Sensitization of pancreatic carcinoma cells for chemotherapy-induced apoptosis using small-molecule inhibitors of X-linked inhibitor of apoptosis protein (XIAP)

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Table of contents

Abbreviations .................................................................................................................. V

1. Introduction .................................................................................................................. 7
   1.1. Cancer - A definition ............................................................................................. 7
   1.2. Pancreatic cancer .................................................................................................. 9
      1.2.1. Definition ......................................................................................................... 9
      1.2.2. Epidemiology .................................................................................................. 9
      1.2.3. Etiology and pathogenesis .............................................................................. 10
      1.2.4. Symptoms ....................................................................................................... 13
      1.2.5. Diagnostics ..................................................................................................... 13
      1.2.6. Therapy ........................................................................................................... 14
      1.2.7. Prognosis ....................................................................................................... 15
   1.3. Apoptosis ............................................................................................................... 15
   1.4. Proteins involved in process of apoptosis .............................................................. 18
      1.4.1. Caspases ......................................................................................................... 18
      1.4.2. Inhibitor of apoptosis proteins (IAPs) ............................................................ 19
      1.4.3. Bcl-2 protein family ....................................................................................... 20
      1.4.4. The endogenous IAP-antagonist SMAC ....................................................... 21
   1.5. X-linked inhibitor of apoptosis protein (XIAP) ....................................................... 22
   1.6. SMAC-mimicking IAP-antagonizing molecules ................................................... 24
   1.7. Aim of the study .................................................................................................... 26

2. Materials and Methods ............................................................................................... 27
   2.1. Materials ............................................................................................................... 27
      2.1.1. Cell lines ......................................................................................................... 27
      2.1.2. Solutions ......................................................................................................... 27
      2.1.3. Substances ...................................................................................................... 28
         2.1.3.1. Cytostatics ............................................................................................... 28
         2.1.3.2. XIAP antagonists ................................................................................. 28
         2.1.3.3. Caspase Inhibitor ................................................................................ 29
      2.1.4. Equipment ...................................................................................................... 29
   2.2. Methods ................................................................................................................ 30
      2.2.1. Cell culture ..................................................................................................... 30
2.2.2. Seeding of cells .......................................................................................... 30
2.2.3. Treatment of pancreatic cancer cell lines ............................................. 30
2.2.4. Examination for cell viability: MTT viability assay ............................... 31
2.2.5. Determination of the role of caspases with pan-caspase inhibitor ........ 31
2.2.6. Examination for apoptotic cell death: Analysis of DNA fragmentation ...... 32
2.3. Statistics ...................................................................................................... 32

3. Results .......................................................................................................... 33

3.1. Small-molecule XIAP inhibitors lead to an enhanced cytotoxicity induced by chemotherapeutic agents only in few pancreatic carcinoma cell lines ............. 33
3.2. Small-molecule XIAP inhibitors lead to a caspase-dependent cell death in pancreatic cancer cell lines .................................................................................. 47
3.3. Elucidating the mode of cell death with evidence of DNA fragmentation ...... 50

4. Discussion ...................................................................................................... 57

4.1. Drug resistance in pancreatic ductal adenocarcinoma ............................... 61
4.2. Suppression of XIAP with SMAC mimetics seems to be effective only in few selected pancreatic carcinoma cells treated with cytostatics ......................... 66
4.3. Involvement of caspases in drug-induced cell death in PDAC .................. 72
4.4. Drug-induced cell death in XIAP-inhibited PDAC cell lines .................... 74
4.5. Perspectives for future therapy of pancreatic ductal adenocarcinoma ......... 76

5. Summary ........................................................................................................ 79

6. References ..................................................................................................... 80

7. Acknowledgment ............................................................................................ 94

8. Curriculum vitae ............................................................................................ 95
Abbreviations

- 18-FDG-PET: 18 - Fluorodeoxyglucose positron emission tomography
- Akt: Serine / threonine protein kinase B (PKB)
- ALL: Acute lymphocytic leukemia
- ALK: Anaplastic lymphoma kinase
- AML: Acute myeloid leukemia
- ARTS: Apoptosis-related protein in the TGFβ-signaling pathway
- Bak: Bcl-2 antagonist killer 1
- Bax: Bcl-2 associated x protein
- Bcl-2: B-cell CLL / lymphoma-2;
- Bcl-XL: B-cell lymphoma-extra large
- Bcr-abl: Breakpoint cluster region-abelson
- BIR: Baculoviral IAP repeat
- BIRC: Baculoviral IAP repeat containing
- BMI: Body mass index
- BRCA1 / 2: Breast Cancer gene 1 / -2
- CA 19-9: Carbohydrate antigen 19-9
- CDKN2A: Cyclin-dependent kinase inhibitor 2A
- CLL: Chronic lymphocytic leukemia
- CML: Chronic myeloid leukemia
- CT: Computer tomography
- CXCR4: Chemokine (C-X-C motif) receptor 4
- DIABLO: Direct inhibitor of apoptosis-binding protein with low-pl
- DMSO: Dimethyl sulfoxide
- DNA: Desoxyribonucleotide acid
- EGFR: Epidermal growth factor receptor
- ER: Estrogen receptor
- FACS: Fluorescence-activated cell sorting
- FADD: Fas-associated protein with death domain
- FCS: Fetal calf serum
- FOLFIRINOX: Combination of 5-FU, leucovorin, irinotecan and oxaliplatin
- HENT1: Human equilibrative nucleoside transporter-1
- HER2/neu: Human epidermal growth factor receptor-2
- Hid: Head-involution defective
- HTRA2: High temperature requirement protein A2
- Grim: Cell-death gene in Drosophila melanogaster
- IAP: Inhibitor of Apoptosis Protein
- KRAS: Kirsten rat sarcoma
- Mcl-1: Myeloid cell leukemia 1
- MLH1: MutL-homolog 1
- ML-IAP: Melanoma IAP
- MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium-bromid
- NAIP: Neuronal apoptosis inhibitory protein
- NF-kB: Nuclear factor kB
- NSCLC: Non-small cell lung cancer
- OMI: Inhibitor of apoptosis protein in Drosophila melanogaster
- p16: Tumor suppressor protein
- p53: Tumor suppressor gene / - product
- PDAC: Pancreatic ductal adenocarcinoma
- PI3K: Phosphatidylinositol 3-kinase
- Puma: P53-upregulated modulator of apoptosis
- PTEN: Phosphatase and tensin homolog
- RAS: Rat sarcoma
- Reaper: Cell-death regulating gene in Drosophila melanogaster
- RING: Really interesting new gene
- RRM1: Ribonucleotide reductase M1
- SCLC: Small-cell lung cancer
- SEPT4: Septin 4 gene
- SMAC: Second mitochondria-derived activator of caspase
- Src: Oncoprotein ‘Sarcoma’
- TNF-α: Tumor necrosis factor α
- TRAIL: Tumor necrosis factor-related apoptosis inducing ligand
- UBA: Ubiquitin-associated domain
- Wnt: Gene family comprising 19 genes
- XAF1: XIAP-associated factor 1
- XIAP: X-linked inhibitor of apoptosis protein
1. Introduction

1.1. Cancer - A definition

The term “cancer” was coined by Hippocrates 400 years before Christ for the first time, referring to a disease, which presented macroscopically with features of a crab (in Greek karkinoma) engraved in the sand: Characterizing unknown illnesses, he described solid formations (‘the body of the crab’) entrenched in human flesh[181].

More than hundred years ago, von Hansemann and Boveri detected for the first time microscopically chromosomal abnormalities in cancer cells[17, 70]. Nowadays, we know that the disturbance of chromosomal integrity is a result of the acquisition of a variable amount of mutations generally accumulating over a longer period of time that drive healthy cells into tumorous ones[179]. But not all of the acquired mutations present in a cell contribute to formation of tumors, those, which do, are called “driver mutations” providing the corresponding cell with advantages in respect to growth and proliferation compared to the surrounding tissue, analogue to Darwin it is alike “proliferation of the fittest” [180].

Two main kinds of genes have to be highlighted talking about cancer: oncogenes and tumor suppressor genes. P53 is maybe the most known tumor suppressor gene, it encodes for a protein that is embedded in an intracellular network preventing cell proliferation by arresting cell cycle, if certain defects are recognized[19]. A loss-of-function mutation within a tumor suppressor gene inactivates its tumor suppressing potency[97]. On the other hand, there are genes, which have tasks in regulation of growth, differentiation and proliferation of normal cells: Mutations within these genes can result in a permanent activation of the now called oncogenes that considerably contribute to tumor formation[97]. KRAS of the ras gene family is one of the most common oncogenes found in tumors[106].

Based upon these genetic changes, Hanahan and Weinberg postulated different “hallmarks of cancer” that describe the biology of tumors[69]:

In the first place, a constitutively activated proliferative signaling, that is, cancer cells either change intracellular response to growth signals, increase response to
limited signaling or take care that there is more than enough of various growth signals (autocrine stimulation). Second hallmark is the ability to circumvent orders, often mediated by products of tumor suppressor genes, with intention to suppress (normally unwanted) growth. To the third hallmark are we going to dedicate us in this thesis, it is resistance to cell death and fourth is to establish immortality by preventing telomeres from being shortened in the end of each replicative cycle. The fifth trait distinguishing cancer from normal cells is induction of angiogenesis in order to keep up a sufficient nutritive and oxygen supply independently of tissue homeostasis and balance. The sixth hallmark stated is opposing contact inhibition what can be regarded as gain of capability to leave native tissue and settle down in other organs or invade surrounding structures[69].

![The Hallmarks of Cancer](image)

**Figure 1  The Hallmarks of Cancer**

The transformation of a normal into a cancerous cell is amongst others the result of a change in a variable number of genes and consecutively a set of differing cellular proteins providing the cancer cell with certain survival and proliferation advantages. In 2011, these six principles were complemented by two further hallmarks (influencing energy metabolism and evading destruction by the immune system) and form the basis of tumorigenesis, disease progression and resistance to therapeutic efforts. Reprinted from Cell, 144, Hanahan, Douglas, Weinberg, Robert A., Hallmarks of cancer: the next generation, 647-674, Copyright © 2011, with permission from Elsevier.
In 2011, the authors widened the six capabilities firstly written down in 2000 by two further characteristics for cancer cells: Escape from immunosurveillance and as second, from Hanahan and Weinberg named “additional emerging hallmark”, the ability to alter energy metabolism favoring aerobic glycolysis [69].

1.2. Pancreatic cancer

1.2.1. Definition

Pancreatic cancer is a malignancy that occurs with a vast majority in the exocrine part of the organ, more exactly in the epithelium of the ducts, whereas neoplasms only rarely originate in cells with endocrine function[74]. Based upon this histological definition, the term ‘pancreatic carcinoma’ or ‘pancreatic ductal adenocarcinoma’ (PDAC) is more exact because it alludes to the underlying tissue.

As a result of a rather aggressive growth, pancreatic cancer is in most cases only diagnosed at a very advanced state and within the last 4 decades, only little therapeutic improvements concerning survival could be achieved[186].

1.2.2. Epidemiology

Pancreatic cancer is one of the tumors with the poorest prognosis: Worldwide more than 200,000 people die of it every year[40], about 37,000 in the United States in 2010, where it is the 4\textsuperscript{th} most common cause of cancer related death[87, 186], although it is only the 10\textsuperscript{th} most frequent diagnosed type of cancer[87]. The incidence rate is approximately 1 in 10,000 people[99] and mortality rates do not show a great difference[40], because 80-85% of all patients present in an inoperable state[73]: After diagnosis median survival time is 5-8 months and the overall 5-year survival rate is below 5%[82]. These numbers hardly changed within the last decades for pancreatic malignancies, while there was a marked improvement with respect to therapeutic efficacy in most other tumors[73].
The median age of patients at time of diagnosis is 71 years\cite{85}, men suffer more often from pancreatic cancer than women (ratio 1,5 : 1)\cite{99}, and among the black population there is a higher incidence rate compared to white people\cite{7}.

### 1.2.3. Etiology and pathogenesis

Many risk factors and conditions have been suggested to contribute to pancreatic cancer, some are accepted as indisputable like tobacco smoking, which accounts for up to 20% of PDAC and apart from that, in specimen from smokers more genetic defects can be detected compared to non-smokers\cite{13}. Another potentially avoidable risk factor is high-energy uptake resulting in a high BMI\cite{4}.

But there are also factors that cannot be influenced, like age or genetic lesions and increased susceptibility due to inherited diseases as well as first-degree relatives suffering from PDAC\cite{155}.

In addition, many lifestyle-associated diseases, like diabetes mellitus or chronic pancreatitis (because of excessive alcohol consumption) lead to an increased risk\cite{4, 156}. Remarkably, studies could not show an association between alcohol consumption itself or sugar uptake and the development of pancreatic cancer\cite{71, 116}. Some authors also put into question, if diabetes or inflammation of the pancreas are reason for or a complication of PDAC\cite{4}.

There are inconsistent results concerning effect of dietary composition, for example if red meat or caffeine promote tumorigenesis of the pancreas or in how far vegetables and fruits have the potential to prevent it and which of them show the highest potency\cite{4}.

Inflammation seems to play an important role for tumor initiation: Many different pathological events finally lead to activation of different kinds of immune cells, which in turn act via cytokines like TNF-α, interleukines and cyclooxygenase-2, to provoke an inflammatory milieu\cite{120} resulting in the prolonged production of potentially DNA-damaging free radicals\cite{80}. In recent times, inflammative processes have been considered to be decisive for the carcinogenic potential of smoking or diseases eventually leading to pancreatic cancer, like obesity, diabetes mellitus or chronic pancreatitis\cite{82}. 


Solid cancers show a plethora of mutations that form the basis of tumor development: Genetic profiling disclosed several thousands of mutated genes to be present in cancerous cells, though not each mutation bears the potential to transform normal into tumor cells[171]. Recent research postulated that mutations of driver genes are most decisive, leading to advantages in respect to growth and proliferation, which are characteristic for cancer[180]. Authors claim, in some tumors only two to eight mutated driver genes are required for transformation into a malignant cell[169].

Like the progression of colorectal cancer from precancerous adenomas[179] pancreatic cancer also emerges from precursor lesions, so called ‘pancreatic intraepithelial neoplasias’ (PanINs) and ‘intraductal papillary mucinous neoplasm’ (IPMNs) to an invasive growing tumor, because PanINs and IPMNs already have undergone genetic changes that can be seen in invasive neoplasms, too[18, 191]. These changes can affect oncogenes, which are activated, or tumor suppressor genes, that are inactivated in the process of tumorigenesis and furthermore, there are many defective signaling pathways in pancreatic cancer leading to uncontrolled growth, invasiveness and metastasis[82].

In respect to oncogenes, the ras oncogene is detected in more than 90% of pancreatic cancers: In no other human tumor KRAS expression levels are as high as in this malignancy[3].

Important inactivated tumor suppressor genes are for example p53, PTEN, p16, BRCA2 or SMAD4[82].

As mentioned above, apart from a surplus of extracellular stimuli intracellular signaling is deregulated in pathways that are important for maintenance of physiological functions and regulation of apoptosis in normal cells, for instance EGFR[164] and Hedgehog[167] signaling as well as Notch-, cyclooxygenase-2-, SRC-, PI3K-, CXCR4-, Wnt- and Insulin-like growth factor-I signaling or Akt-[145] and NF-κB pathway amongst others[82].

Another reason for defective genes and their expression form epigenetic variations: Those genes are not mutated or deleted but have undergone modifications like genomic methylation[142] or histone-(de-) acetylation[133]. These alterations lead to a loss of function (‘silencing’) of the corresponding gene or an overexpression, respectively[27, 198].
Moreover, it has been suggested that more than 10% of individuals with pancreatic cancer have a hereditary predisposition[67], so that people with a family history of pancreatic cancer are considered as higher risk in respect to cancer development as well as people suffering from one of the following diseases:

A 53-fold risk for contracting pancreatic cancer is described for patients with hereditary pancreatitis: Inherited mutations afflicting the cationic trypsinogen gene PRSS1 result in defective trypsinogen, which causes inflammation, sometimes already in children[32]. The risk in families with familial atypical multiple mole melanoma syndrome (FAMMM) is 15-38 times higher as in the normal population because of defects in tumor suppressor gene p16 or CDKN2A[67].

People with hereditary ovarian and breast cancers as a result of mutated BRCA1 and BRCA2 share an increased risk and some authors even propose that the rate of BRCA2 mutations in cases of PDAC, which are supposed to be sporadic, is almost as high as in breast or ovarian cancer[58]. Malfunctioning BRCA1 is associated with a relative risk of 2 to 3 for PDAC development[168], with defective BRCA2 it is even double as high[173].

Hereditary nonpolyposis colorectal cancers (HNPCC) syndrome due to failure in DNA mismatch repair genes (most often MLH1) occurs in 2 forms of which the second one shows a higher risk for PDAC, too[93].

Probably the highest relative risk (132-fold) is associated with the Peutz-Jeghers syndrome bearing a mutation of STK11[56].

Only recently Tomasetti & Vogelstein published new aspects of tumorigenesis: They stated two thirds of all cancers emerge as a result of random mutations in the course of DNA replication of stem cells, which did not show cancerous traits so far, assuming that environmental factors or inherited conditions may not play that major role[170].
1.2.4. Symptoms

Pancreatic cancer generally grows without provoking any symptoms for a long period of time. The first disorders that eventually lead to diagnosis are jaundice, loss of weight, abdominal and back pain; in some cases, a newly diagnosed diabetes mellitus or a pancreatitis can indicate the presence of a pancreatic tumor[94, 192].

Apart from that, paraneoplastic thrombosis, especially if occurring recurrently, may be a sign of this neoplasm, too[72].

It is to be said that as soon as it presents with symptoms, it is already in an advanced stage and in most cases not resectable anymore[10].

1.2.5. Diagnostics

As symptoms of pancreatic cancer are unspecific, the first procedure in diagnostics generally is the abdominal ultrasound[117]. The sensitivity in detecting pancreatic cancer is highly dependent on the experience of the examining doctor: A sensitivity of up to 97% is achieved by multislice CT, MRI is not used regularly in the diagnostic process, it might be taken into consideration in case of unspecific or small findings[117]. Finally, endoscopic ultrasound (EUS) offers the possibility for further imaging and staging in respect to lymph node status and to perform fine-needle aspiration for histological specimen[117]. 18-FDG-PET can be seen as a supplementary device, if there is still lack of clarity after the methods specified above[148].

In clinical practice, CA 19-9 is the only tumor marker used in the diagnostic process of PDAC, however, its very low positive predictive value make it an inappropriate screening tool for asymptomatic patients[95] as well as for people at a higher risk for PDAC[32]. The relatively low sensitivity (approximately 80%) and specificity (it ranges from 80-90%[9]) limit its use in the detection of early stages. Several benign diseases or states, like biliary obstruction, as well as other adenocarcinomas, can increase CA 19-9 and thus give false positive results [60]. False negative results can occur in Lewis-negative patients (5-10% of the population) because CA 19-9 is connected to the presence of Lewis blood group antigen[91].
1.2.6. Therapy

Therapy of PDAC is quite difficult because conventional therapies showed deceiving results in the past.

Up to now, the only potentially curative therapy for PDAC is a complete resection via pylorus-preserving pancreaticoduodenectomy (PPPD) for head cancers or left pancreatic resection with splenectomy for cancers of the tail of the organ[190]. In centers that perform many of these interventions, the mortality rate due to operation is less than 3 %[190]. But on the one hand, only 15-20% of newly diagnosed patients are resectable and on the other hand, long-term survival can be seen in only less than 3 % even when R0-resected: the reason therefore are occult metastases which are concealed for imaging methods used for staging[10].

PDAC is one of the most resistant malignancies in respect to conventional antitumor treatment, chemotherapy and radiation have not shown to be highly effective, the indications are limited on palliative and adjuvant settings[82].

In 1997, 5-FU was replaced by Gemcitabine monotherapy as chemotherapeutic agent of choice for first-line therapy of unresectable PDAC: the result was a longer survival with tolerable side effects[103]. However, within the last years of research, no remarkable improvements could have been achieved any more using systemic therapies, not even with drug combinations: the therapeutic regimen of gemcitabine with erlotinib only lead to a slight increase in survival and the rather toxic regimen with FOLFIRINOX is not appropriate for all patients[29]. A new approach was initiated introducing nab-paclitaxel in PDAC therapy: combined with gemcitabine, it significantly increased survival compared to gemcitabine alone[1].

In addition to surgery, chemoradiation is the treatment of choice in the United States after an operation, whereas in Europe gemcitabine monotherapy (or 5-FU) is recommended in adjuvant settings[186].

For neoadjuvant strategies there is not enough evidence so far, some studies suggest a downstaging of locally advanced tumors using chemoradiation in a selected population[57].

Radiotherapy alone showed to be inferior in respect to overall survival in locally advanced PDAC compared to chemoradiation, but it can be used to alleviate symptoms caused by local disease progression like pain or obstruction of the biliary duct[186].
1.2.7. Prognosis

Due to its late diagnosis and high resistance to chemotherapy and radiation, PDAC has been and still is a malignancy with one of the poorest prognosis and with the least improvement of survival rates within the last 3 to 4 decades: In 1975, the 5-year survival of all stages of PDAC was 3 % on average, at the beginning of the new millennium it still is merely 3 to 5 %[10, 87].

Median survival after diagnosis is 5 to 8 months, even when diagnosed at a local stage, about 80 of 100 patients die within the first 5 years following operation and adjuvant chemotherapy[87]: the benefit of adjuvant therapy is limited to 6 to 10 months, and it is to be said that there is no profit in long-term survival in R0-resected people[10].

All in all, not even 3 % of PDAC patients can be considered cured[10].

1.3. Apoptosis

In multicellular organisms tissue homeostasis is maintained by a genetically determined, tightly regulated and evolutionary highly conserved program of death and renewal of cells[46]. The most common form of programmed cell death is called apoptosis and its function is to eliminate damaged or aberrant cells upon different stimuli in a controlled manner without causing remarkable side effects like, for example, an inflammatory reaction[136]. It is, to some extent, like a cellular suicide and is characterized by morphological changes of the cellular structure like DNA fragmentation, condensation and shrinking of the nucleus, membrane blebbing to apoptotic bodies and loss of the cellular shape[48, 81, 136, 199]. Last step of an apoptotic cell is to present biochemical 'eat me signals' on the 'new' cell surface that can be recognized by phagocytes, which finally disintegrate the apoptotic bodies[81]. Of about $10^{14}$ cells in human organism, $10^9$ die daily from apoptosis[43].
There are two main pathways which are able to trigger apoptosis, an extrinsic and an intrinsic one: the extrinsic pathway starts with binding of corresponding ligands to the extracellular part of so called death receptors, whereas the initiation of the apoptotic program via intrinsic pathway is initiated by mitochondria[46]. It is known that there are crosstalks between the two pathways in order to amplify an apoptotic signal[81].

A crucial role play furthermore cysteine proteases called ‘caspases’, which are present in an inactive form in the cytosol[66]. Once stimulated via extrinsic or intrinsic pathway signaling, different caspases cleave target proteins in a cascade that eventually leads to the morphological changes characteristic for apoptosis[42].

The IAP (inhibitor of apoptosis protein) family and some of the Bcl-2 proteins function as counterpart of pro-apoptotic caspases and have important functions in apoptosis regulation; they are currently target of many potential novel therapies[43, 50].

Dysregulation of apoptosis leads to cellular imbalance and thus to pathological states, i.e. autoimmune and degenerative diseases and of course cancer, too[43]. On the other hand, apoptosis is also very important in respect to therapeutic anticancer strategies: most cytotoxic drugs and radiation therapy act via induction of apoptosis and treatment failure can be explained to a huge part by innate or acquired resistance of tumor cells to apoptosis[48].
Apoptosis is mediated via extrinsic or intrinsic cascades or pathways, respectively. Both converge to effector caspase-3, which triggers the morphological changes of cellular integrity by engagement of target substrates like caspase-activated DNase (CAD) that is bound by inhibitor of CAD (ICAD), whereupon fragmentation of the nucleus, chromatin condensation, membrane blebbing and rearrangement of the cytoskeleton emerge as traits of apoptotic cell death.

The extrinsic pathway starts with binding of corresponding ligands to death receptors and subsequent formation of death-inducing signaling complex (DISC) comprising FADD (Fas-associated protein with death domain) and pro-caspase-8. FLIP (FLICE-inhibitory protein) is able to inhibit generation of caspase-8.

If activated, caspase-8 can either directly cleave and activate (pro-) caspase-3 or crabwise by involvement of intrinsic cascade signaling via truncation of pro-apoptotic Bcl-2 (B-cell lymphoma-2) protein Bid to t-Bid (truncated Bid).

The intrinsic pathway furthermore can be initiated by DNA damaging impacts like chemotherapy, irradiation or reactive oxygen species (ROS) and culminates in pore formation of outer mitochondrial membrane releasing pro-apoptotic factors (like second mitochondria-derived activator of caspases (SMAC), cytochrome c and others) into the cytosol. This permeabilization of the outer mitochondrial membrane is tightly controlled by members of the Bcl-2 protein family with either pro- (Bax, Bak) or, like Bcl-XL (B-cell lymphoma-extra large) and Bcl-2, anti-apoptotic function.

Once liberated, SMAC blocks inhibitor of apoptosis proteins (IAPs), whereas cytochrome c forms, together with the apoptotic protease activating factor-1 (Apaf1) and pro-caspase-9, a complex called 'apoptosome', whose task is to activate initiator caspase-9, which itself cleaves and activates caspase-3.

There is also a caspase-independent apoptosis pathway mediated by apoptosis inducing factor (AIF) and endonuclease G (EndoG).

Reprinted from Front Cell Neurosci., 8, Marzban Hassan, Del Bigio Marc R., Alizadeh Javad, Ghavami Saeid, Zachariah Robby M., Rastegar Mojgan, Cellular commitment in the developing cerebellum, 2015; 8:450. Open Access publication distributed under CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.
1.4. Proteins involved in process of apoptosis

1.4.1. Caspases

Caspases, or more exactly “cysteine-aspartyl-specific proteases”, are key enzymes in the apoptotic process[81]; up to now, 12 human caspases have been described and they fulfill essential functions not only in apoptosis but also in inflammatory processes[43].

The caspase family comprises initiator (caspase-8, -9 and -10) and effector caspases (caspase-3, -6 and -7) that influence apoptosis, whereas caspase-1, -4 and -5 are mainly involved in inflammation[43].

Caspases are regularly present in the cytosol in an inactive state. Upon different stimuli the intrinsic pathway is initiated by permeabilization of the outer membrane of mitochondria[48]. From this point on, the decisive event for execution of apoptosis is the formation of the so called ‘apoptosome’, a complex of proteins previously released from the mitochondrial intermembrane space, which comprises and activates the pro-form of initiator caspase-9[46]. Active caspase-9 in turn cleaves and activates the effector caspases 3 and -7[197].

If the extrinsic pathway is initiated, caspase-8 gets activated at the DISC, the multiprotein complex of this signaling cascade, resulting again in activation of the effector caspases mentioned above[11].

The two pathways converge at effector caspase level and finally lead to chromatin condensation, shrinking of the nucleus, DNA fragmentation and degradation of proteins of the cytoskeleton so that cell morphology is completely abrogated and apoptosis takes place[81].

Caspases are often targeted and inhibited by, for example, viruses in order to extend cell survival, as they need cellular metabolism themselves for replication, indicating that during evolution, the apoptotic machinery has been a promising attacking point of many diseases including cancer as well[36].

The effects of caspases can be abolished either by removal from the cytosol via proteasomal degradation after ubiquitination or by inhibiting their enzymatic activity[36]. As we will see in the next section, most IAPs show ubiquitination capabilities underlining an evolutionary conservation of this mechanism whereas direct interaction to antagonize caspases is only described for XIAP[36, 154].
1.4.2. Inhibitor of apoptosis proteins (IAPs)

Apoptosis is regulated by many molecules with either pro- or anti-apoptotic effects[46]. Firstly discovered in baculoviruses as proteins with essential functions for viral replication and thus survival, the evolutionary significance of IAPs became evident after identification of similar molecules in various species like nematodes, flies, yeasts and of course humans about twenty years ago[140].

There are eight human IAPs, formerly known as BIRC1-8 (BIRC1 / NAIP, BIRC2 / c-IAP1, BIRC3 / c-IAP2, BIRC4 / XIAP, BIRC5 / survivin, BIRC6 / BIR-containing ubiquitin-conjugating BIR domain enzyme apollon, BIRC7 / ILP-2 and BIRC8 / ML-IAP, also known as ‘livin’) [50].

All share a characteristic feature: they possess at least one baculovirus IAP repeat (BIR) domain, which is crucial for interaction with other proteins, especially caspases[50]. Some IAPs also have a RING domain for ubiquitination and other binding sites like the CARD domain, whose function is not quite clear yet[34, 50].

It is known that IAPs do have functions others than regulation of apoptosis, too, for example, concerning protein degradation[43], cell division[140] and in other intracellular pathways[50].

In respect of apoptosis, XIAP is the best examined and most effective IAP, it is the only one to antagonize caspases directly by interaction of its BIR domains with corresponding structures of different effector and initiator caspases whereas other IAPs, like c-IAP1 and -2, exert their anti-apoptotic function rather indirectly via SMAC-binding or stimulating the expression of anti-apoptotic proteins[50].

Within the last years, c-IAPs were awarded further anti-apoptotic functions: they are supposed to suppress the formation of a pro-apoptotic TNF-α receptor-mediated signaling complex and to be involved in NF-kB signaling and thus contributing to pro-survival efforts[181].

Another IAP, survivin, has only one BIR domain, however, it is able to inhibit the effector caspases 3 and -7[151].

There is evidence that high levels of IAPs in cancer cells are associated with more aggressive tumors, higher resistance to therapies and shorter overall survival[50].

But, interestingly, IAPs can also have favorable effects in respect to the course of a malignant disease: Livin / ML-IAP, for example, is not present in most normal
cells, and in melanomas and renal cell cancer, high livin levels turned out to be prognostically favorable in some studies[68, 123].

Apart from some conflicting results of different studies, therapeutic targeting especially of XIAP seems to be a promising approach for future therapies[50].

1.4.3. Bcl-2 protein family

The Bcl-2 family is another group of key regulators[30], especially regarding the intrinsic pathway involving mitochondria: they determine the extent of permeabilization of the outer mitochondrial membrane (OMM) and thus regulate the initiation of apoptosis mainly via the intrinsic pathway[24].

There are many anti-apoptotic as well as pro-apoptotic members which act within their family binding and influencing each other and thus adjusting the threshold for apoptosis[30, 137]. Similar to IAPs, the Bcl-2 proteins share a chemical structure that is identifying for these molecules: the Bcl-2 homology (BH) domains (BH1 to BH4) that are necessary for protein-protein interactions within the Bcl-2 family[137].

The anti-apoptotic Bcl-2 members like Bcl-2, Bcl-XL and Mcl-1 possess four BH domains of which the region between BH1-3 is decisive for binding of BH3 domains of BH3-only pro-apoptotic proteins[6]. It is the outer mitochondrial membrane where the anti-apoptotic members are located and exert their effects: upon different forms of intracellular stress[30, 137] they try to prevent membrane permeabilization triggered by pro-apoptotic members of their family by binding and inhibiting them[6].

The pro-apoptotic Bcl-2 family members can be subdivided in two functionally different groups: first, the BH3-only proteins like Bad, Bid, Noxa, Puma amongst others and the multidomain group which contains Bak and Bax, a kind of “effector proteins”, which also possess the BH domains one to four[6].

Bax and Bak need to be activated by BH3-only proteins in order to be able to form pores in the OMM[6]. The precise underlying molecular events have not been fully elucidated yet, it is either a direct activation of these effector molecules or a rather indirect by binding to anti-apoptotic members with the result of a higher
concentration of Bax and Bak, respectively[46]. One could argue that BH3-only proteins are in the center of the Bcl-2 family as they inhibit the anti-apoptotic members and lead to activation of the pro-apoptotic effector molecules Bak and Bax, which in turn are responsible for release of pro-apoptotic factors from mitochondria[6]. In malignancies, there is often a disturbed balance of pro- and anti-apoptotic Bcl-2 proteins and it is a further therapeutic approach to antagonize the anti-apoptotic members using small molecules or antisense oligonucleotides to especially inhibit Bcl-XL, which is expressed very strongly in many cancers, for example pancreatic cancer, too[24].

1.4.4. The endogenous IAP antagonist SMAC

In the late 1990s, Vucic et al. showed that there are certain proteins in Drosophila melanogaster, which are able to block IAPs[184, 185]. Based on these findings, murine (DIABLO) and human IAP antagonists (like second mitochondria-derived activator of caspases, in short ‘SMAC’, HTRA2 (OMI), ARTS (SEPT4) and XAF1) were discovered and their chemical structure as well as those of their target proteins have been described[23, 35, 178].

In non-apoptotic cells, SMAC is located within the mitochondria as a dimeric precursor protein of 239 amino acids[181]. After cellular stress, it gets cleaved at position 55 resulting in a liberation of the functionally relevant IAP-binding motif (IBM), which is a sequence of only four amino acids (alanine, valine, isoleucine and proline) homologous to corresponding proteins of other mammals or drosophila[50]. Translocated to the cytosol, it seizes caspase-binding motifs of IAPs, so that these cannot inhibit caspases anymore, because of a “competition” of SMAC and caspases for the IAP binding sites[140]. Most important is binding to XIAP because it inhibits caspases directly[36]. The BIR-3 domain of this potent IAP is to interact with and inhibit caspase-9, which has a homologous tetrapeptide IBM in common with SMAC so that the way, this inhibition functions, becomes clear: SMAC prevents XIAP-caspase-9-interaction by binding to the IAP at the molecular target, the BIR3 domain[108, 158, 193].
Furthermore, IAP antagonists lead to an increase in proteasomal degradation of c-IAPs as they stimulate the E3 ubiquitin ligase activity of various IAPs and thus their autoubiquitylation and degradation[36, 176]. Again, BIR domains are required for ubiquitylation, but interestingly, XIAP (and also livin) only get ubiquitylated but not degraded[36].

There is data that IAP antagonists also have pro-apoptotic functions apart from IAP-binding and are furthermore involved in inhibition of proliferation of tumor cells, whereas a physiological function has not been defined yet[181].

In cancer cells, SMAC levels are associated with tumor stage, grading, metastasis and survival rates in different cancers[50]. As SMAC and IAP-binding proteins in general act at central points of the apoptotic machinery and are present in cancer cells excessively, mimicking of the tetrapeptide IAP-binding motif is a promising approach in respect to novel therapies[50].

It is of great interest that the tetrapeptide residue, originally named Reaper-Hid-Grim (RHG) domain according to the corresponding structure in drosophila, is sufficient to exert the effects of the IAP inhibitors and this sequence seems to be strongly conserved in the course of evolution which indicates the importance within the signaling cascade and highlights its potential as therapeutic target[140].

1.5. X-linked inhibitor of apoptosis protein (XIAP)

Among IAPs, X-linked inhibitor of apoptosis protein (XIAP, figure 3) is the best characterized, in the course of evolution it has gained the capability of directly binding caspases as only IAP at all, whereas other IAPs lack this function and exert their anti-apoptotic effect in a different manner[36]. XIAP interacts with members of both, the executioner and the initiator caspases, stressing its central position in regulation of apoptosis[132].

The chemical structure of XIAP features three BIR domains and, at the carboxy-terminus, a RING domain[34].
XIAP is a protein of 497 amino acids (aa) and includes three BIR (baculovirus IAP repeat) domains where physical interactions with the corresponding caspases take place. Moreover, it contains a UBA (ubiquitin-associated) and a RING (really interesting new gene) domain, the latter confers E3 ligase activity for ubiquitination of target proteins. C-IAP1 and c-IAP2 furthermore share a caspase recruitment domain (CARD), whose role is unclear, however.

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Inhibition of initiator and effector caspases follows different modes: XIAP BIR-3 domain harbors a peptide binding groove that interacts with the processed pro-form of initiator caspase-9 and inhibits it at the same time[50]: At the apoptosome, pro-caspase-9 gets cleaved and liberates its four amino acid residue located at the neo-N-terminus of the small subunit, giving way for XIAP interactions which in the end prevent caspase homodimerization and thus its activation[50, 101].

In contrast, inhibition of effector caspases 3 and -7 takes place at the BIR-2 domain, where XIAP reacts with the previously cleaved (pro-) caspases, and at another important sequence upstream of BIR-2, the so called ‘linker region’ [77, 139, 153], resulting in inhibition of the executioners of apoptosis: the active substrate binding site of the caspases gets hidden taking away their catalytic power[11].

The carboxy-terminal RING domain is responsible for many other protein-protein interactions of IAPs in general including XIAP[50]. It confers ubiquitin ligase activity for proteasomal degradation of proteins and, in respect to XIAP, there is evidence that the RING domain plays a role in regulation of cell motility, i.e. metastasis in tumor cells[107].
1.6. SMAC-mimicking IAP-antagonizing molecules

IAPs and their inhibitory proteins like SMAC regulate apoptosis at a central point influencing the activation levels of the executioners of programmed cell death, the caspases[50]. Basic research on molecular structures of naturally present IAP-inhibiting proteins like Hid in Drosophila or human SMAC as well as of IAPs and an understanding of how they interact facilitated within the last decade research for promising molecules imitating the binding of SMAC to XIAP above all, as this IAP is involved in several levels of the apoptotic process including its final leg, where many pathways converge[193].

Because the four amino acid residue of SMAC is sufficient for IAP-binding, the pharmacological industry initially focused on generating small molecules containing the alanine-valine-proline-isoleucine-motif resulting in SMAC mimetics that address - as monovalent substances - either solely the BIR-3 domain of XIAP, or, taking bivalent peptides into consideration, act via interference with BIR-2 and BIR-3[161]. Bivalent peptides, which consist of two IBM connected via a linker-sequence, show much higher binding properties but lack pharmacological features of monovalent peptides like satisfying oral bioavailability[50].

The binding of XIAP with its antagonists is achieved by protein-protein interactions like formation of hydrogen bonds and van der Waals forces as well as the proximity of hydrophobic pockets with corresponding chemical residues[36, 161].

Oost and colleagues screened various alterations of the AVPI-motif and they could show that it is even possible to enhance the binding affinity by replacing one of the four amino acids by another one [132]. SMAC-derived peptides yielded encouraging results in vitro but did not show good properties in pharmacokinetic aspects, so different substances have been created with better features in respect to stability and cellular penetration[132].

It was in 2002 when Vucic and Fulda could show for the first time that inhibition of IAPs using competing molecules for caspase-binding sites can sensitize tumor cells for anticancer therapies, they were formerly resistant to[51, 182].
Introduction

Up to now, there are monovalent as well as bivalent molecules that inhibit either several IAPs or only selected ones and exert antitumor effects themselves or act as sensitizers when combined with other anticancer therapies like chemotherapy, radiation or the use of TRAIL[51, 55, 104, 160, 175, 177, 201].

As already mentioned, XIAP is one of the best examined and the most potent IAP concerning caspase inhibition, so that XIAP-targeting antagonists are a broad field of current research, though without countable results so far[46].

It has been shown that high levels of anti-apoptotic factors like IAPs or low levels of pro-apoptotic members, like SMAC, are associated with bad prognosis, more aggressive and higher graded tumors, lower recurrence-free survival rates and poor response to antitumor therapies [37, 39, 63, 119, 138, 194]. Although there are some studies surprisingly showing a benefit in survival at patients with higher levels of XIAP in NSCLC cells[41] or a lower recurrence rate of people suffering from prostate cancer[50], IAPs, and particularly XIAP, seem to be a promising target for novel therapies[50].

So for interference with IAPs, several strategies have been developed: one is to mimic the IAP-binding motif (IBM) of SMAC, which is also our approach in this study[183].
1.7. Aim of the study

We know that apoptosis is deregulated in almost all tumor cells and that upregulation of IAPs in general and XIAP in particular as well as downregulation of endogenous IAP inhibitors like SMAC might be a decisive mechanism in tumorigenesis[63, 119]. It has also been shown that high levels of IAPs and low levels of SMAC are associated with bad prognosis, more aggressive and higher graded tumors, lower recurrence-free survival rates and poorer response to antitumor therapies[37, 39, 138, 194].

Studies revealed XIAP, the most potent inhibitor of apoptosis proteins, as promising target for future therapies blocking this crucial protein that is highly expressed in PDAC, too[92].

With pancreatic ductal adenocarcinoma we addressed to an, up to now, highly resistant tumor concerning conventional therapies trying to sensitize several PDAC cell lines for chemotherapy-induced apoptosis using small molecule SMAC-mimicking inhibitors of XIAP in part I of this thesis.

In a second section we tried to elucidate the apoptotic nature of the observed effects in an indirect way: we inhibited caspases, which are the main characters in the apoptotic spectacle, with a pan-caspase inhibitor.

Finally, DNA fragmentation, which is a defining morphological change during apoptosis, should be pointed out in part III of the study.
### 2. Materials and Methods

#### 2.1. Materials

##### 2.1.1. Cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPC1</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
<tr>
<td>BxPC3</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
<tr>
<td>Capan1</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
<tr>
<td>MiaPaCa2</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
<tr>
<td>Panc1</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
<tr>
<td>PancTu1</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
<tr>
<td>PaTuI</td>
<td>Pancreatic carcinoma cell line</td>
<td>provided by R.M. Schmid (TU Munich)</td>
</tr>
</tbody>
</table>

##### 2.1.2. Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Invitrogen, Germany</td>
</tr>
<tr>
<td>DMSO</td>
<td>Merck, Germany</td>
</tr>
<tr>
<td>FCS</td>
<td>Biochrom, Germany</td>
</tr>
<tr>
<td>Hepesbuffer</td>
<td>Biochrom, Germany</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Biochrom, Germany</td>
</tr>
<tr>
<td>MTT</td>
<td>Sigma Aldrich Chemie GmbH, Germany</td>
</tr>
<tr>
<td>Nicoletti buffer</td>
<td>(0.1% sodium citrate pH 7.4 + 1 % Triton X-100 + 50 μg/ml propidium iodide)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Biochrom, Germany</td>
</tr>
</tbody>
</table>
Materials and Methods

RPMI 1640
Streptomycin
Trypan blue
Trypsin/EDTA

2.1.3. Substances

2.1.3.1. Cytostatics

5-Fluorouracil (5-FU)
Doxorubicin (Adriamycin)
Gemcitabine (Gemzar®)
VP-16 (Etoposide)
Taxol® (Paclitaxel)

2.1.3.2. XIAP antagonists

IDUN 13100
IDUN 13483
IDUN 13497
IDUN 13496
IDUN 13097
IDUN 13098

IDUN Pharmaceuticals / Pfizer, USA
IDUN Pharmaceuticals / Pfizer, USA
IDUN Pharmaceuticals / Pfizer, USA
IDUN Pharmaceuticals / Pfizer, USA
IDUN Pharmaceuticals / Pfizer, USA
IDUN Pharmaceuticals / Pfizer, USA

Sigma Aldrich Chemie GmbH, Germany
Sigma Aldrich Chemie GmbH, Germany
Lilly Deutschland GmbH, Germany
Sigma Aldrich Chemie GmbH, Germany
Sigma Aldrich Chemie GmbH, Germany
2.1.3.3. Caspase Inhibitor

zVAD.fmK  
Bachem, Germany

All further substances were provided by Sigma Aldrich Chemie GmbH, Germany.

2.1.4. Equipment

Pipettes  
Abimed, Germany

Multipipette Plus  
ThermoLabsystems, USA

Pipetboy acu  
IBS Integra Biosciences, USA

CO₂-Incubator  
NuAire, Plymouth, USA

Incubator BBD 6220  
Heraeus Instruments, Germany

Varifuge 3.0 RS  
Heraeus Instruments, Germany

Microscope CK30  
Olympus GmbH, Hamburg, Germany

FACScan  
BD Biosciences

FACScalibur  
BD Biosciences

Plate reader Wallac 1420 Victor multilabel counter  
Perkin-Elmer, USA

Combitipps  
Eppendorf, Inc., Germany

Falcons  
Becton Dickinson, USA

Safe-lock tubes  
Eppendorf, Inc., Germany

Polypropylen conical tube  
Becton Dickinson, USA

(15ml, 50ml)

Tissue culture flasks  
Becton Dickinson, USA

(75cm², 150cm²)

Well plates  
Becton Dickinson, USA

(24 wells, 96 wells)

Haemocytometer  
Brand, Germany
2.2. Methods

2.2.1. Cell culture
All of the cell lines mentioned above - apart from Capan-1, which were kept in RPMI - were cultivated in DMEM cell culture medium with addition of 10% FCS, 1 mM L-glutamine, 1% penicillin/ streptomycin and 25 mM hepes buffer in an incubator at 37°, 5% CO₂ and 95% air humidity. The density of the cells within the flasks was controlled regularly and if necessary, cells were split to prevent a too high density of the carcinoma cells.
In order to harvest the cells for in vitro experiments, they were washed with PBS and detached using trypsin/EDTA.

2.2.2. Seeding of cells
All experiments were done with a cell density of 5 x 10⁵ cells/ cm². Therefore, 10µl of cell suspension was mixed with 10µl trypan blue, which only marks damaged or dead cells, viable cells could not be labeled; with a haemocytometer we determined, how many viable cells can be expected in 1 ml cell suspension according to the amount of cells per 32 squares in the Neubauer counting chamber. Based upon this, we calculated how many milliliters we need to get the favored amount of cells per well.
96 well-plates were used for MTT experiments, for Nicoletti experiments we took 24-well plates.

2.2.3. Treatment of pancreatic cancer cell lines
After seeding of the cells into the corresponding well plates, cell culture medium with the additions mentioned above was added including different doses of corresponding cytostatic drugs: In separate experiments before, the maximum tolerable dose of all cytostatics was determined, based upon that, cells were treated with doses ranging from 0 (no cytostatic drug added) to the maximum tolerable concentrations of the corresponding drugs in doubling doses.
Afterwards, IDUNs[132] were added in a concentration of 10µM and equally DMSO - the solvent of the IDUNs as negative control. As there were no significant differences between the various active IDUNs used in the first experiments (figure 4), we focused on only one active XIAP inhibitor (IDUN 13483) for the following experiments and renounced a particular specification of each single compound[132].

For zVAD.fmK experiments, the pan-caspase inhibitor was added in concentrations of 50 µM.

When treatment was done, we kept the well plates in the incubators for 72 h hours.

2.2.4. Examination for cell viability: MTT viability assay

The MTT viability assay uses the metabolic potential of viable, i.e., proliferating cells. Adding initially yellow colored MTT, metabolically active cells reduce this tetrazolium to purple colored formazan.

According to the manufacturer’s instructions, the pre-treated cells were stimulated with 1mg/ml MTT buffer in RPMI 1640 medium without phenol red for 3 h at 37°C. Subsequently, isopropyl alcohol was given for 30 min in order to precipitate the reduced formazan before the well plates were put into the ELISA reader getting analyzed at 550 nm.

2.2.5. Determination of the role of caspases with pan-caspase inhibitor

Pan-caspase inhibitor zvad.fmk was added to the pancreatic carcinoma cells additionally to cytostatics and IDUNs in 96-well plates.

After an incubation period of 72 hours at 37°C, cell viability measurement was performed using the MTT assay as mentioned above.
2.2.6. Examination for apoptotic cell death: analysis of DNA fragmentation

Proof of apoptotic cell death was provided by demonstration of DNA fragmentation using propidium iodide staining[49]. Cells undergoing apoptosis discharge DNA in a controlled manner with leaving less genetic information left in the nucleus to be detected. For this reason, DNA content of a cell can distinguish between normal and apoptotic cells: the latter have a reduced quantity of DNA.

To visualize DNA in the course of apoptosis we first centrifuged the cells, then washed them with PBS and incubated them for 24 hours at 4°C with lysis buffer (‘Nicoletti buffer’) that contained 0.1% Triton-X 100, 0.1% tri-sodium citrate dihydrate and 50 µg/ml propidium iodide. During this process the cells had to be kept at 4°C and without exposition to light. As propidium iodide cannot permeate membranes of living cells, it can only get to the nucleus in damaged or apoptotic cells, respectively.

DNA fragmentation was determined by fluorescence-activated cell sorting analysis in the flow cytometer.

2.3. Statistics

Unless otherwise noted, all experiments were done in triplets. Of these results 3 means were summarized and standard error of mean was determined. Student’s t-test was used in Microsoft® Excel for assessment of statistical significance to reject the null hypothesis. For p-values, which are smaller than the determined threshold for statistical significance (in this thesis 0.05, 0.01 and 0.001 were used), an effect or a difference between the two groups can be postulated with statistical significance and alternative hypothesis, that assumes results in two groups differ not only by chance, is favored[61].
3. Results

3.1. Small-molecule XIAP inhibitors lead to an enhanced cytotoxicity induced by chemotherapeutic agents only in few pancreatic carcinoma cell lines

First experiments were done to elucidate, whether chemoresistance of pancreatic carcinoma cell lines can be reduced, when the potent caspase inhibitor XIAP is targeted and thus inhibited itself. We took pancreatic carcinoma cell lines ASPC1, BxPC3, Capan1, MiaPaCa2, Panc1, PancTu1 and PaTuII and treated each of them with different cytostatic drugs: 5-FU, doxorubicin, gemcitabine, taxol and VP16 (figures 4 - 10).

First part was to determine drug concentrations to achieve a gradual dose response (derived from figures 4 - 10, specific experiments not shown). It turned out that most cell lines showed a dose response when treated with increasing drug concentrations, solely application of gemcitabine was associated in most experiments with a marked chemoresistance, even when concentrations were risen up to factor 250 of the starting dose: This was observed most clearly in Panc1, PaTuII and PancTu1 cell lines (figures 6, 8 and 9).

Small-molecule XIAP inhibitors (IDUNs 13100, 13483, 13496, 13097 and 13098), provided from IDUN-pharmaceuticals, were used to target XIAP (figures 4 - 18). For pharmacokinetic reasons they were dissolved in DMSO, which served as negative control in our experiments. All IDUNs but IDUN 13100 were agonists at the BIR-3 domain of XIAP (i.e. active IDUNs) and supposed to inhibit it intensely, imitating SMAC binding to this anti-apoptotic protein. In contrast, IDUN 13100 was structurally analogue to the other IDUNs but exerted only very weak binding properties and functioned as control, too.
Results

As the physico-chemical binding properties of all active IDUNs were supposed to be quite similar, we did the first experiments in order to check the effect of the compounds in respect to their ability to increase cytotoxicity induced by doxorubicin and 5-FU according to MTT-viability assays (figures 4a and 4b).

The first pancreatic carcinoma cell line we tested was ASPC1 (figure 4). Cells were equally seeded in well plates as mentioned above, then, IDUNs and DMSO (in concentrations of 10 µM each) and afterwards the drugs were added in the predetermined doses, before incubating the well plates for 72 hours. In fact, IDUNs 13483, 13496, 13097 and 13098 all showed a clear and similar reduction of viable cells in ASPC1 cell line compared to DMSO control and less active IDUN 13100 after stimulation via doxorubicin and 5-FU (Figures 4a and 4b).

Because of comparable efficacy, we focused for further experiments on only one active compound: IDUN 13483. Herewith we performed all following experiments compared to the less active compound IDUN 13100 and to DMSO-treated cells as controls (figures 5 - 10).
Results

Figure 4  Small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein) increase drug-induced cytotoxicity in ASPC1

ASPC1 pancreatic carcinoma cells were stimulated with drugs in corresponding doses and additionally treated either with DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUN 13100 as controls or active compound IDUN 13483 (for all experiments) at a dose of 10µM each. In doxorubicin- and 5-FU-treated cells additionally IDUNs 13496, 13097 and 13098 as active compounds. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.1.1 Small-molecule XIAP inhibitor IDUN 13483 increases drug-induced cytotoxicity in ASPC1 pancreatic carcinoma cells most effectively when combined with doxorubicin and 5-FU

As already shown in the previous experiments (see section 3.1 and figure 4), the active IDUNs, represented by IDUN 13483, were very effective in additionally reducing viability of ASPC1 pancreatic carcinoma cells that were stimulated by doxorubicin and 5-FU: Examining viable cells with MTT assay, we detected in the active arm a further decrease in metabolic activity as indicator of viability to only 10% (compared to 52% in the control setting) for combination with 5-FU (figure 4b) and from 54% in the control arm to 9% in the treatment arm when doxorubicin was applied (figure 4a), which indicated a significant enhancement of drug-efficacy when XIAP is inhibited by the small-molecule XIAP inhibitor IDUN 13483 compared to control cells, whose XIAP was not antagonized. IDUN 13100 had, as it was supposed to by its nature as a weak antagonist, only little effect on reducing viable ASPC1 carcinoma cells in combination with drug treatment (figure 4).

The distinct response of active IDUN 13483 in ASPC1 cells stimulated with doxorubicin and 5-FU, however, could not be transferred completely to treatment with other cytostatics we tested: VP16, gemcitabine and taxol (figures 4c - 4e). Cytotoxic stimuli with these drugs lead to a rather constant and dose-independent decrease of viable cells between 24% (gemcitabine) and 18% (VP16), whereas taxol treatment reduced viability by 22% compared to DMSO-control. Another remarkable aspect was the resistance of ASPC1 cells in the control setting of the experiments towards partly very high drug doses: Still 50% of cells were viable after taxol dose was risen from 1.56 nM to 400 nM (figure 4e) and also gemcitabine treatment did not result in a further decrease of metabolically active cells in the wake of increasing concentrations from 1.56 µg/ml to 50 µg/ml (figure 4c).
3.1.2 XIAP inhibition by small molecules increases effectiveness of different cytotoxic agents in MiaPaCa2 pancreatic carcinoma cells

MiaPaCa2 cells were prepared as stated in part 2 of this thesis and kept over 72 hours in an incubator after stimulation and treatment with doxorubicin, 5-FU, gemcitabine, VP16 and taxol.

According to MTT assay, IDUN 13483 again showed - at least partly - a significant reduction of viable cells in combinational treatment with these cytostatics compared to negative controls (figure 5).

The most effective reduction of viable MiaPaCa2 cells (minus 35% compared to controls) was observed with gemcitabine co-stimulation at a dosage of 0.78 µg/ml (figure 5c), almost the same for VP16 at a dose of 1.56 µg/ml (minus 34%, figure 5d). Doxorubicin, 5-FU and taxol also lead to a considerable diminution of metabolically active MiaPaCa2 cells by at least 24% (figures 5a, 5b and 5e).

It is conspicuous that we needed relatively very low dosages of the cytotoxic drugs to get the results mentioned above or a dose response of the cells in general.

But the higher we have chosen the concentrations of the drugs, the lesser was the additional effect of IDUN 13483 as more cells in the control trial turned out to reduce metabolic activity as sign of cell death, too.
**Figure 5** XIAP (X-linked inhibitor of apoptosis protein) antagonist sensitizes MiaPaCa2 for chemotherapy-induced cytotoxicity

MiaPaCa2 cells were stimulated with drugs in corresponding doses and additionally treated either with DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUN 13100 as controls or IDUN 13483 at a dose of 10µM each. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplicates, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.1.3  Panc1 pancreatic carcinoma cells show weak response to XIAP inhibition via small molecules in combination treatment with different cytostatics

Reduction of viable Panc1 cells by small-molecule inhibition of XIAP using IDUN 13483 can be augmented in a range of 23% (VP16) to 36% (doxorubicin) compared to treatment with DMSO as a control (figure 6). However, we additionally see almost the same effectiveness of the “weaker” IDUN 13100. The dosages of the cytostatics to get a dose response are partly low and partly moderate, but especially with gemcitabine, even with very high concentrations, still more than 85% of treated Panc1 cells are viable (figure 6c). Similar to these results we still have 60% viability in cells stimulated with 100nM taxol and in absence of an effective XIAP-antagonism (figure 6e). For treatment with 200µM 5-FU, a decrease in viability can be detected in only 48% (figure 6b). And again: the higher the concentration of the drug, the more diminishes the effectiveness of XIAP-inhibition by IDUN 13483 (and IDUN 13100, too).
**Results**

**Figure 6** Small-molecule inhibitor of XIAP (X-linked inhibitor of apoptosis protein) slightly increases drug-induced cytotoxicity in Panc1

Panc1 cells were stimulated with drugs in corresponding doses and additionally treated either with DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUN 13100 as controls or IDUN 13483 at a dose of 10µM each. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.1.4 Inhibiting XIAP is not effective in reducing viability when combined with cytotoxic agents in BxPC3 pancreatic carcinoma cells

BxPC3 cells show a proper dose response on all added cytotoxic drugs, but there was hardly any gain in viability reduction using small-molecule inhibitors of XIAP (figure 7).

![Graphs showing 72-h-effects of various cytotoxic agents with and without XIAP inhibitors on BxPC3 cells.](image)

Figure 7 Small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein) do not increase drug-induced cytotoxicity in BxPC3

BxPC3 cells were stimulated with drugs in corresponding doses and additionally treated with either DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUN 13100 as control or IDUN 13483 at a dose of 10µM each. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
Stimulation with different cytostatics resulted in the XIAP-antagonized setting with basically no additive reduction of viable BxPC3 carcinoma cells compared to controls. At some concentrations, there were adverse effects with more cancer cells being detected viable after IDUN supplementation than without the XIAP antagonist (figure 7).

3.1.5 PaTull cells can hardly be sensitized to chemotherapy-induced cell death using small-molecule inhibitors of XIAP

Results of PaTull cells treated with our 5 cytotoxic agents and XIAP-inhibiting IDUNs did not show a great influence on cell viability compared to negative controls (figure 8).

Though there was, apart from gemcitabine-stimulated PaTull cells, a dose response with doxorubicin, 5-FU, VP16 and taxol, additional treatment with XIAP-inhibitors did not reduce viable PaTull carcinoma cells for more than 17% (VP16, figure 8d), the least effect was observed with 5-FU at a relatively high dose (11% reduction with a dose of 200µM, figure 8b).

As in experiments before, the effect was dose-dependent with only a small range of concentrations that lead to a surplus of cell death when parallel XIAP inhibiting was performed and a vanishing of the effect with higher doses and in consequence a stronger toxicity of the chemotherapeutic drug itself.

It is remarkable that in PaTull pancreatic carcinoma cell line, IDUN 13100 showed efficacy in reducing viability, too, in part to the same extent as active IDUN 13483 or - for some of the experimental settings - even beyond (figures 8a - e).
Figure 8  PaTuII cells can only weakly be sensitized for drug-induced cell death by antagonization of XIAP (X-linked inhibitor of apoptosis protein)

PaTuII cells were stimulated with drugs in corresponding doses and additionally treated with either DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUNs 13100 as controls or IDUN 13483 at a dose of 10µM each. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean.

Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.1.6 Chemoresistance in PancTu1 can be influenced moderately using small-molecule inhibitors of XIAP

Our experimental settings with application of IDUN 13483 on chemotherapeutically stimulated PancTu1 pancreatic carcinoma cells (figure 9) only show moderate effects in reducing viable tumor cells when combined with VP16 (relative reduction of viability by 21% at a dose of 12.5 µg/ml, figure 9d) and doxorubicin (relative reduction of viability by 20% at a dose of 1 µg/ml, figure 9a), whereas the other drugs only exert weak effects between 6% (taxol, figure 9e) and 10% (gemcitabine, figure 9c).

Also in PancTu1, the efficacy of antagonizing XIAP initially increases with higher doses to a point, when further rise again leads to its weakening: The most distinctive effect of XIAP inhibition can be observed, when 77% (VP16 at a concentration of 12.5 µg/ml, figure 9d) or 47% (doxorubicin at a concentration of 1 µg/ml, figure 9a), respectively, of cells in the control trial survive.

PancTu1 cells are, as it has been described in several pancreatic carcinoma cell lines above, partly resistant towards higher doses of gemcitabine, as no further dose response can be attained at concentrations up to 400 µg/ml (figure 9c).
Results

**Figure 9** XIAP (X-linked inhibitor of apoptosis protein) antagonist increases drug-induced cytotoxicity in PancTu1 only marginally

PancTu1 cells were stimulated with drugs in corresponding doses and additionally treated with either DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUN 13100 as controls or IDUN 13483 at a dose of 10µM each. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.1.7 Capan1 pancreatic carcinoma cells can not be sensitized for chemotherapy-induced cell death by inhibiting XIAP via small molecules

Last cell line we tested, Capan1, turned out to be highly resistant towards antagonizing XIAP with the small-molecule XIAP inhibitor IDUN 13483 (figure 10).

**Figure 10** Small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein) do not increase drug-induced cytotoxicity in Capan1

Capan1 cells were stimulated with drugs in corresponding doses and additionally treated with either DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitor IDUN 13100 as controls or IDUN 13483 at a dose of 10µM each. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
Though a dose response upon treatment with cytotoxic agents can be achieved with similar concentrations like in other cell lines, the XIAP-inhibiting compound does not lead to further cell death in Capan1 (figure 10).

3.2. Small-molecule XIAP inhibitors lead to a caspase-dependent cell death in pancreatic cancer cell lines

Caspases are known mediators of an apoptotic cell death caused by cytotoxic agents and XIAP is an acknowledged inhibitor of caspases 9, -3 and -7. In our second part of this thesis we tried to investigate the nature of the observed sensitization of pancreatic carcinoma cells resulting in a decrease of viable cells after XIAP had been inhibited and an apoptotic stimulus had been given via 7 different cytostatics (figures 4 - 10). Therefore, we checked for restoration of chemoresistance using the broad-range caspase inhibitor zVAD.fmk in a dose of 50µM in ASPC1 and MiaPaCa2 (figures 11 and 12): These two pancreatic carcinoma cell lines had shown the most promising results in respect to additional reduction of viable tumor cells in our first experiments after XIAP had been antagonized (figures 4 and 5).

3.2.1 Inhibiting caspases abrogates increased chemosensitivity induced by small-molecule XIAP inhibitors in ASPC1 pancreatic carcinoma cells

Based upon our cell viability results in part I of the thesis (figures 4 - 10), we examined the role of caspases and to which extent they contribute to overcome chemoresistance in ASPC1 cells. The most potent reduction of viable tumor cells could be detected with doxorubicin and 5-FU (figure 4a and 4b), so we added caspase inhibitor zVAD.fmk (50 µM) to ASPC1 cells stimulated with doxorubicin (drug concentrations 0.75 µg/ml and 1 µg/ml) and simultaneously treated with 10µM IDUN 13483 (figure 11a). After 72 hours, we observed in zVAD.fmk-treated cells an abolition of the chemosensitizing effects exerted by XIAP-inhibiting IDUNs: This indirectly showed the involvement of caspases in the apoptotic process of pancreatic carcinoma ASPC1 cells and that IDUNs were supposed to liberate them from XIAP binding.
Without zVAD.fmK only 10% of ASPC1 cells were alive after 72 hours when treated with 0.75 µg/ml doxorubicin and IDUN 13483, the inhibitory effect on XIAP obviously lead to a surplus of caspases, which subsequently induced or exerted (apoptotic) cell death, respectively (figure 11a).

If we now compared these results to those with addition of zVAD.fmK, the expected inhibition of liberated caspases took place and could be documented as more cells remained viable, because the executioners of death were missing (figure 11a). So caspase-inhibition goes along with less cell death / - apoptosis, just as we had presumed.

The same was detected when these cells were stimulated with 5-FU in concentrations of 200µM and 400 µM (figure 11b): Both experiments showed that the effects, gained by liberating caspases via IAP inhibitors, were abrogated, if caspases were addressed by zVAD.fmK and thus inhibited themselves (figures 11 and 12).

We even saw that in cells treated with the less active IDUN 13100 and DMSO, too, cytotoxic drug effects were suspended to a certain degree by zVAD.fmK, indicating a low basic caspase activity.

Figure 11 Caspase inhibition restores anti-apoptotic properties of X-linked inhibitor of apoptosis protein (XIAP) in ASPC1

ASPC1 pancreatic carcinoma cells were stimulated with drugs in corresponding doses and additionally treated either with DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitors IDUN 13100 and 13483 at a dose of 10µM each and additionally with pan-caspase inhibitor zVAD.fmK in a dose of 50 µM. Bars show functional pairs. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.2.2 Inhibition of caspases restores chemoresistance in MiaPaCa2 pancreatic carcinoma cells treated with XIAP inhibitors and chemotherapeutic agents

Similar to ASPC1 cell line, our experiments with MiaPaCa2 cells provided an indication of caspase involvement in cell death induced by cytotoxic medication and simultaneous treatment with XIAP inhibitors (figure 12).

As doxorubicin, gemcitabine and VP16 had shown the strongest reduction of viable MiaPaCa2 pancreatic carcinoma cells in our former experiments (figure 5), we chose these three drugs for further investigation with the caspase inhibitor zVAD.fmk.

![Figure 12](image-url) Caspase inhibition in MiaPaCa2 restores anti-apoptotic properties of XIAP (X-linked inhibitor of apoptosis protein)

MiaPaCa2 cells were stimulated with drugs in corresponding doses and additionally treated with either DMSO (dimethyl sulfoxide) and small-molecule XIAP inhibitors IDUNs 13100 and 13483 at a dose of 10µM each and additionally with pan-caspase inhibitor zVAD.fmk in a dose of 50 µM. Bars show functional pairs. Cell viability was measured by MTT assay and indicated as percentage of unstimulated controls. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
Results

It was easy to detect that in all experimental settings, the addition of the pan-caspase inhibitor reduced the effects of XIAP inhibition (figures 12a - c). So we concluded that in these tumor cells, caspase-dependant cell death played a major role and inhibition of the executioners of death by zVAD.fmk lead to a loss of the cytotoxic effects obtained by XIAP inhibition:

In MiaPaCa2, pre-treated with IDUN 13483 and gemcitabine (at a concentration of 1.56 µg/ml), caspase inhibition by zVAD.fmk resulted in a reduction of cell death by 35% compared to control setting, in which caspases could act without being antagonized (figure 12b).

In doxorubicin-stimulated cells, the difference still was approximately 30% (figure 12a), almost the same in VP16-treated MiaPaCa2 carcinoma cells with a reduction of dead cells by almost 30%, too, when caspase inhibitor zVAD.fmk was added (figure 12c).

To some extent DMSO and IDUN 13100 also showed little caspase-mediated cytotoxicity as zVAD.fmk in part restored chemoresistance here, too, even if it was to a far lesser extent than in the IDUN 13483 trial (figures 12a - c).

3.3. Elucidating the mode of cell death with evidence of DNA fragmentation

Our results in the previous experiments showed for two out of seven pancreatic carcinoma cell lines treated with different cytostatic drugs an increased chemosensitivity as a result of additional XIAP inhibition using small molecules (figures 4-10). Now, it was to further clarify, whether the treated cells underwent apoptosis or if there were other mechanisms leading to a reduced viability according to the results observed in the MTT-experiments in section one.

Therefore, we performed staining of nuclei with propidium iodide in order to proof apoptosis by showing DNA fragmentation as a hallmark of apoptotic cell death.
3.3.1 Doxorubicin induces apoptosis in ASPC1 pancreatic carcinoma cells when XIAP is blocked by small-molecule XIAP inhibitor IDUN 13483

In ASPC1, as well as in MiaPaCa2, results of DNA staining with propidium iodide went along with our MTT viability assays (figures 4 - 10) and the zVAD.fmk results (figures 11 and 12) as they pointed out clearly an apoptotic cell death for combinational treatment: At doxorubicin concentrations of 0.75 µg/ml in ASPC1, of 81% non-viable cells according to MTT measurement (figure 4 in section 3.1.2), 62% showed a lowered DNA quantity like it can be seen in apoptotic cells when measured with fluorescence cytometer, compared to 33% of control cells (figure 13).

Figure 13  ASPC1 cells show enhanced apoptosis after treatment with doxorubicin and IDUN 13483

ASPC1 cells were stimulated with doxorubicin and additionally treated either with DMSO (dimethyl sulfoxide) or small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein), IDUNs 13100 and 13483, at a dose of 10µM each. Bars show DNA fragmentation in percentage measured by FACS (fluorescence-activated cell sorting) analysis. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.3.2 In ASPC1 pancreatic carcinoma cells treatment with 5-FU in combination with the small-molecule XIAP inhibitor IDUN 13483 only causes low levels of apoptosis

When we have a look at experiments in part 3.1.1 of this thesis, 5-FU seemed to be a potent suppressor of cell viability in combination with XIAP antagonization via small-molecule inhibitor IDUN 13483 in ASPC1 (figure 4b). But this reduction in viability of 50% compared to the control setting was apparently not due to apoptosis, as only 20% of the cells (8% in the control arm) showed characteristic DNA fragmentation (figure 14). This was far less as we would have expected from preceding MTT results and the findings of the doxorubicin experiments pointing to an efficient apoptotic machinery in this cell line (see 3.3.1, figure 13).

![Figure 14](image-url)  
**Figure 14** ASPC1 cells show hardly enhanced apoptosis after treatment with 5-FU and IDUN 13483

ASPC1 pancreatic carcinoma cells were stimulated with 5-FU and additionally treated with either DMSO (dimethyl sulfoxide) or small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein), IDUNs 13100 and 13483, at a dose of 10µM each. Bars show DNA fragmentation in percentage measured by FACS (fluorescence-activated cell sorting) analysis. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean.
Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.3.3 In combination with gemcitabine treatment XIAP inhibition does not result in an enhancement of ASPC1 pancreatic carcinoma cells undergoing apoptosis

Experiments to examine the number of cells undergoing apoptosis after stimulation with gemcitabine and parallel treatment with small-molecule XIAP inhibitor IDUN 13483 were conflicting in ASPC1 cell line: based on MTT results in part 3.1.1 (figure 4c), we would have expected a significant difference of apoptotic cells in the active and in the control arm. However, the difference was only marginally and of weak significance and even in DMSO and IDUN 13100-treated cells we observed many cells presenting DNA fragmentation (figure 15). It was additionally remarkable that there was an immanent toxicity of 17–22% in control trials with no drug administered.

Figure 15 ASPC1 cells show high apoptosis level after treatment with gemcitabine and IDUN 13483 independent of XIAP (X-linked inhibitor of apoptosis protein) inhibition

ASPC1 cells were stimulated with gemcitabine and additionally treated either with DMSO (dimethyl sulfoxide) or small-molecule XIAP inhibitors, IDUNs 13100 and 13483, at a dose of 10µM each. Bars show DNA fragmentation in percentage measured by FACS (fluorescence-activated cell sorting) analysis. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.3.4 XIAP inhibitor IDUN 13483 promotes apoptosis in MiaPaCa2 carcinoma cells treated with doxorubicin

Results underlined that it was apoptosis. MiaPaCa2 cells exerted after treatment with doxorubicin and IDUN 13483 (figure 16).

In the previously performed cell viability assays, 71% of the cells stimulated with doxorubicin in a concentration of 0.125 µg/ml did not show any metabolic activity (figure 5). With propidium iodide staining it turned out that 65% of these carcinoma cells actually underwent apoptosis, whereas only 30% of DMSO-treated control cells showed an apoptotic activity (figure 16). Yet, even without stimulation we saw spontaneous apoptosis ranging from 7% in the DMSO-treated cells to 15% (IDUN 13100) and 24% apoptosis in cells treated solely with IDUN 13483 in the absence of any cytotoxic stimulus.

Figure 16   MiaPaCa2 cells show enhanced apoptosis level after treatment with doxorubicin and IDUN 13483

MiaPaCa2 cells were stimulated with doxorubicin and additionally treated with either DMSO (dimethyl sulfoxide) or small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein), IDUNs 13100 and 13483, at a dose of 10µM each. Bars show DNA fragmentation in percentage measured by FACS analysis (fluorescence-activated cell sorting). Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean.

Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.3.5 VP16 stimulation in MiaPaCa2 pancreatic carcinoma cells leads to a significantly higher rate of apoptosis when XIAP is inhibited by small-molecule inhibitor IDUN 13483

Although cell viability assays showed a greater influence of XIAP inhibition in MiaPaCa2 (figure 5d), experiments trying to proof apoptosis solely demonstrated an - at least significant - difference in the active treatment arm compared to controls but not to the expected range (figure 17).

At VP16 doses of 4.5 µg/ml 61% of cells showed DNA fragmentation as evidence of apoptotic cell death when treated with DMSO. This number could only be raised to 71% in the presence of the XIAP inhibitor IDUN 13483. XIAP inhibition itself contributed to 23% of apoptosis without stimulation by any cytostatic drug and we even found 13% MiaPaCa2 cells executing apoptosis in the absence of any compound.

Figure 17 MiaPaCa2 cells show enhanced apoptosis levels after treatment with VP16 and the X-linked inhibitor of apoptosis protein (XIAP) inhibitor IDUN 13483

MiaPaCa2 cells were stimulated with VP16 and additionally treated with either DMSO (dimethyl sulfoxide) or small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein), IDUNs 13100 and 13483, at a dose of 10µM each. Bars show DNA fragmentation in percentage measured by FACS (fluorescence-activated cell sorting) analysis. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
3.3.6 Gemcitabine treatment induces high rates of apoptosis in MiaPaCa2 cell line almost independent of XIAP inhibition

Surprisingly, we could not detect much difference in apoptosis of MiaPaCa2 cells stimulated with gemcitabine (figure 18), if we have a look at XIAP-inhibited cells (treatment with IDUN 13483) or negative controls (DMSO): The amount of carcinoma cells eventually undergoing apoptosis in the treatment arm was similar to the amount of cells showing no metabolic activity according to MTT results in 3.1.3 (figure 5c). On the other hand, DNA fragmentation was detected in far more cells of the control arm than we could have expected from corresponding viability assays.

Figure 18 MiaPaCa2 cells show independent of XIAP (X-linked inhibitor of apoptosis protein) inhibitor IDUN 13483 enhanced apoptosis levels after stimulation with gemcitabine

MiaPaCa2 cells were stimulated with gemcitabine and additionally treated either with DMSO (dimethyl sulfoxide) or small-molecule inhibitors of XIAP (X-linked inhibitor of apoptosis protein), IDUNs 13100 and 13483, at a dose of 10µM each. Bars show DNA fragmentation in percentage measured by FACS (fluorescence-activated cell sorting) analysis. Mean values of - unless otherwise noted - 3 independent experiments, done in triplets, are shown with standard errors of mean. Significance level was determined using t-test (* = p < 0.05, ** = p < 0.01, *** = p < 0.001).
4. Discussion

In the current study, we aimed to improve the response of pancreatic ductal adenocarcinoma cell lines to chemotherapy-induced apoptosis by inhibiting XIAP, a protein that acts at a central point in regulation of apoptosis and thus is supposed to contribute to resistance to current anticancer therapies[50]. We addressed one of the most lethal diseases in the western world as incidence and mortality rates are almost the same for this tumor entity[40], because it is only diagnosed in an advanced state and apart from late diagnosis, PDAC moreover has been showing such a high capacity of evading therapies as it is only seen in very few other cancers[82].

During the last 25 years, treatment progress did merely improve within this time period[186], which is quite remarkable when we have a look at what changed in medicine in this time corridor: HIV, once a death sentence became a chronic disease[33], mortality rates from patients with CML almost crashed in many cases after (bcr-abl-) tyrosine kinase inhibitors were introduced in leukemia therapy[163]. The discovery of the latter is like a morning star in targeted antitumor therapies and serves as shining example indicating that successful and specific treatment of malignant diseases is possible under certain circumstances.

But also in many solid tumors, improvements in respect to survival were achieved during years in which therapy of PDAC was marking time:

For colorectal cancers, as an example, mortality rates are only half of incidence rates[84] and from 1960 on, survival rates significantly improved[86].

In patients suffering from lung cancer as the tumor entity with - in absolute numbers - highest rates of cancer-related deaths worldwide, 5-year survival rates doubled within the last 25 years for limited-stage SCLC[83]. In NSCLC, another resistant neoplasia, median survival rates also slightly increased with introduction of novel therapies after identification of distinct mutations in selected tumors[141].

Systemic therapies remain the only option for the largest part of patients suffering from PDAC, as in most cases, it is diagnosed in an advanced or spreaded state[114]. So it is worth focusing on new potential drugs aiming to improve the results of therapy protocols acting systemically.
Based on studies from Burris et al. in 1997, gemcitabine still is the standard in systemic treatment of pancreatic cancer in an advanced and inoperable state[29]: It improved rates of median survival compared to monotherapy with 5-FU, which is the mostly used cytotoxic agent in gastrointestinal cancers[172], from 4.41 to 5.56 months and additionally decreased disease-associated symptoms[21].

In 2011, Conroy et al. demonstrated that using the polychemotherapy regimen FOLFIRINOX in a certain collective of patients, overall survival rates doubled in contrast to the gemcitabine treated group[28]. However, this improvement was achieved at the expense of higher toxicity so that not everyone is appropriate for this therapeutic option[28].

Similar results were shown in a meta-analysis in 2014, in which 23 studies were involved including almost 10.000 pancreatic cancer patients and 19 different treatment protocols[62]: Diverse combinations of chemotherapeutic drugs, partly with monoclonal antibody bevacizumab against vascular endothelial growth factor or tyrosine kinase inhibitor erlotinib pointed out a progress in postponing death by the disease as progression-free survival as well as overall survival could be improved significantly [29, 62]. These findings also show that novel strategies have entered the approach to pancreatic cancer and it is worth following because conventional treatment alone has failed to prevail as satisfying therapy.

If we have a look at these new strategies targeting molecules and pathways detected in the recent past, apoptosis, as central suicide programme still active within transformed cells and its regulation and switching off in cancers is spotlighted.

Apoptosis is not only a dysregulated programme in neoplasia but the common final path of conventional anticancer treatment, so it was promising to concentrate on defective apoptosis pathways as key mechanisms in resistance to treatment protocols of cancers in general and PDAC, too[46]. Many signaling cascades have been elucidated and decisive proteins and peptides involved are known so far[47]. After a set of anti-apoptotic proteins, the inhibitors of apoptosis proteins, or ‘IAPs’ in short, were identified and IAP inhibition itself and the way of blocking anti-apoptotic proteins has been shown in Drosophila
melanogaster for the first time[185]. Those IAPs were found in humans, too and thus emerged into the focus of cancer research[113]. Studies have shown an overexpression of IAPs in many human cancers[101] and especially one IAP was thought to be most potent in preventing apoptosis, the so called X-linked inhibitor of apoptosis protein (XIAP): It is supposed to be the best examined of all IAPs because of its unique ability to directly inhibit caspases by binding them to its different BIR domains[46].

Upregulation and overexpression of XIAP is associated with a dismal prognosis and worse response to therapies in many cancers[50]:

In colorectal cancer, high levels of XIAP go along with poorer survival and invasiveness corresponding to an advanced Duke’s stage[194], in clear cell renal carcinoma it is linked to aggressive growth, an advanced state of the disease and a lower 5-year survival rate, too[118, 138]. XIAP is also correlated with poorer survival rates in hepatocellular carcinoma[8] as well as a higher rate in metastasis and recurrence of the tumor[150], and in breast cancer cells, Bockbrader et al. indicated a correlation between the level of XIAP and sensitization to cytotoxic agents[14].

But X-linked inhibitor of apoptosis protein is not only relevant in solid tumors: In hematologic neoplasms like AML, ALL and CLL higher levels were connected to poor response to treatment as well as worse rates of overall survival[79, 165] and progressive disease[63].

In PDAC, too, XIAP is highly upregulated compared to normal pancreatic tissue[92, 110, 152] and thus it seemed to be a promising approach to find new therapies targeting this protein.

Many ways have been described to block the anti-apoptotic functions of XIAP, ranging from antisense oligonucleotides / RNA interference to repress XIAP expression[50] to mimicking the SMAC peptide and thus physically addressing and antagonizing XIAP[50]. In 2002, only shortly after the discovery of small molecules, Vucic et al. for the first time described that cancer cells can be sensitized using new antagonizing agents[182].

The pharmaceutical industry generated several different substances, for example small-molecule peptidomimetics imitating the N-terminal residue of SMAC with
AVPI-IBM as key- and BIR3 domain as target structure[50]. Another group of compounds are phenylurea-based, they address BIR2 domain of XIAP thus restoring effector caspase-3[46].

Both modes of directly blocking XIAP showed activity against a broad range of tumors[101, 144], phenylurea-based compounds turned even out to be able to induce apoptosis without an additional stimulus[92].

In recent years, also bivalent SMAC mimetics entered the stage, exerting much higher binding properties through binding the two functionally important BIR regions on XIAP, BIR3 and BIR1/2[113].

A further potential candidate is embelin, a natural product of the Japanese Ardisia, which turned out as XIAP-antagonizing small molecule, too[128].

In our experiments, we used compounds (kindly provided by Idun pharmaceuticals / Pfizer) derived from Hid, a pro-apoptotic and IAP-inhibiting protein discovered for the first time in lower organisms[132]. It was found to have higher binding properties than human SMAC because of a differing chemical structure that enhances its affinity for BIR3 domain of XIAP[132].

XIAP was regarded as kind of an “Achilles’ heel of cancers”[76], suggesting great potential in antagonizing this anti-apoptotic protein and indeed, numerous tumor cells were shown to be susceptible addressing XIAP, amongst them with colorectal, prostate and breast cancer some of the most common types of tumors in humans[144].

For pancreatic carcinoma also multiple studies have been designed with promising results in vitro in respect to overcoming apoptosis resistance using antagonization of XIAP:

Pre-clinical trials with RNA interference to keep down XIAP levels showed sensitization to different chemotherapeutic drugs[110]; one of these antisense oligonucleotides already entered phase I in combination with gemcitabine in metastatic pancreatic cancer[111].

Concerning SMAC mimetics, multiple pre-clinical studies provided evidence of their effectiveness in combination with TRAIL[159], gamma irradiation[55] or cytostatic drugs[46], even if a real milestone could not be set by now.
4.1. Drug resistance in pancreatic ductal adenocarcinoma

The first part of our work was to overcome innate resistance of the seven cell lines by testing increasing dosages of doxorubicin, 5-FU, gemcitabine, VP16 and taxol (results not shown separately). What we found out was a heterogeneity in responding to the different agents used and moreover, the cells turned out to be quite resistant as is indicated by in part very high concentrations needed in order to get a dose response (figures 4 - 10). Chemoresistance is a challenging feature of pancreatic ductal adenocarcinoma and formed the first barrier in our experiments as IDUNs[132] do not work properly without a death-inducing stimulus[47, 181].

Many determinants seemed to contribute to resistance of pancreatic malignancy: If we follow the way of the drug from point of injection to its final destination, i.e., the apoptotic machinery, we face many factors on multiple levels that are able to impair the efficiency of a cytostatic ranging from altered drug uptake to differing metabolization outside and within the target cells to enhanced expression of competitive genes, which lead to an excessive supply of survival mediating proteins in comparison with pro-apoptotic ones[110, 149].

It is worth mentioning that gemcitabine, the standard chemotherapeutic agent in PDAC, was one of the most less effective drugs concerning diminution of cell viability in our seven cell lines tested (figures 4 - 10), in Panc1 there was hardly any measurable effect (figure 6). In most gemcitabine-treated cell lines we detected from a certain drug concentration on no more additional effect with higher dosages, neither in the controls nor in the IDUN-treated group (figures 4 - 10, sub-item c in each case), so that we had to assume limited cytostatic concentrations in the biophase. Drug uptake via specific transporting molecules can be disturbed and may play a decisive role in resistance of cancer cells: Several studies showed hENT1 to be essential for intracellular drug uptake of nucleosides, like gemcitabine, which are hydrophilic, and thus cannot pass membranes by diffusion [5, 129, 149, 172]. Differential expression of hENT1 may be the reason for resistance to gemcitabine
as we could see in our experiments most clearly in ASPC1, Panc1, PaTuII and PancTu1 (figures 4, 6, 8 and 9), what was already shown in part by Masanori Tsuji[172].

Talking about resistance, we also have to think of efflux transporters, like p-glycoprotein to name one of the most famous ones[22]. They are present in virtually every (cancer) cell and confer resistance to a vast amount of structurally differing cytotoxic drugs[22]. It is very probable that these efflux pumps played a role in our experiments and contributed to the fact that there were partially very high dosages necessary to get a dose response or to circumvent this barrier, respectively (figures 4 - 10).

However, it is not only transmembrane transport mechanisms but also intracellular metabolism that could be decisive for treatment failure: For gemcitabine, as prodrug, enzymes metabolizing it to its active di- or triphosphate form, are important pharmacodynamic factors and may be limiting concerning efficacy of the drug[149]. Deoxycytidine kinases activate gemcitabine, meaning that a lack or low expression of this enzyme could contribute to chemoresistance[129].

In addition, overexpression of target proteins of the cytostatics used in these experiments, might lead to resistance: Gemcitabine targets RRM1, an enzyme involved in DNA synthesis and -stability in tumor cells: elevated levels can go along with therapy failure and consequently less favorable survival rates[124]. Fryer et al. demonstrated high expression of pERK to be associated with higher gemcitabine resistance in Panc1 cells compared to BXPC3 cells, whereas MiaPaCa2 were, probably because of relatively low pERK expression levels, most sensitive to this drug[45]. In our experiments, Panc1 cells indeed did show hardly any response to gemcitabine treatment (figure 6), whereas MiaPaCa2 were comparably easy to sensitize (figure 5) so that we could confirm these data.

KRAS mutations are very common in pancreatic ductal adenocarcinoma[134], Panc1 with mutated KRAS was described to be rather resistant towards gemcitabine[96], which goes along with results of a rather less sensitive cell line in our experiments (figure 6), whereas BxPC3, a known wild-type KRAS pancreatic carcinoma cell line[78], responded relatively well to gemcitabine treatment showing a regular sensitization in this study (figure 7). This highlighted KRAS as additional factor contributing to chemoresistance of gemcitabine treated cells.
Kalluri and Weinberg pointed out cancer cells with phenotypic features of epithelial-mesenchymal transition are supposed to be less differentiated and more resistant to chemotherapeutic or gemcitabine treatment, respectively[90]. Based on this and research from Kim Y., Panc1, a cell line expressing rather mesenchymal features, was, as already mentioned, one of the most resistant cell lines tested here (figure 6)[90, 96].

To which extent the different mutations and alterations contribute to chemoresistance, is not quite clear, it is possibly the complexity and diversity of all the mechanisms mentioned above that finally lead to a highly resistant cancer.

As an increase of mitochondrial outer membrane permeabilization is necessary in order to induce the intrinsic apoptotic pathway[50], deregulated pro- and anti-apoptotic proteins of the Bcl-2 family form a further complex in resistance mechanism of transformed cells: Upregulation of Bcl-2 or Bcl-X\textsubscript{L} go along with higher resistance towards anticancer treatment[15] as well as a shorter survival of patients[44]. In contrast, overexpression of Bax turned out to be an indicator for treatment response of ASPC1 to gemcitabine and 5-FU[149].

Compared to gemcitabine, the majority of our cell lines were less resistant towards 5-FU (figures 4 - 10), another nucleoside analogue and potentially hENT1-using drug. This could on the one hand be explained by findings of Masanori TsuJe, who claimed 5-FU seems to be more relevant for de-novo-synthesis of nucleosides whereas gemcitabine is supposed to act primarily upon the nucleoside salvage pathway and thus could be more dependent on transmembrane uptake of DNA-forming units[172].

On the other hand, function of 5-FU is influenced by differing transporting proteins like multidrug resistance proteins 5 and -8, whereas p-glycoprotein does not seem to play a pivotal role in 5-FU resistance[65].

Furthermore, there is a complex of important enzymes for 5-FU metabolism: Thymidilate synthase, dihydropyrimidine dehydrogenase, which catabolizes the biggest part of 5-FU during liver passage, and methylenetetrahydrofolate reductase as well as thymidine phosphorylase, which primarily activates 5-FU, or capecitabine to 5-FU, in tumor tissue[187]. These are known key enzymes, whose
up- or downregulation in the wake of tumorigenesis can not only decide whether there is an appropriate treatment response, but also if serious side effects may occur because of too high dosages of the drug acting in non-cancerous tissue[130, 131, 143, 187].

In each of our cell lines we got a sensitization with 5-FU (figures 4 - 10), this could mean that pancreatic cancer cell lines used in this study favor uptake of this nucleoside, but these results may not be very representative for people suffering from this type of cancer, as in vitro models exclude various pharmacokinetic aspects like the first pass effect that may weaken a 5-FU therapy considerably.

Another reason for cell lines to react differently on treatment with diverse cytostatics may be that each drug seems to induce a certain response following DNA damage, which finally leads to activation of different pro-survival cascades: Liu et al. showed in prostate cancer cells, PI3K/Akt-pathway selectively influenced cytotoxicity induced by microtubule-addressing agents like paclitaxel / taxol, whereas the effect of other drugs, in this work, for instance, doxorubicin, were not altered[109]. After inhibition of the PI3K/Akt-pathway, the prostate cancer cells were sensitized towards paclitaxel treatment whereas the other drugs did not generate better results: This suggests that different modes of cytotoxic action favor different pro-survival pathways, in case of paclitaxel possibly because its mechanism differs considerably from the way, many other drugs work as it addresses the microtubules[109].

In our experiments, paclitaxel was one of the weakest drugs in inducing apoptosis, this could be a hint for an augmented activation of PI3k/Akt-pathway in our cell lines or a loss-of-function mutation in genes encoding for its counterpart: PTEN[16]. Bondar et al. already stated the role of PI3K and Akt in chemoresistance of pancreatic cancer cells[16].

Xiaolei Wang showed in his work from 2013 in ovarian cancer cells several mechanisms leading to resistance to paclitaxel, some were similar to those described in connection with other drugs and some were specific for this microtubule-stabilizing drug[188]. A differential expression of the class III β-tubulin isotype seemed to be a major reason for resistance to paclitaxel[188], whether this was the case in our PDAC cell lines, too, might be clarified by further experiments.
In ovarian cancer, mutation of BRCA1 gene was also reported to be engaged in resistance to paclitaxel / taxol[162, 200]. As this gene is not only associated with ovarian and breast, but also prostate cancer and pancreatic carcinoma, it might be another reason for impaired response rates upon treatment with paclitaxel in our experiments (figures 4 - 10).

Further research is needed to check for BRCA1 mutations and to identify patients with BRCA1 wild-type that might be more susceptible for taxane treatment.

For resistance to etoposide / VP16, we found explanation in a work from Nath S. et al.: They showed in Capan1 and BXPC3 cell lines the importance of the mucinous transmembrane glycoprotein MUC1 (CD227), which is overexpressed in many pancreatic cancers and connected to regulation of multidrug resistance genes[125]. Yet, we detected especially for gemcitabine in Capan1 a kind of resistance that could be easily explained with upregulated efflux pumps, as from relatively low dosages up to extensively high drug concentrations, there was no additional cytotoxic effect (figure 10c). The fact that VP16 treatment in this cell line lead to a clear dose response (figure 10d) suggests that it is probably not only transmembrane transporting but also drug-specific effects that have to be taken into consideration explaining resistance.

Many different studies examined resistance of pancreatic cancer cells on doxorubicin, for example Lopes et al., who pointed out NF-kB as one of the most important reasons for chemoresistance in pancreatic cancer: NF-kB upregulates different IAPs leading to resistance to doxorubicin, paclitaxel and 5-FU amongst others[110]. This study also suggests that regulation of apoptosis is a hallmark of PDAC and that this phenomenon is not dependant on an upregulation of a peculiar IAP, but originates on a very heterogeneous expression level of the known human IAPs in this type of cancer[110].

Doxorubicin is an anthracycline antibiotic used for anticancer therapies[38]. This reminds us that resistance is an evolutionary developed mechanism probably having helped (micro-) organisms to survive for many years despite of efforts to fight them, and as anticancer treatment has many parallels with antimicrobial therapies, cure of cancer seems a quite challenging goal.
Taken together, we can state that resistance to chemotherapy, which is a necessary precondition for additional treatment with our small-molecule XIAP antagonists (IDUNs), is a multifactorial process involving drug delivery to its intracellular targets, metabolism of the cytostatics, action of the activated drug on intracellular pro-survival pathways and the expression levels of a vast majority of target- as well as anti-apoptotic proteins within a tumor cell.

Apart from that, in our in vitro models another important aspect has not even been taken into consideration: the tumor microenvironment, which is addressed lower in this work (section 4.5).

4.2. Suppression of XIAP with SMAC mimetics seems to be effective only in few selected pancreatic carcinoma cells treated with cytostatics

The second section was on efficiency of the small-molecule XIAP inhibitors, called IDUNs[132], when PDAC cells were treated with these compounds after having been stimulated with five drugs differing in mode of action, amongst which is the up-to-date standard systemic therapy gemcitabine[29] and with 5-FU the most widely used drug in gastrointestinal cancers[172] as nucleoside drugs, doxorubicin as intercalating substance and topoisomerase-II-inhibitor etoposide / VP16 as well as taxol / paclitaxel - a microtubule-interfering agent.

The task was to clarify the efficacy of combining conventional drugs with a form of targeted therapy aiming to overcome the innate resistance of pancreatic ductal adenocarcinoma cells and to select compounds that exert transferable results for clinical interventions by enhancing apoptosis.

Vogler et al. showed that XIAP is expressed in the seven pancreatic carcinoma cell lines we used in our work, too[181]. This study demonstrated, IDUNs are able to sensitize PDAC cell lines for apoptosis, but only if they are stimulated with TRAIL, there was no sensitization when chemotherapy was applied in PaTuII and PancTu1 cell lines. On the other hand, Giagkousiklidis et al. stated that IDUNs can sensitize pancreatic cancer cell line ASPC1 for irradiation-induced apoptosis[55].
If we have a look at our first experiments (figures 4 - 10), we observed that combination of chemotherapy and XIAP antagonists showed promising results only in few selected pancreatic cancer cell lines: in ASPC1, that Giagkousiklidis et al. already sensitized to gamma irradiation-induced apoptosis using IDUNs[55] and in MiaPaCa2, a cell line formerly described as rather sensitive[45, 110]. These two turned out to be susceptible for treatment with XIAP-antagonizing IDUN 13483 combined with varying cytotoxic agents (figures 4 and 5), whereas the majority of the other cell lines tested did not respond at all or not in a way that was worth following further investigation (figures 6 - 10).

ASPC1 was most sensitizable when treated with doxorubicin or 5-FU and IDUNs (figures 4a and 4b). We observed a dose-dependent effect of doxorubicin and 5-FU suggesting that XIAP took a central role in apoptosis resistance in this cell line: once the threshold was exceeded, pro-apoptotic effects of the cytostatics emerged entirely and the anti-apoptotic barrier was passed.

We assumed that upstream working anti-apoptotic Bcl-2 proteins like Bcl-2 and Bcl-XL did not seem to be highly upregulated in ASPC1 or MiaPaCa2, respectively, because the concentration of XIAP had to be considered as the limiting resistance factor as after its specific inhibition, the drugs could work according to their concentrations (figures 4 and 5). To proof the weak role of the Bcl-2-family in these two cell lines, concentrations of factors from outer mitochondrial membrane space, like SMAC or cytochrome c, could be determined, however, it was not part of this study.

Although there are many publications that do not ascribe XIAP the critical role in apoptotic resistance[50, 110], we could show that in a subset of cell lines, XIAP can be crucial and in consequence, inhibition of this key molecule could mean that balance inclines towards apoptosis because other important anti-apoptotic factors like c-IAPs are obviously not present in a sufficient amount to stop programmed cell death.

If we follow this point of view, it seems to be like mathematics if a cell undergoes apoptosis or if it is able to circumvent this fate and goes on proliferating and dividing.

In literature, the conclusion of high levels of XIAP remains conflicting as there are
many studies that do not show a clear and unique relationship between high concentration of XIAP and treatment success in different types of malignancies[50]. In prostate cancer, it was even contrary because higher levels of XIAP correlated with less rates of tumor recurrence[147].

As already mentioned, IDUNs antagonize BIR3 domain of XIAP liberating initiator caspase-9 but not effector caspases (figure 2). The effect that we observed in ASPC1 and MiaPaCa2 when treated together with certain drugs, implicates a sufficient SMAC level in the cytosol. This goes along with statements from Seeger et al., who demonstrated that it is not the absolute levels of XIAP expression that determine chemoresistance or -susceptibility but a plethora of molecules that are supposed to influence activity of this central IAP[146].

Beyond that, the ratio SMAC / XIAP has already been described as marker for progression in renal cell carcinoma highlighting the relevance of different levels of pro- and antiapoptotic molecules within a cell[196].

The assumption of adequate SMAC concentrations is another indirect evidence for the weakly present upstream apoptosis inhibitory proteins of the Bcl-2 family, as SMAC is located in the intermembrane space of mitochondria and released dependant on interactions of Bcl-2 member proteins (figure 2).

For clinical practice we could assume from our results that determination of XIAP and SMAC expression or -levels, respectively, could give evidence for response of ASPC1 and MiaPaCa2 to doxorubicin, 5-FU and VP16 (figures 4 and 5), but as we already know from Seeger et al., there are many other factors influencing XIAP and making diagnostics difficult, because a potpourri of participants have to be taken into consideration and the mere level of XIAP is not a reliable marker[146].

In addition, it might be promising to combine different cytostatics like it is performed in many tumor therapy protocols in order to improve treatment response and prevent the formation of resistant cells, because none of the drugs administered was efficacious in all cell lines and gemcitabine, the standard of care for present day treatment, even showed the worst results (figures 4 - 10).
Apart from ASPC1 and MiaPaCa2, addition of IDUNs could not sensitize other cell lines to an extent, from which we could derive a promising therapeutic approach (figures 6 - 10). We either saw no sensitization at all, like in BxPC3, which contrariwise was one of the least resistant cell lines tested as drug concentrations were comparably low (figure 7), or only a weak reduction in cell viability. This underlines the - maybe most important - supposition that XIAP is not the central IAP in apoptosis regulation in pancreatic carcinoma[50, 110].

This goes along with what was already postulated: other IAPs, e.g. c-IAPs, seem to be equally or even more important than XIAP[50, 110]. Lopes et al. described survivin and c-IAP2 being most often upregulated in PDAC[110]. If we take into consideration results from Vogler et al., who illustrated that in ASPC1 survivin is expressed to a far lesser extent, if it is expressed at all[181] and have a closer look at most of our cell lines that did not respond to IDUNs (figures 4 - 10), the importance of XIAP as central IAP is weakened additionally and we have to raise the question, if it is sufficient to antagonize solely this IAP and leave other apoptotic proteins aside.

Wang Z. et al. pointed at another aspect of survivin and its role in apoptotic cell death: it is to suppress caspase-9 and ML-IAP via different modes[189]. So if we call in mind the mechanism of action of IDUNs, liberating initiator caspase-9, cells lacking survivin should respond highly on agents addressing the BIR3 domain of XIAP in a competitive way[189].

C-IAPs, as another group within the IAP family, were already shown to be probably more important in apoptosis resistance compared to XIAP[126]. MiaPaCa2, the second cell line with promising results in respect to sensitization for chemotherapy-induced cell death via small-molecule XIAP inhibitors in our study (figure 5), was characterized to express c-IAP2 at very low levels, whereas Capan1, here widely resistant to XIAP inhibition (figure 10), seemed to show rather elevated c-IAP2 levels according to Lopes et al. pointing on the relevance of this IAP[110]. Moreover, c-IAP2 expression was connected to 5-FU and doxorubicin resistance[110] and in ASPC1 and MiaPaCa2, we could detect a quite good dose response with these two drugs suggesting rather low c-IAP2 levels
within these cell lines (figures 4 and 5).

Taken together, we could confirm results from other studies that claim tumor cells in general, and PDAC as special case, are mostly not dependant on upregulation of a specific IAP but overexpress many IAPs making them far more difficult to attack via these pathways[98, 110].

Vogler et al. and Lopes et al. stated, pancreatic cancer cells harbor high levels of caspases, which represent innate suicide programmes as feature of (formerly) normal cells, but those are counteracted by upregulated IAPs in transformed cells making these survive[110, 181]. This could emphasize that IAP-addressing may be a promising goal, but, as our results did show, too, it is hardly to be reached by focusing on only one IAP.

As a consequence and derived from our results by single targeting strategy of IDUNs addressing solely one BIR domain, a more effective inhibition is supposed to be obtained by newly designed, bivalent compounds, which have already entered clinical trials[50]: These molecules are formed by two connected peptides mimicking natural SMAC and having pharmacokinetic and pharmacodynamic advantages because they bind to BIR3 and BIR2 with higher affinities and in turn lead, in case of XIAP, to liberation not only of initiator caspase-9 but also effector caspase-3 and -7. This would theoretically make cells more independent of SMAC concentrations or high levels of anti-apoptotic Bcl-2 proteins, because the point of action is localized downstream of mitochondria and DNA damage.

Yet, whether bivalent compounds are more effective in combination with chemotherapies than monovalent XIAP inhibitors has to be clarified in further experiments. At least for TRAIL-induced apoptosis, there should be a potentially higher efficacy not only in cells, in which XIAP forms a main anti-apoptotic protein but also if we take into account that these compounds are to reduce c-IAP levels significantly, what could enlarge their potential considerably[50, 176].

Because of the high chemoresistance of PDAC, it might also be more promising, if compounds induced apoptosis themselves without agents that initiate the intrinsic apoptotic pathway: this has been shown for phenylurea-based compounds, which acted without an external stimulus[46]. This approach is based on studies of Wang
Z. and Schimmer A. D., who demonstrated that those compounds liberate effector caspase-3, meaning they act more downstream in contrast to IDUNs and thus can initiate apoptosis more easy than compounds dissociating initiator caspase-9[144, 189].

We know that endogenous IAP inhibitor SMAC or its expression, respectively, correlates with prognostic markers in different tumor entities[50]. As IDUNs are dependent on a co-factor dissociating effector caspases from BIR1/2 domain, it is possible that differentially expressed SMAC is another reason for some cell lines not to be sensitizable for IDUN treatment in our experiments while others, possibly more SMAC-containing cell lines, consequently showed higher rates of apoptosis (figures 4 - 10). Quantitative analysis of different proteins or expression on mRNA level could be helpful to back this assumption.

For future therapies against PDAC, individualization will be helpful or even necessary, if we want to get better survival rates and therapy response. We observed within 7 cell lines very heterogeneous results, Bayer Lopes et al. already named PDAC a malignancy with various molecular abnormalities[110] and if we do not want to stay for another several decades in the role of spectators facing this type of cancer but improve therapy, we have to find out more about tumor biology on molecular level and maybe for each patient separately, in order to address the proteins that dominate a certain subtype or a specific phenotype[26].

In a study of Collisson et al., at least two subtypes of PDAC (classical and quasi-mesenchymal and possibly an exocrine-like as third one) were described according to expression of certain subtype-defining genes[26] delivering further possible explanations, why results in our experiments were not quite promising. The authors also emphasized the importance of getting deeper insights into the genome of pancreatic carcinoma cells within a tumor network, as they derived therapeutic relevance from different subtypes: Correlating their results to a work from Moore M.J. et al. in 2007, pancreatic carcinoma cells attached to the classical subtype seemed to be more responsive to targeted therapy with tyrosine kinase inhibitor erlotinib whereas the mesenchymal subgroup of cells yielded better results after conventional gemcitabine treatment[26, 122].
In their work “comparative proteomic profiling of pancreatic ductal adenocarcinoma” Kim Y. and colleagues detected more than 1900 proteins of whom over 700 were differentially expressed in Panc1 and BXPC3 cells highlighting again the complexity of pancreatic carcinoma[96]. Based on these findings it seems almost impossible to treat this highly devastating disease with only 1 drug or 1 compound, respectively, at least when there is no profile of the genomic alterations or not sufficient information about the phenotype.

Given the theory of different drug responsive phenotypes of cancer cells according to their genetic configuration, we could easily explain our experiments herewith as none of the pancreatic carcinoma cell lines reacted like the other (figures 4 - 10), some showed response to all drugs, some were hardly chemosensitive no matter what drug we used and in between the two extremes, we observed a potpourri of different survival rates as a consequence of co-treatment with XIAP-antagonizing IDUN compounds and cytostatics.

4.3. Involvement of caspases in drug-induced cell death in PDAC

We demonstrated that XIAP inhibition increases drug effects at least in few selected pancreatic ductal adenocarcinoma cell lines, whereas the majority did not respond to small molecules aiming to inhibit XIAP (figures 4 - 10).

In ASPC1 and MiaPaCa2 we wanted to clarify, whether caspases are involved in drug-induced cell death in vitro.

Vogler et al. already pointed out that in certain PDAC cell lines (PaTuII and PancTu1), drugs seem to induce modes of cell death different from apoptosis, which might not be the major death pathway in this tumor entity and thus cell death is neither dependant on caspases nor can it be influenced by XIAP antagonization weakening the role of these death executioners and XIAP as relevant factor and potential therapeutic approach, respectively[181].
As ASPC1 and MiaPaCa2 showed a good response on XIAP inhibition in our starting experiments (figures 4 and 5), we examined for caspase involvement in these cell lines (figures 11 and 12).

Our results indicate that caspases seemed to contribute significantly to cell death by cytotoxic agents as in these two cell lines, the IDUN[132] effect could be cancelled, if caspases were inhibited by the pan-caspase inhibitor zVAD.fmk (figures 11 and 12). Based upon these results we can claim that ASPC1 and MiaPaCa2 cell lines exert cell death upon drug treatment in a caspase-dependant manner, however, other forms of cell death seem to play a bigger role accounting for more than 50% of dead cells in ASPC1 (figure 11).

In MiaPaCa2, caspase-dependant cellular death was also present in a significant or even highly significant number, and in some experimental settings even accounted for the same rate of cell death like other forms (figure 12).

So, even in these two quite well sensitizable cell lines, other forms of cell death obviously also take place, as by zVAD.fmk the cytotoxic effect could not be restored completely (figures 11 and 12). The non-apoptotic forms were in most of the settings in our experiments even present to a higher degree compared to apoptosis itself.

These findings go along with what many authors already stated: in PDAC, non-apoptotic modes of cell death play a decisive role[54, 64, 174, 181]. It is only two out of seven tested cell lines supposed to yield usable results in this context and in so far, we had to realize that the majority of PDAC cell lines do not undergo caspase-mediated cell death after XIAP inhibition following stimulation by drugs.

But at least for in vitro experiments, successful treatment with small-molecule XIAP inhibitors could be achieved in pre-selected settings, however, criteria for sensitivity or markers for responsiveness are not defined so far.

Even if our results are not transferable into clinical trials, they may be helpful in future research to enlighten, why some cell lines do respond on XIAP inhibition and in how far it could be possible to pre-select patients for therapy using diagnostic markers to filter them from the mass of people suffering from PDAC.
4.4. Drug-induced cell death in XIAP-inhibited PDAC cell lines

To further investigate, whether the detected caspase-driven cell death leads to morphological changes connected to apoptosis, we tried in the next section of this study to check for DNA fragmentation (figures 13 - 18). Again, we limited our experimental setting on the two cell lines that were sensitizable for XIAP inhibition: ASPC1 and MiaPaCa2.

What we detected was rather conflicting than uniform: In ASPC1, doxorubicin and 5-FU seemed to lead to apoptotic cell death (figures 13 and 14) as we would have expected from results of our previous experiments with caspase involvement and significant reduction in viability (figures 4 and 11). We can derive that in 5-FU treated cells, non-apoptotic cell death dominates (figure 14), whereas doxorubicin-induced cell death is in most part of apoptotic nature according to the corresponding morphological features (figure 13). This goes along with studies that claimed, inhibition of caspase activity can initiate a switch from apoptotic- to necrotic cell death[102]. Results from ASPC1 treated with 5-FU and IDUNs[132] showed a similar outcome, as apoptosis is not the dominant feature. The reason could be a lack of ATP based upon a low oxygen supply of the cancerous cell, as programmed cell death is supposed to require more energy carriers[53].

Contrary to ASPC1, we only got results indicating specific apoptosis in MiaPaCa2 cells when treated with doxorubicin (figure 16), treatment with VP16 and gemcitabine (figures 17 and 18) also lead to apoptotic cell death or DNA fragmentation, respectively, but this effect did not seem to be dependent on XIAP inhibition, as the control cells ran into DNA fragmentation, too, and to nearly the same extent.

One explanation might be that, according to Kroemer et al., apoptosis can occur in vitro by non-caspase-dependant mechanisms or in the wake of multiple intracellular stress conditions, so that the specific reaction on XIAP inhibition could be masked by unspecific effects[100]. Another possibility is that by freezing and thawing of the cells for experiments, they undergo a kind of accidental cell death because of harsh environmental conditions, and, apart from that, it is also imaginable that crosstalks between pro-survival and pro-apoptotic signaling cascades lead to induction of cell death and morphologic traits of an apoptotic
What we also know is that cells being in a state of mitotic catastrophe, for example, in part show morphological features of apoptotic and necrotic cells, too[53].

Moreover, cells presenting with DNA fragmentation are not necessarily dependant on caspases: So even if MiaPaCa2 cells treated with VP16 or gemcitabine did not show remarkable caspase involvement, there could nevertheless occur DNA fragmentation to a considerable degree: several authors showed caspase-independent fragmentation of DNA induced by AIF or endonuclease G[53, 88, 105], pointing out again that different ways can lead to apoptotic cell death.

Yet, in MiaPaCa2 stimulated with VP16 and gemcitabine, we would have expected more reduction in cell viability in the previous experiments (figures 5 and 12) so that this explanation is rather not applicable on VP16 or gemcitabine treatment additionally to XIAP inhibition in this cell line.

For doxorubicin it is interesting that according to Eom et al., different drug concentrations are supposed to lead to different modes of cell death[38]: Rather high doses induce apoptosis, while lower doses tend to initiate a senescence-like state that finally leads to mitotic catastrophe.

So we can conclude, it is not only different agents that lead to a different cellular response and make experimental results hard to interpret, but also within the same drug, there could be differences in the way a tumor cell reacts upon varying concentrations.

And in the end, Galluzzi et al. only recently postulated, morphological traits established in dying cells as marker for a certain mode of cell death become critically questioned as new definitions of cellular demise according to biochemical features emerge[53]. In so far, we have to interpret critically our results from the Nicoletti experiments examining for DNA fragmentation as feature of apoptotic cell death (section 3.3, figures 13 - 18).
4.5. Perspectives for future therapy of pancreatic ductal adenocarcinoma

Future therapies of PDAC will have to consider molecular or genetic profiles, like it is already state-of-the-art for other tumors like breast cancer (HER2/neu, ER, BRCA)[25], colorectal cancer (KRAS, EGFR) or NSCLC (EGFR, ALK)[89], in order to get indications to dominant resistance-granting factors and to specify therapy to individual phenotypic or molecular biological features.

A suitable approach for pre-treatment diagnostic are liquid biopsies: Montagut et al. showed that in patients with colorectal cancer, circulating tumor DNA extracted from blood can be examined and thus an evaluation in respect to probability of response to therapy is possible[121]. And if we take into account findings from Pantel K. et al., who pointed out that genetic profiles of distant metastases differ from the one present in the primary tumor, the necessity of further improvement of pre-treatment diagnostics becomes more and more evident[135]. Solid biopsies of a primary tumor, which has been performed for decades, are on the one hand often easier to get because of size and localization of the primitive tumor, but on the other hand, they have to be considered as not that significant in progressive cancers[135].

So it will probably be only a question of time, when liquid biopsies fully enter and revolutionize tumor diagnostics.

Gene profiling and subdivision based upon genetic variations have already been an established and obligatory criterion for classification and sub-classification in hematologic malignancies for years[2]: Prognostic assessment, risk stratification and therapeutic consequences improved considerably with the detection of bcr-abl gene in the wake of a t(9;22) translocation in chronic myeloid leukemia as shining example[115, 166].

In PDAC, the subtypes labeled by Collisson et al. are also supposed to have prognostic relevance challenging the present-day classification according to tumor size, lymphonodal and systemic metastasis as well as differentiation[26]. Establishing genetic profiles in PDAC as suggested in a study, in which expression levels of S100A2 calcium-binding protein were investigated, could even evaluate postoperative outcome in advance of a pancreatectomy[12].
If expression levels of XIAP can function as biomarkers in PDAC like Shi et al. postulated for hepatocellular carcinoma, is unclear but represents another effort to improve pre-therapeutic diagnostics[150].

In respect to therapy based on genetic findings, we know that BRCA1 or -2 mutations can lead to PDAC, and we also know that breast- and ovarian cancers harboring these mutations, are supposed to be susceptible for platin-based therapies and maybe PARP-inhibitors, too: A phase II study is on its way, testing gemcitabine and cisplatin in combination with the PARP inhibitor veliparib[20].

What we further have to keep in mind especially for in vitro research is what Hanahan and Weinberg pointed out: Tumors have to be considered not as an aggregation of cells with the same capabilities in respect to proliferation, autonomous growth and evading apoptosis but as a heterogeneous construct with different subpopulations of cancer cells that emerged by spontaneous mutations and a tumor promoting microenvironment[69]. The fibrotic surrounding is supposed to be another, in vitro not present resistance mechanism, which, on the other hand, can offer further therapeutic options, as soon as we have understood the interactions with all its participants and communication features like cytokines, receptors and the stromal cells[69].

With its immunomodulator simtuzumab, biotechnology enterprise Gilead already tested an agent addressing stromal cells in phase II trial, unfortunately with deceiving results so far[20], but this approach has certainly to be kept in mind.

If we take into consideration that pancreatic cancer is a tumor with one of the strongest surrounding and protection by stromal cells[195], it will moreover be helpful to increase concentrations of any cytotoxic agent at its point of action in order to overcome chemoresistance: Maksimenko et al. showed that the gemcitabine-prodrug conjugated to squalenoyl resulted in nanoassemblies of formed vesicles in blood, which were protected from being metabolized by blood deaminases, which normally inactivate the drug considerably[112]: An increased antitumor activity compared to gemcitabine applied conventionally was shown in resistant MiaPaCa2 xenograft models. In how far the metabolization component was responsible for better results or if the chemically modified gemcitabine just showed a stronger binding to the target structures, yet remains unclear.
Encapsulation of drugs, like it has already been performed with doxorubicin incorporated in liposomes[22], is an upcoming branch in research. Nowadays, it is performed by nanoparticles containing adsorbed cytostatics and has already shown to increase bioavailability and decrease toxicity[31]. In a phase III trial, albumin-bound paclitaxel (so called nab-paclitaxel), has proven to be another potential combination for gemcitabine to improve overall survival, a goal, that was up to now only reached by erlotinib, as another form of targeted therapy, combined with the standard cytostatic in metastatic pancreatic carcinoma [75].

Moreover, chemokine receptor CXCR4 is upregulated in PDAC and it is known that stromal cells produce its ligand, CXCL12, excessively, which is in turn responsible for failure of gemcitabine-based therapy[157]. Thus, antagonizing CXCR4 could be a further point of attack in PDAC therapy.

In recent years, another class of delivery systems was found when communication of tumor cells was investigated: Cancers use microparticles to transfer biologic information without direct cell-to-cell contact, similar to plasmides in bacteria transporting resistance genes to other microbes. These microparticles are another new approach in fighting chemoresistance, as their expression level also correlates with lower drug concentrations in the tumor cells[59].

So based on many different approaches for anticancer treatment, future will hopefully lead to effective new therapies.
5. Summary

Pancreatic cancer is highly resistant to current chemotherapy based treatment protocols. X-linked inhibitor of apoptosis protein (XIAP) is in the center of apoptosis resistance by its properties to inhibit both effector and inhibitor caspases.

In this study we focused on sensitizing pancreatic cancer cells for chemotherapy-induced apoptotic cell death using small-molecule inhibitors (‘IDUNs’) of XIAP. These peptide based inhibitors were added to seven cell lines in combination with five different chemotherapeutic agents that were previously shown to be only marginally effective in execution of apoptosis.

With MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromid) tetrazolium reduction assay we investigated cell death in seven chemoresistant cell lines treating them with a combination of five different cytostatics and IDUNs additionally. To further distinguish the mode of cell death executed, pan-caspase inhibitor zvad.fmk highlighted caspase involvement, and finally, DNA staining was performed by propidium iodide and measured via flow cytometry to point out a hallmark of apoptosis.

Our results showed that small-molecule inhibitors of XIAP only sensitize for drug-induced cell death in very few cancer cell lines. At least, we could demonstrate that in those cell lines, in which XIAP inhibition seems to be a promising approach, caspases are involved in the process of cell death and these cells present with morphological features of apoptosis, too.

On the other hand, a large part of non-caspase mediated and non-apoptotic cell death also seems to take place.

We concluded that the heterogeneity of pancreatic cancer and its resistance to therapies still is and probably will be a huge problem for future treatment and that more precise pre-therapeutic diagnostics and individualisation or personalization of treatment, respectively, will be necessary to adequately fight this devastating disease.
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