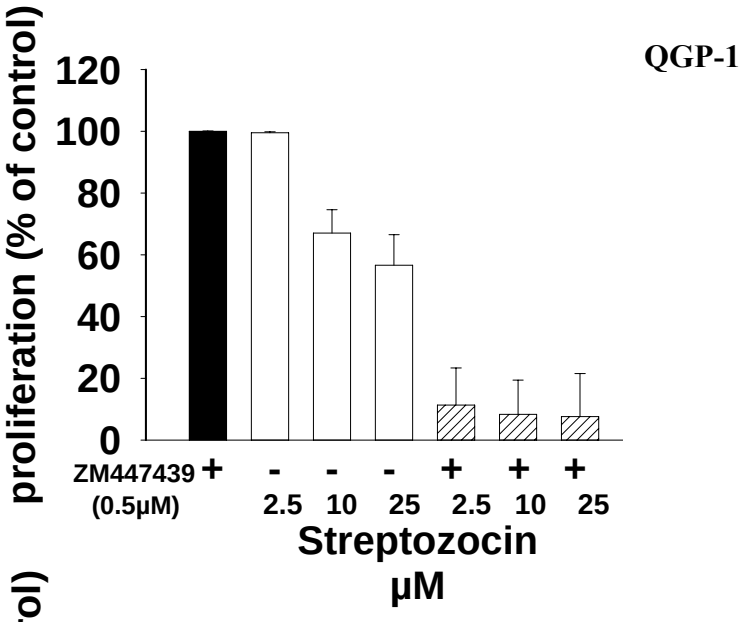


Fig. 32. ZM447439 induced DNA fragmentation in QGP-1, MIP-101 and DU-145 neuroendocrine tumor cells, but failed to induce DNA fragmentation in BON cell line. DNA isolated from 2 x 10^5 cells, treated for 48 h with rising concentrations of ZM443479. After elution DNA was subjected to agarose gel electrophoresis and stained by ethidium bromide. Representative tracings of three independent experiments are shown. C - untreated control, M - molecular weight marker (100 bp), P - positive control. The concentrations are shown in μM.

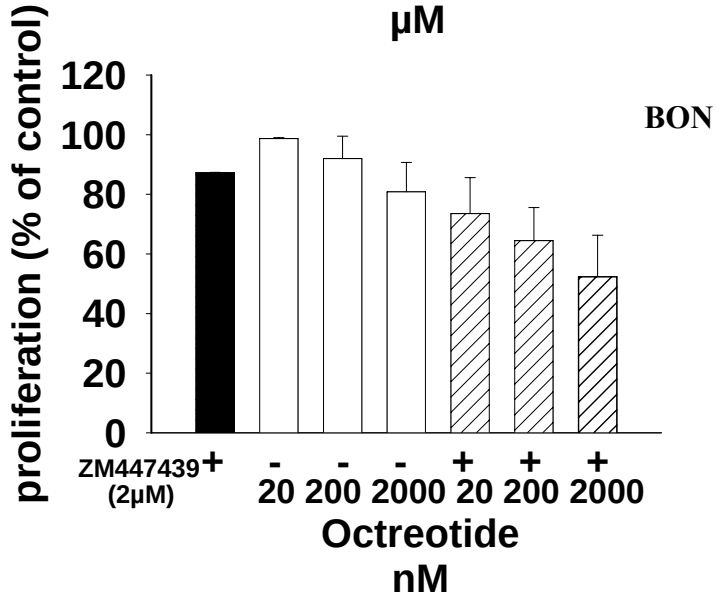
4.4.5. Effects of ZM447439 in combination with conventional bio- and chemo-therapeutic drugs on the cell growth and viability in GEP-NET cell lines

Furthermore, we investigated the effects of a combined treatment of GEP-NET cells with ZM447439 and biotherapeutic/cytotoxic drugs that are commonly used in the clinical practice: octreotide and SOM230 (in BON), streptozocin and doxorubicin (QGP-1) and cisplatin and 5-FU (in MIP-101). Interestingly, ZM447439 and 5-FU showed an antagonizing effect to each other, and 5-FU alone had a stronger effect on the MIP-101 cell growth than the combination with ZM447439 (Fig. 33C). However, additive antiproliferative effects were observed in MIP-101 cells, when sub-IC50 concentrations of ZM447439 were combined with the clinically relevant chemotherapeutic agent cisplatin (83.56 % versus 68.14 % for 5 µM cisplatin alone (Fig. 33E)). When treating QGP-1 cells with sub-IC50 concentrations of ZM447439, ZM447439 plus doxorubicin displayed additive growth inhibitory effects (Fig. 33D). Streptozocin in combination with sub-IC50 ZM447439 reduced the cell growth of QGP-1 cells with 43.4 % versus 12.4 % of streptozocin alone (Fig. 33A). Applying octreotide alone for 3 days to BON cells decreased their growth by up to 24.2 % (2000 nM). When treating BON cells with a sub-IC50 concentration of ZM447439 plus octreotide (20-2000 nM) this resulted in additive growth inhibitory effects up to 47.7 %, compared to the treatment with ZM447439 alone (Fig. 33B).

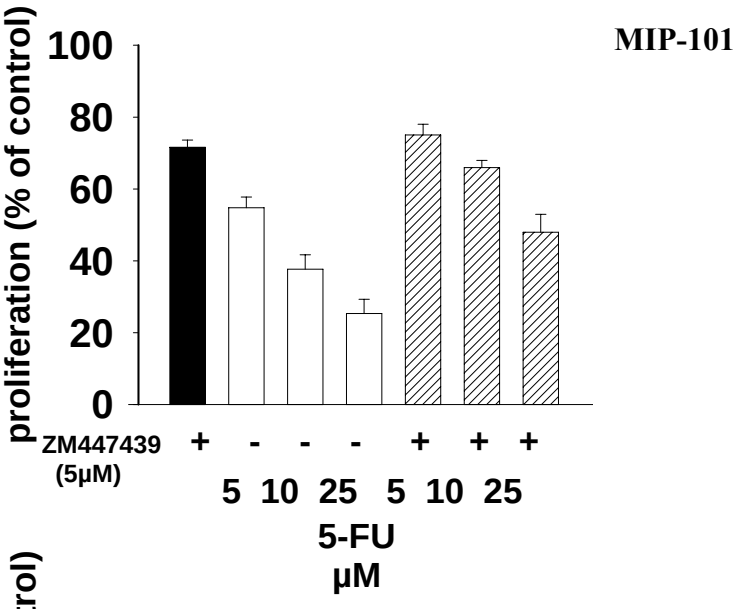
A


QGP-1
proliferation (% of control)
120
100
80
60
40
20
0
ZM447439 + - - - + + +
(0.5µM)
2.5 10 25 2.5 10 25
Streptozocin
µM

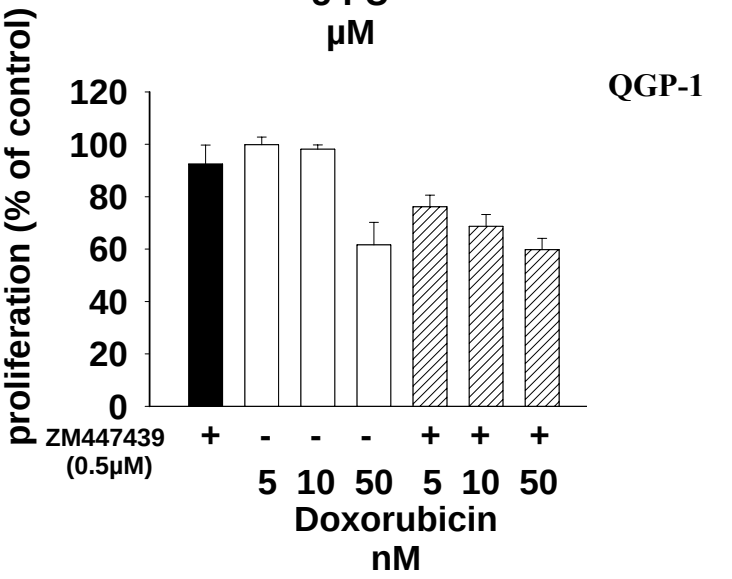
B


BON
proliferation (% of control)
120
100
80
60
40
20
0
ZM447439 + - - - + + +
(2µM)
20 200 2000 20 200 2000
Octreotide
nM

C


MIP-101
proliferation (% of control)
100
80
60
40
20
0
ZM447439 + - - - + + +
(5µM)
5 10 25 5 10 25
5-FU
µM

D


QGP-1
proliferation (% of control)
120
100
80
60
40
20
0
ZM447439 + - - - + + +
(0.5µM)
5 10 50 5 10 50
Doxorubicin
nM

E

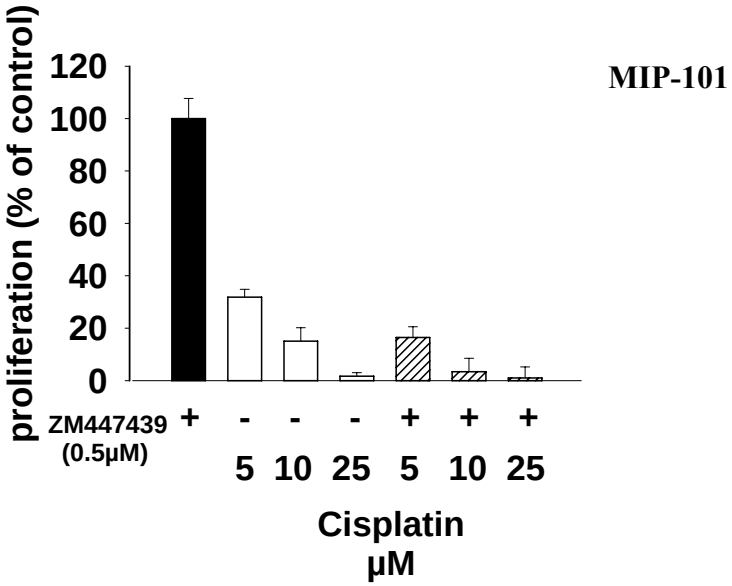

MIP-101
proliferation (% of control)
120
100
80
60
40
20
0
ZM447439 + - - - + + +
(0.5µM)
5 10 25 5 10 25
Cisplatin
µM

Fig. 33. Antiproliferative effects of ZM447439 alone (black bars), a bio-/chemo-therapeutic drug alone (white bars) or in combination of ZM447439 with cytotoxic drugs (hatched bars). QGP-1 treated with streptozocin (A) and doxorubicin (D), BON with octreotide (B), MIP-101 treated with 5-FU (C) and cisplatin (E). End-point - Data was obtained after 72 hours of incubation and plotted as percentage of controls (means), which were set at 100 %. Each plot is representative of at least three independent experiments.

5. Discussion

GEP-NETs represent a heterogeneous group of tumors with varying clinical manifestation depending on tumor type (functioning or non-functioning), origin and extension, but also on histological differentiation and proliferative capacity. Although much research has been carried out in order to better discriminate patients with disease progression and a worse prognosis, treatment possibilities of GEP-NETs are still not satisfactory and the prognosis of these patients is not clearly predictable.[115]

5.1. Cell line models for GEP-NET disease

There are a few human cell line models for foregut neuroendocrine tumor disease (BON, INS-1), but the existing cell line models for midgut tumors are even fewer. Rat RIM-1 and mouse STC-1 cell lines are usually used as a model, however, the results from these cell lines are not transferable to human GEP-NETs. Furthermore, until now just one cell line is used as a model for PDECs – LCC-18. Previous studies described the LCC-18 cell line as positive to synaptophysin, vasoactive intestinal polypeptide (VIP) and glucagon.[96,97] In our experiments the LCC-18 cell line was positive to both synaptophysin and chromogranin A, but not to SNAP25, synaptobrevin and syntaxin. DeBrüne et al. described NCI-H716 as a poorly-differentiated colon carcinoma line with possible neuroendocrine differentiation.[98,99] NCI-H716 cells expressed large amounts of high affinity receptors for gastrin, serotonin and somatostatin and thus, NCI-H716 cells seemed a suitable model for the study of neuroendocrine differentiation in intestinal epithelium and of auto- or paracrine growth regulation in intestinal neoplasia. In our experiments this line showed positivity for synaptophysin, chromogranin, synaptobrevin, syntaxin and negativity just to SNAP25. But in our experience, and as reported by other authors (M. Höpfner, personal communication) the cell line was only slow growing with generation times of several days. Therefore, it seemed not suitable as a model for an aggressive PDEC cell line. In the present study we propose for the first time the colon cancer cell line MIP-101[100], which was positive to synaptophysin, synaptobrevin and

syntaxin, as a model for poorly-differentiated neuroendocrine cancers. Furthermore, we propose that LCC-18 and MIP-101 cell lines could be used as cell line models for the PDEC disease, but we preferred MIP-101 cell line as it was positive for three out of five markers for neuroendocrine differentiation, but also because it grows fast and needs simple growth conditions. Similarly, the QGP-1 cell line could be used for WDEC disease models. The Caco2 cell line also showed some neuroendocrine characteristics, but this was not sufficient to be included in this study. Until now, there is no commercially available neuroendocrine cell line suitable model for midgut tumors.

5.2. Strategies to inhibit the growth of GEP-NET cell lines using conventional bio- and chemotherapeutic agents

Chemotherapy, using cisplatin and etoposide or 5-FU and oxaliplatin (currently ongoing clinical studies) is the preferred method of treatment of poorly differentiated and somatostatin-resistant gastrointestinal neuroendocrine carcinomas[6,32,33] and has proved to have some clinical benefit for the patients. For well-differentiated pancreatic neuroendocrine carcinomas, the combination of 5-FU (or doxorubicin) and streptozocin is used as a standard treatment.[6,23] Despite the fact that those tumors (pNETs) have some chemosensitivity, disease control is limited (8 – 10 months). The antiproliferative effects of somatostatin analogues like octreotide is still a matter of debate, whereas the improvement of related clinical symptoms is undisputed.

In our study we tried to emulate the clinical methods in our neuroendocrine tumor cell lines as a basis for further combination experiments with new and experimental drugs. We directed our attention especially to irinotecan, a drug which in combination with platinum derivates is used currently with success in the treatment of SCLC. Therefore, it was proposed that this combination could be used in the treatment of WDEC and PDEC disease. As showed by the Kulke group, the initial studies on WDEC patients showed no cases of partial or full response, but they revealed a high level (78 %) of stable disease.[6,33] The biotherapeutic- and cytotoxic agents studied inhibited the cell growth of the evaluated neuroendocrine cell lines in quite a different time- and dose- dependent

manner. In our study we showed that the growth of QGP-1, a cell line established from well-differentiated neuroendocrine carcinoma of the pancreas, is extremely sensitive to doxorubicin and SN-38, but that it has a low to moderate sensitivity to streptozocin, 5-FU, oxaliplatin and etoposide. This was confirmed in the cell cycle experiments, where a substantial block in the cell cycle progression was observed after incubation with doxorubicin and SN-38. Although it is difficult to obtain conclusions based on experiments with a single cell line and even though they could hardly be applied to the human organism, we have to admit that these results are in contrast to clinical experience, where 5-FU is used in combination with streptozocin.[6,33] However, the fact that SN-38 induces a substantial cell cycle block and growth inhibition, which is not accompanied by correspondingly high induction of caspases and substantial DNA fragmentation might be a possible explanation of the fact that irinotecan induced exceptionally high level of disease stabilization, but not tumor regression in recent studies.[6,33] On the other hand doxorubicin induced a much higher level of caspases activation and DNA fragmentation, and disease regression is more likely when this drug is included in the chemotherapy protocols. Moreover, streptozocin induced low levels of cell cycle block and growth inhibition this drug induced the highest level of caspases activation. This might be explained with a possible direct cytotoxic rather than cytostatic effect on QGP-1 cells. This might be a possible explanation for the high response rate of WDEC tumors when streptozocin is used, especially in combination with doxorubicin – exceeding 47 % and this therapy has the longest duration of response, about 19.7 months (median).[118] However, we cannot explain the fact that 5-FU induced considerable block of the cell cycle progression with a sub-G1 quotient over 40 %, but failed to induce substantial apoptosis and growth inhibition in QGP-1 cells.

MIP-101, a cell line established from poorly-differentiated colorectal carcinoma, was highly sensitive to SN-38, but just moderately sensitive to cisplatin and etoposide, which are the standard treatment for PDEC disease.

Upon treatment with etoposide and SN-38 we found that the cell cycle progression in MIP-101 cells was blocked in the G2/M phase - the so called taxol-like block. However, just cisplatin and to some extend etoposide induced significant caspases-3 and -7 activation. This could explain the good response to these drugs in clinical practice.

However, it was surprising that SN-38 did not induce substantial levels of apoptosis even though it had a substantial effect on the cell growth and cell cycle in MIP-101 cells.

The effects of SN-38 on the cell proliferation, cell cycle and induction of apoptosis in MIP-101 cells lead to the conclusion that it might be considered as a very good option in the treatment of aggressive poorly-differentiated colon NETs. In clinics, the probably very similar behaving small cell carcinomas of the lung (SCLC) are treated with cisplatin and irinotecan (the active metabolite is SN-38) in a multicenter Phase III with some effect[101], although a French study in gastrointestinal neuroendocrine carcinomas failed to show a therapeutic benefit with this combination.[102] Nevertheless, SN-38 should be taken into consideration, maybe in other drug combinations. Interestingly, the experimental treatment with 5-FU and oxaliplatin, a combination well established in colorectal adenocarcinomas, which has been introduced to clinical studies because of the possible same tumor stell cell as proposed by Wright[103], exerted poor effect on the cell growth and on the induction of apoptosis in our cell line. However, this could be an effect dependent on the cell type context and will still have to be considered as a treatment option in this tumor entity. Surprisingly, cisplatin - although it induced caspases-3 and -7 and cell cycle arrest - was less effective in inhibiting the cell growth compared to SN-38. This might be explained by the fact that low differentiated cell lines overexpress ABC transporters (drug pumps) such as MRP2, and on the other hand, cisplatin is a well known substrate of MRP2 and ERCC1[104], as SN-38 is not prone to such a drug resistance mechanism. Further studies are needed for evaluating the multidrug resistance status of MIP-101 cell line.

In BON cells, a pancreatic NET cell line and a cell model for WDET disease, tumors which are resistant to conventional cytotoxic drugs, a tendency of growth inhibition by octreotide was observed in the high μM range. SOM230, a newly synthesized octreotide analogue, which is supposed to block all five octreotide receptors and has proved to be an promising therapeutic in endocrine tumor disease, especially in the case of a hypophyseal tumor[26,27], did not exert a significant growth inhibition effect on BON cells even in the mM range. Unexpectedly, BON cells were very sensitive to SN-38, yet no significant activation of caspases-3 and -7 or DNA fragmentation was observed when using either

cytotoxic or biotherapeutic drug. Nevertheless, a possible apoptosis activation in BON cells through a pathway independent of caspases-3 and -7 could not be excluded.

5.3. Strategies to counteract the growth of GEP-NET cell lines targeting the chromosome passenger complex

Both survivin and aurora B are important regulators of the mitotic spindle checkpoint system, the surveillance mechanism of cells which prevents progression of mitosis until all chromosomes are properly aligned during metaphase.[67]

In the present study we proved that it is technically possible to downregulate survivin expression, as well as to inhibit the function of aurora kinase B in neuroendocrine cell lines. Moreover, we propose that expression of survivin splice variants might be correlated to the tumor differentiation in GEP-NET tumors.

5.3.1. Expression of survivin and survivin splice variants in GEP-NET cell lines and DU-145 prostate carcinoma cell line

Survivin-2B and survivin ΔEx3 appear to play different roles in cancer development.[39,40,105,106] Recent studies also showed that the expression ratio of survivin-2B/survivin was significantly higher in adenocarcinoma samples than in the normal ones.[107] Nevertheless, a higher ratio of survivin-2B/survivin correlated with a better prognosis and the expression ratio of survivin-2B/survivin for Stages III and IV was lower than that for Stages I and II, suggesting a negative role of survivin-2B in cancer progression.[40] These studies indicated that high levels of survivin-2B are associated with patients showing ‘‘no relapse and alive’’, while high levels of survivin ΔEx3 are associated with patients who have died following a relapse of non-small cell lung cancer.[40]

In order to characterize the survivin isoforms expression in GEP-NET cell lines we subjected nuclear and cytoplasmatic extracts from GEP-NET cell lines and DU-145 cell

line to immunoblotting with polyclonal antibodies to survivin-2B and survivin ΔEx3. Protein bands corresponding to the correct size of survivin-2B were detected only in the nucleus of the well-differentiated BON and to a much lesser degree in QGP-1 cells. This is very interesting as previous studies reported survivin-2B expression in the perinuclear zone and to some extent on the outer mitochondrial membrane[40], but no significant survivin-2B expression was reported in the nucleus so far. Moreover, survivin-2B expression was detected in exceptional cases in BON cells obtained from well-differentiated benign pancreatic tumor and to some extent in QGP-1 cells obtained from a well-differentiated neuroendocrine pancreatic tumor, but not in the poorly-differentiated MIP-101 and in DU-145 cells. These observations are consistent with the recent reports that transient expression of survivin-2B inhibited lung cancer cell growth and induced apoptosis, although the underlying mechanism remains to be elucidated.[108] However, Mahotka et al. used self-produced antibodies as we used established antibodies by Abcam Inc. and this raises the question of antibody specificity.

Even more intriguing were the results with survivin ΔEx3. Several laboratories have already shown that exogenously expressed survivin ΔEx3 is localized in the nucleus. However, although the majority of survivin ΔEx3 was in the nuclear fraction, there was some survivin ΔEx3 in the mitochondrial, but not in the cytoplasmic fraction.[105,107] In the present study survivin ΔEx3 was detected as a 17 kDa monomer in the cytoplasm of DU-145, MIP-101 and QGP-1 cell lines, but not in the well-differentiated benign BON cell line. However, in the nucleus of DU-145, BON and especially in MIP-101 cell lines a 37 kDa dimer (possibly homodimer) was also observed. Additionally, in the nucleus of the poor-differentiated MIP-101 cell line and to much lesser extend in the cytoplasm of QGP-1 cell line another survivin ΔEx3 dimer was observed, possibly heterodimer with the full-length survivin. This heterodimer had a weight of 32-33 kDa. It is known that survivin homodimerizes in solution. Previously, a model had been proposed in which survivin-2B or survivin ΔEx3 may homodimerize or heterodimerize with survivin to modulate the role of survivin in controlling mitosis and/or apoptosis.[39]

In the present work we propose that survivin ΔEx3 might be expressed not only in the nucleus, but in some cell lines also in the cytoplasm. Moreover, survivin ΔEx3 might be expressed in cancer cell lines, but also in benign tumors as monomer and as homo- and/or

heterodimer. However, we detected a protein band on the Western Blot, which in our opinion might be a heterodimer of survivin ΔEx3 with full-length survivin. This possible heterodimer was observed just in the nucleus of poorly-differentiated MIP-101 cell line. It would be interesting to know if survivin ΔEx3 and survivin-2B when dimerize with wild type survivin have an impact on the function of both the wild type survivin and its splice variants. The fact that survivin ΔEx3 was detected mainly in MIP-101 and DU-145 (low-differentiated cell lines) and the possible survivin ΔEx3/wild type survivin heterodimer only in MIP-101 cell line, suggests that their expression might be confined to poorly- and low differentiated neuroendocrine tumors. However, to prove that the expression of survivin ΔEx3 alone or as heterodimer with full-length survivin could be conversely correlated with the cell differentiation and possibly may be correlated with the tumor aggressiveness, further studies are needed. Another interesting alternative for future studies explaining all these new phenomena could be the invention of a siRNA specific to each splice variant.

5.3.2. Strategies to counteract the growth of GEP-NET cell lines using RNAi-based methods targeting survivin expression

In recent years considerable efforts have been made by researchers to develop strategies for modulating apoptosis in cancer and other human diseases.[38,109] In this context, approaches to counteract survivin in tumor cells have been proposed with the dual aim of inhibiting tumor growth through an increase in spontaneous apoptosis, and of enhancing tumor cell response to apoptosis-inducing agents (Chapter 1.2.5).

In the present study we demonstrated that it is possible to reduce markedly the expression of the cytoprotective factor survivin in human neuroendocrine cancer cells by survivin targeted siRNA. The survivin expression was downregulated in cell lines with relatively high expression of this protein – QGP-1, MIP-101 and DU-145. As in QGP-1 and DU-145 the survivin expression was downregulated 24 - 48 hours after transfection with siRNA, it took 72 hours to downregulate substantially survivin in MIP-101 cell line, possibly because of the high basic level of survivin expression. On the other hand, there was no effect of the siRNA transfection in BON cell line, a cell line originating from a

well-differentiated pancreatic tumor (WDET) although it had the same rate of transfection efficiency as all other cell lines used in this study. A possible reason could be that in this cell line survivin was expressed at relatively low levels. However, co-localization to the specific target site within cells might be the main hurdle that has to be overcome in such cell lines for an efficient inhibition of survivin gene expression. It is very tempting to contemplate that survivin expression might be downregulated by siRNA predominantly in cancer cells, as non-cancer cells (benign tumors and normal somatic cells) are spared. Another possible mechanism might be that there could be some protective factor of survivin expression (as p53), which does not functionate properly in cancer cells. Furthermore, survivin protein expression might function by a switch off-on mechanism, which means that even though the survivin RNA level was downregulated by the siRNA, it still did not reach the point where its expression could be switched off.
In our opinion siRNA provides a powerful tool for targeted inhibition of survivin gene expression.

5.3.3. Strategies to counteract the growth of GEP-NET cell lines and DU-145 prostate carcinoma cell line using aurora kinase-specific blockade

The aurora kinases, a family of mitotic regulators, have received much attention as potential targets for novel anticancer therapeutics. Previous studies found that aurora A and B were aberrantly expressed in a variety of solid tumors (Chapter 1.3.2 and 1.3.3). This is the first study that investigates aurora protein expression in GEP-NET cell lines and in the neuroendocrine prostate cancer DU-145 cell line. Here, we provide evidence that the aurora kinases A and B dual inhibitor ZM447439 may be a promising anticancer agent for GEP-NETs treatment alone and especially in combination with conventional cytotoxic agents. Furthermore, ZM447439 had an exceptionally good effect on the growth of the hormone resistant prostate cancer cell line DU-145.
In the GEP-NET cell lines studied, aurora kinases A and B was localized in the nucleus, in line with previous reports in other tumor types.[69,74] In the present study we raised the question whether the aurora kinase inhibitor ZM447439 exerted dominant effects on cell

division, spindle checkpoint control or cell viability. First, we tested whether exceeding concentrations of ZM447439 inhibited cell division. Cells expressing the aurora kinases exhibited normal cell-cycle profiles. By contrast, in the populations treated with ZM447439, the cell-cycle profiles differed widely, with the exception of MIP-101, where the effect was lower. All NET cell lines studied exhibited a prominent G2/M arrest, showing a large 4N peak (N stands for haploid number chromosomes) and in exceptional cases peak corresponding to cells with DNA contents > 4N. However, polyploidisation became apparent after 24 – 36 hours. Consistently, after 48 hours, the treated cells began to undergo apoptosis. The reason for this phenomenon may be because the ZM447439-treated cells exited mitosis without dividing and thus lost their viability.

Since aurora B is localized in the nucleus and is a chromosomal passenger complex member, the induction of caspases-3 and -7 by ZM447439 in BON and QGP-1 cells was surprising. The most plausible explanation is that the aurora kinase B deregulation causes polyploidity and chromosome instability, which in turn switch on the p53 gene, the major genome guardian, and cells undergo apoptosis. This idea seems reasonable since the recent discovery of the connection between aurora kinases and p53.[63,73] However, further studies with the p53 status in the present cell lines are warranted.

Even more intriguing were the results of the Western Blot analysis following aurora kinase B inhibition. In BON and QGP-1 cells, aurora kinase inhibition caused a decrease of its protein level, which coincided with simultaneous decrease of survivin protein level and induction of caspases-3 and -7. In DU-145 cells, the aurora kinase B inhibition caused increased protein expression followed by a survivin overexpression and very low levels of caspases-3 and -7 activation. MIP-101 was the only cell line where no change of survivin expression was observed, though aurora kinase B was overexpressed after ZM447439 incubation. Again, low activation of caspases-3 and -7 was detected. It seems that aurora kinase B could somehow influence positively the expression of survivin. Furthermore, survivin, but not aurora kinase B expression, might have a direct influence on the viability of the neuroendocrine cells, as in the cases where survivin was not downregulated, the down- or up-regulation of aurora kinase B had little effect on the caspases-3 and -7 activation. Nevertheless, it could not be excluded that ZM447439 exerts some unspecific effects in the apoptotic pathway. Alternatively, cell cycle arrest

and inhibition of cell proliferation may lead to apoptotic stimuli via uncertain signaling pathways.[37] In recent studies (presented on the AACR/EORTC Conference in San Francisco, 2007) ZM447439 was shown to induce polyploidisation (over 4N) and prominent apoptosis levels, however in these experiments the ZM447439 concentration was above 5 μM. In the present studies we incubated GEP-NET cell lines with ZM447439 concentrations not exceeding 5 μM as we know that excessive concentrations could exert an unspecific effect on other kinases (information provided by the manufacturer Tocris). Nevertheless, here we provide compelling evidence that aurora kinase B is the target of ZM447439 and that aurora kinases targeting might be a promising anti-cancer drug strategy. However, as was recently published (Feb. 2008) it seems that the newly synthesized aurora kinase inhibitor PHA-739358 has a substantially higher pro-apoptotic and antiproliferative effect on tumors cell lines[117] and will soon be investigated by our work group, too.

5.3.4. Strategies to counteract the growth of GEP-NET cell lines using aurora kinase blockade in combination with other conventional bio- and chemo- therapeutic drugs

Targeted therapies alone and in combination with cytotoxic therapy have recently been successfully introduced in the treatment of NET tumors (reviewed by Kulke et al[33]). In the present study we provide evidence that the highly specific dual aurora kinase A and B inhibitor ZM447439, in combination with octreotide, cisplatin or streptozocin potently inhibits the growth of MIP-101, BON and GEP-NET carcinoma cells. The main idea behind the present experiments was that some cytotoxic agents target the DNA molecule and thus drive the cells in G2/M phase, where the cells undergo DNA repair. It is known that aurora kinases A and B, together with survivin expression peak during this phase possibly taking part in this self-repair process.[44,45,72,74,110,111] Thus inhibiting aurora kinases A and B and/or survivin in this phase could drive the cancer cells to apoptosis. Sub-IC50 concentrations of ZM447439 enhanced the antiproliferative effect of cisplatin, doxorubicin or streptozocin alone. The most pronounced effect was observed in the QGP-1 cell line where the addition of ZM447439 streptozocin reduced the cell

growth by almost 90 %. With the addition of ZM447439 to doxorubicin the resistance to the latter was overcome. This is very interesting because the advanced WDEC disease, for which the QGP-1 cell line is proposed to be a cell model, is often resistant to aggressive protocols including streptozocin and doxorubicin. Here, with the addition of the new aurora kinase inhibitor ZM447439, a cytotoxic effect may be re-introduced.

The somatostatin analogue octreotide, which has poor antiproliferative effects alone, in combination with ZM447439 induced an inhibition of the cell growth in the BON cell line. SOM230, a new somatostatin analogue which engages five somatostatin receptors, did not exhibit such effects in the BON cell line. As expected, cisplatin had a strong growth inhibiting effect on MIP-101 cell line, proposed here as a model for PDEC tumors, but the addition of sub-IC50 concentrations of ZM447439 inhibited the cell growth even more profoundly. The present evidence might be helpful in practice, allowing reduction of cisplatin administration doses and thereby avoiding the side effects of this highly cytotoxic agent, but also of ZM447439, and in that way reducing the side effects of both drugs. Intriguing and unanticipated was the indication that the co-incubation of ZM447439 and 5-FU, a drug very commonly used in the treatment of gastroenteropancreatic and GEP-NET tumors, had an antagonizing effect on the cell growth in MIP-101 cell line. A possible explanation is the fact that 5-FU is a pyrimidine analogue, which inhibits the thymidilate synthase and thus RNA synthesis, but does not affect the DNA molecule. On the other hand ZM447439 induces a G2/M cell cycle block inhibiting the function of aurora kinases A and B, which on the other hand might take part in the DNA repair. It has still to be proven if the G2/M phase blockade induced by ZM447439 might enhance the function of the thymidilate synthase and thus diminish the effect of 5-FU. However, the DNA-intercalating agents - streptozocin, cisplatin and especially doxorubicin-induced a G2/M phase arrest[104,112], a phase during which the cells overexpress aurora kinases A and B in order to overcome the G2/M block, so the addition of ZM447439 had a profound additive to supra-additive effect on the tumor cell growth. The combination of sub-IC50 concentrations of ZM447439 and DNA-interacting cytotoxic agents could be extremely valuable in practice, allowing achievement of a good therapeutic effect at a low level of adverse reactions to either agent.

5.3.5. Expression of aurora kinase B and survivin after aurora kinase-specific blockade in GEP-NET cell lines

Aurora kinase B activity is essential for chromosome segregation and execution of cytokinesis. To fulfill these critical functions, the kinase needs to be in its active conformation at the right place at the right time. It is clear that activity and localization of aurora B is tightly controlled by its interaction partner survivin.[44,113] Suppression of aurora B activity by addition of a small molecule inhibitor ZM447439 to the cells caused hyperstabilization of survivin at the inner centromeres. This was accompanied by a gradual loss of survivin from this location even in the presence of a proteasome inhibitor that prevents cells from entering anaphase. This finding suggests that aurora B activity is required for maintenance of proper survivin dynamics.[44,67]

There are so far no studies of the effect of ZM447439 on the expression of aurora kinase B and survivin in GEP-NETs. Blocking the function of aurora kinase B using ZM447439 led to its compensatory overexpression in MIP-101 and DU-145 cells, which was followed by survivin overexpression in DU-145 cells, but not in MIP-101 cells. In BON and QGP-1 cells, the aurora kinase B inhibition led to dose-dependent aurora kinase B protein degradation followed by survivin down-regulation in both cases. This means that ZM447439 may induce either overexpression or downregulation of aurora kinase B depending on the cell type. Intriguingly, aurora kinase B downregulation was almost always followed by survivin downregulation. It seems that one mechanism for dynamic regulation of survivin may be the modulation of aurora kinase B, which might be additionally modulated by the p53 status.[67] That could be one possible explanation why in MIP-101 cells, where aurora kinase B was upregulated, no survivin upregulation was observed. Another explanation may be a possible “switch on/off” mechanism of the interaction between survivin and aurora kinase B. However, further studies are needed.

5.4. Survivin as a predictive value in patients with a GEP-NET tumor

In the present study we established a method by which the level of survivin could be used as a predictive value in the treatment outcome by patients with GEP-NET tumors and hematological malignancies (HM). A recent similar study showed slightly elevated survivin levels in the urine and serum of breast cancer patients.[114] We used a "TiterZymeEIA human Total Survivin Kit" for the determination of survivin in serum from patients with GEP-NET tumors. Furthermore, we included also patients with other types of solid cancers and HM in order to compare the serum survivin levels in a heterogeneous group of malignancies and prepare a template for future studies.

We observed a statistically significant increase of the survivin protein levels in patients with HM and solid cancers compared to the healthy controls. However, there was a subgroup of patients from the HM/solid cancers group, in whom the survivin level was comparable to that in the healthy controls. Most of these patients were patients who had solid cancer disease with low tumor burden or had HM, but had recently received a cytoreductive therapy. However, there was a breast cancer patient who had very high survivin plasma levels. In the course of the following examinations we encountered multiple bone metastases in this patient. This is not surprising as it was recently proven that there could be a leukemic phase in the course of development of non-leukemic diseases, during which tumor cells could be detected in the systemic circulation. Interestingly, the patients who had AML and those with aggressive NHL had relatively high survivin plasma levels compared to the median of the whole HM/solid cancer group. Here again, a high tumor cell burden as well as a proposed high cell turnover has to be postulated in the blood of those patients (leukocytes: 100.000/µl and elevated LDH level (IPI Score over 3-4), respectively).

Furthermore, we detected an increase of the survivin protein level in the group of patients with GEP-NET tumors compared to the healthy controls. Overall, patients with stable GEP-NET disease had low survivin levels, except those who experienced a disease relapse or rapid progression. However, the difference in survivin plasma levels between healthy controls and patients with GEP-NETs did not reach statistical significance. A possible explanation might be the fact that most of the patients, from whom blood samples were obtained, had WDET and WDEC disease, tumors with low cell turnover, which means small survivin fractions have been released. It seems that the level of serum

survivin depends mainly on the tumor cell turnover and the tumor mass as in patients with aggressive lymphomas, as well as on extensive (metastatic) solid tumor disease have much higher levels of survivin expression than those with extensive, but stable disease. Interestingly, we observed that healthy controls who had recently suffered a virus infectious disease and a pregnant healthy woman had considerably elevated levels of survivin. This is not surprising as it is known that in pregnant women many anti-apoptotic factors are overexpressed – including Mcl-1[36] and possibly survivin. However, the origin of the serum survivin (maternal, fetal or placental) still needs to be ascertained and the present data verified.

The serum survivin level appeared, however, not to be a reliable marker for MRD (minimal residual disease). However, it might be used for risk stratification and evaluation of the possibility of a response to a given treatment since survivin is known to be a multidrug resistance factor. It will be interesting what kind of survivin levels would patients have with high cell turnover as the rapid growing malignancies: retinoblastoma, children's ALL (acute lymphoblastic leukemia) and SCLC (small cell lung cancer), the last one being a member of the NET family. In order to answer those questions, cut-off values for survivin using the "TiterZymeEIA human Total Survivin Kit" for patients with HMs, GEP-NETs or other solid tumors, have to be found. Moreover, to ascertain the predictive value of our results, follow-up studies of the patients already included and the gathering of material from a more significant number of patients are currently being conducted.

In the current study we also provide evidence that the serum survivin levels could be used as a marker of the disease activity in patients with solid tumors and hematological malignancies. Moreover, there might be a strong correlation between the level of survivin and the activity of the disease, presence of metastasis, the presence or absence of a disease remission, and chemotherapy response. For all that future extensive studies are needed.

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6. Conclusion

Treatment possibilities of GEP-NETs are still not satisfactory and novel therapeutic options are needed. Currently, only a few human cell line models for GEP-NET disease are available. The present study is based on experiments with BON and QGP-1 cell lines as models for WDET and WDEC disease, respectively. Moreover, on the basis of expression of neuroendocrine markers we propose that MIP-101 cell line could be used as a cell line model for PDEC disease.

In the current study we report that QGP-1 and MIP-101 cell lines were extremely sensitive to doxorubicin and SN-38 with IC50 values in the nanomolar range. We propose that SN-38 might be considered as a good option in the treatment of WDEC and PDEC disease, but further studies are needed. MIP-101 cells, which here are proposed as a model for PDEC disease, were moderately sensitive to 5-FU and cisplatin, but not to oxaliplatin. However, incubation of GEP-NET cell lines with streptozocin, oxaliplatin and etoposide induced growth inhibition at much higher concentrations. In BON cells, a model for WDET disease, the somatostatin analogue octreotide, induces only remarkable growth inhibitory effect, in combination with the aurora kinases blocking agent ZM447439.

In the current work we are suggesting a new approach in the treatment of GEP-NET disease – attacking the chromosome passenger complex members survivin and aurora kinase B. We proved that it is possible to markedly reduce the expression of the antiapoptotic factor survivin in human GEP-NET cancer cells by survivin targeted siRNA. On the other hand, ZM447439, a potent selective aurora kinase inhibitor, reduced the growth of GEP-NET cells in a time- and dose-dependent manner inducing cell cycle arrest in S - phase and accumulation of cells with 4N DNA content, accompanied by apoptosis. The DNA-intercalating agents, streptozocin, cisplatin and especially doxorubicin, induced a G2/M phase arrest and the addition of ZM447439 had a profound supra-additive effect on the tumor cell growth. The combination of sub-IC50 concentrations of ZM447439 and DNA-interacting cytotoxic agents could be extremely

valuable in practice, allowing achievement of a good therapeutic effect at a low level of adverse reactions to either agent.

In the current work we provide preliminary data of a pilot study that serum survivin levels could be used as a biomarker of disease activity in GEP-NET patients. Here, as well as in patients with solid tumors and hematological malignancies, we found pronounced differences in serum levels of survivin depending on the tumor burden and the probability of circulating tumor cells. However, extensive studies are needed in the future.

7. References

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8. Abbreviations

ADP	Adenosine diphosphate
AK	Aurora Kinase
AKI	Aurora Kinase Inhibitor
APC	Adenomatous polyposis coli
ATCC	American Type Cell Collection
ATP	Adenosine triphosphate
BIR	Baculovirus IAP Repeat
BSA	Bovine serum albumin
°C	Celsius grades
CDK	Cyclin- deppendent kinases
CgA	Chromogranin A
cDNA	Complementary DNA
CO2	Carbone dioxide
conc.	Concentration
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulphoxide
dsRNA	Double-stranded RNA
ΔEx3	Delta Exon3
EIA	Enzyme immunometric assay
ETOH	Ethanol
FACS	Fluorescence associated cell sorter
FCS	Fetal Bovine Serum
FITC	Fluorescin Isothycyanante
5-FU	Fluorouracil
H	Hour
GEP-NETs	Gastroenteropancreatic neuroendocrine tumors
GI	Gastrointestinal
HBSS	Hanks' Balanced Salt Solution
HM	Haematological malignancies
IAPs	Inhibitor of Apoptosis Proteins
IgG	Immunoglobulin G
INCENP	Innercentromere protein
Kb	Kilobases
kDa	KiloDaltons
µg	Microgramm

Min	Minute
Ml	Milliter
µl	Microliter
mM	Millimol
µmol	Micromol
MW	Molecular weight
mRNA	Messenger Ribonucleic Acid
NET	Neuroendocrine tumor
Nm	Nanomol
NSCLC	Non-small cell lung cancer
NSE	Neuron -specific enolase
PBS	Phosphate Buffered Saline
PDECs	Poorly-differentiated neuroendocrine cancer
PE	Phycoerytin
PEI	Percutane ethanol instilation
PETs	Pancreatic endocrine tumors
PI	Propidium Iodide
PMSF	Phenylmethylsulfonyl fluoride
PRRT	Peptide radioreceptor therapy
PVDF	Polyvinylidene fluoride
RFTA	Radio frequent ablation
SDS	Sodium dodecyl sulfate
S.E.M.	standart error of the mean
siRNA	small interfering RNA
TACE	Transarterial chemoembolization
Sec	Seconds
U	Units
V	Volts
VIPoma	Vasoactive intestinal peptide
W	Watt
WDET	Well-differentiated neuroendocrine tumor
WDEC	Well-differentiated neuroendocrine cancer
Z	The p-value is obtained by the Wilcoxon rank test
ZM	ZM447439
*	Indicates significance at level $p < 0.05$

9. Summary

Gastro**e**ntero**p**ancreatic **n**euro**e**ndocrine **t**umors (GEP-NETs) are poorly understood lesions that encompass a broad category of neoplasms derived from neuroendocrine cells of the gastrointestinal mucosa and the pancreas. The lesions are grouped based on the acceptance that they have a common cell lineage and produce similar secretory products. Although the overall 5-year survival rate for all carcinoids is 67.2 %, most of the undifferentiated neuroendocrine tumors are presented with evidence of extensive disease and less than 8 % are alive after 2 years. Treatment possibilities of GEP-NETs are still not satisfactory and novel therapeutic options are much needed.

The present study is based on experiments with BON and QGP-1 cell lines presented as cell models for **w**ell-**d**ifferentiated neuro**e**ndocrine **t**umor (WDET) and **w**ell-**d**ifferentiated neuro**e**ndocrine **c**ancer (WDEC) disease, respectively. Moreover, on the basis of expression of neuroendocrine markers we propose that the MIP-101 cell line could be used as a cell model for **p**oorly-**d**ifferentiated neuro**e**ndocrine **c**ancer (PDEC) disease.

In the current study we report that the somatostatin analogue octreotide could induce growth inhibitory effects, when used in combination with the aurora kinases blocking agent ZM447439. QGP-1 and MIP-101 cell lines were extremely sensitive to doxorubicin and SN-38 with IC50 values in the nanomolar range. We propose that SN-38 might be considered as a very good option in the treatment of aggressive WDEC and PDEC disease. MIP-101 cells were moderate sensitive to 5-FU and cisplatin, but not to oxaliplatin. However, incubation of GEP-NET cell lines with streptozocin, oxaliplatin and etoposid induced growth inhibition at µM-mM concentrations.

In the present work we are suggesting a basically new approach in the treatment of GEP-NET disease – targeting the Chromosome Passenger Complex members survivin and aurora kinase B. We proved for the first time that the survivin main splice variants are expressed in the GEP-NET cells compartments in a unique manner. We showed that it is

possible to reduce markedly the expression of the cytoprotective factor survivin in human GEP-NET cancer cells by survivin targeted siRNAs.

ZM447439, a potent selective aurora kinases inhibitor reduced the growth of GEP-NET cell lines in a time- and dose-dependent manner inducing cell cycle arrest and accumulation of cells with 4N DNA content. The addition of ZM447439 to the DNA-intercalating agents streptozocin, cisplatin and especially doxorubicin, had a profound supra-additive effect to the inhibition of tumor cell growth. The combination of sub-IC50 concentrations of ZM447439 and DNA-intercalating cytotoxic agents could be extremely valuable in the practice, allowing achievement of a good therapeutic effect at a low level of adverse reactions to either agent. ZM447439 induced also overexpression or downregulation of aurora kinase B depending on the cell type. Intriguingly, aurora kinase B downregulation was almost always followed by survivin downregulation and the modulation of aurora kinase B seems to be a possible mechanism for dynamic regulation of survivin.

In the current work we also provide preliminary data of a pilot study that serum survivin levels could be used as a biomarker of disease activity in GEP-NET patients. Here, as well as in patients with solid tumors and hematological malignancies, we found pronounced differences in serum levels of survivin depending on the tumor burden and the probability of circulating tumor cells. However, extensive studies are needed in the future.

10. Zusammenfassung

Gastroenteropankreatische neuroendokrine Tumoren (GEP-NETs) bilden eine sehr heterogene Gruppe von Neoplasien, die aus neuroendokrinen Zellen der Mukosa des Gastrointestinaltraktes und des Pankreas hervorgehen. Diese Läsionen werden eingeteilt nach Lokalisation (Vorderdarm, Mitteldarm, Hinterdarm), Differenzierung, Funktionalität und Größe; alle Faktoren zusammen bestimmen das Malignitätspotential der Tumore. Obwohl die 5-Jahres-Überlebensrate bei gut differenzierten neuroendokrinen Karzinomen 67.2 % beträgt, ist der einzelne Krankheitsverlauf nur schwer vorhersagbar, und die therapeutischen Möglichkeiten für GEP-NETs sind begrenzt. Hier sind dringend bessere und für den einzelnen Patienten passgenauere Therapien notwendig.

Diese Arbeit basiert auf Experimenten mit BON – und QGP-1 Zellkulturen, die ein Modell für gut differenzierte neuroendokrine Tumore (WDET) bzw. für gut differenzierte neuroendokrine Karzinome (WDEC) darstellen. Wir schlagen auf der Basis des Nachweises typischer neuroendokriner Marker die Zelllinie MIP-101 als Modell für ein schlecht differenziertes neuroendokrines Karzinom (PDEC) vor.

In dieser Arbeit untersuchen wir zunächst den Einfluss verschiedener in der Klinik verwendeter Bio- und Chemotherapeutika auf das Wachstum der o.g. Tumorzellinien und die zugrunde liegenden funktionellen Mechanismen wie z.B. den Einfluss auf die Apoptose und Zellzyklus-Regulation. Z.B. sind QGP-1 und MIP 101 Zelllinien extrem sensitiv gegenüber Doxorubicin und SN-38 mit IC50- Werten im nanomolaren Bereich. Wir schlagen daher vor, dass SN-38 (bzw. Irinotecan) eine sehr gute Behandlungsoption bei aggressiven WDEC und PDEC darstellen könnte. Die MIP-101 Zellen sind zudem gegenüber 5-FU und Cispaltin empfindlich, aber nicht gegenüber Oxaliplatin.

Aufgrund unserer Vorarbeiten zur prognostischen Bedeutung des anti-apoptotisch wirkenden und mitose-fördernden Proteins Survivin bei GEP-NET Patienten interessiert uns in dieser Arbeit sowohl die funktionelle Bedeutung von Survivin als auch seines Partner-Proteins im sog. „Chromosomalen Passenger Komplex“, Aurora Kinase B. Hier können wir nun erste Ergebnisse zur funktionellen Herunter-Regulation sowohl von

Survivin (durch spezifische siRNA) bei den o.g. Zelllinien als auch zur pharmakologischen Inhibition von Aurora Kinase B durch die Substanz ZM447439 präsentieren. ZM447439 reduziert das Wachstum von GEP-NET-Zelllinien zeit- und dosisabhängig durch Induktion von Zellzyklusarrest und Akkumulation von Zellen mit 4N DNA-Gehalt. Zusätzlich vermittelt ZM447438 in Kombination mit bereits bekannten Bio- und Chemotherapeutika supra-additive Effekte. So können wir zeigen, dass das Somatostatin-Analogon Octreotid in Kombination mit ZM447439 tatsächlich gering antiproliferativ in BON Zellen wirken kann. Die Gabe von ZM447439 zu den DNA-interkalierenden Agenzien Streptozocin, Cisplatin und besonders Doxorubicin zeigt einen besonders ausgeprägten supra-additiven Effekt auf das Wachstum von QGP-1 und MIP-101 Tumorzellen. Die Kombination von sub-IC50 Konzentrationen dieser Kombinationen könnte in der Praxis extrem wertvoll sein durch gute therapeutische Effekte bei weniger Nebenwirkungen.

In unserer Arbeit bieten wir auch erste Daten einer Pilotstudie an, die zeigen, dass die Konzentration von Survivin im Serum als Biomarker für die Erkrankungsaktivität bei GEP-NET Patienten dienen kann. Hier, genau wie bei hämatologischen und soliden malignen Tumoren, sehen wir ausgeprägte Unterschiede in der Serumkonzentration von Survivin abhängig von der Tumorlast und wahrscheinlich zirkulierenden Tumorzellen. Diese präliminären Ergebnisse sollten in einer klinischen Studie weiter untersucht werden.

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13. Erklärung

Berlin, den 3 December 2008

Ich **Inna Georgieva**, erkläre hiermit, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Functional assessment of gastroenteropancreatic neuroendocrine tumor's (GEP-NET) sensitivity to bio- and chemotherapeutic drugs and the role of survivin and aurora kinase B expression in GEP-NET disease", selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Unterschrift: