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Regulation of Mitochondria-dependent Apoptosis in Non-Small Cell Lung Cancer: Implications for Cancer Therapy

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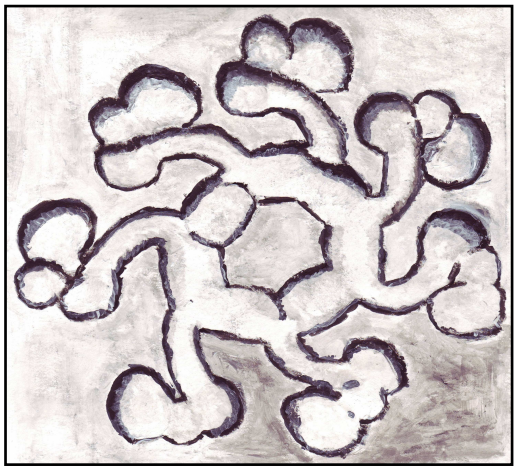

Agnieszka Chęcińska

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Regulation of Mitochondria-dependent Apoptosis in Non-Small Cell Lung Cancer: Implications for Cancer Therapy

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op vrijdag 9 maart 2007 om 15.45 uur
in het auditorium van de universiteit,
De Boelelaan 1105

door

Agnieszka Chęcińska

geboren te Bielawa, Polen

promotor:	prof. dr. G. Giaccone
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	dr. J. Rodriguez

… Nikomu z nas życie, zdaje się, bardzo łatwo
nie idzie, ale cóż robić, trzeba mieć odwagę
i głównie wiarę w siebie, w to, że się jest do
czegoś zdolnym i że do tego czegoś dojść trzeba…

Maria Skłodowska – Curie,
fizyk, nagrodzona dwukrotnie nagrodą Nobla

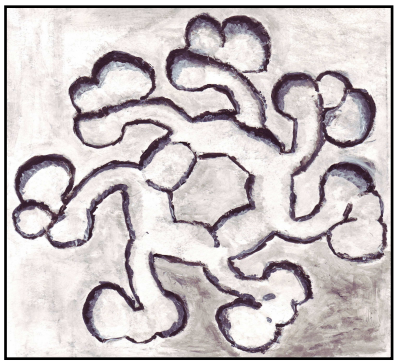
Moim Rodzicom i Dziadkowi Janowi

…You have to have a strength and self-confidence.
You have to believe you have a talent for something
and will achieve results whatever the costs…

Maria Skłodowska - Curie
Polish Physicist,
awarded the Nobel Prize twice

To my Parents and Grandfather Jan

CONTENTS



Chapter 1

Introduction

Summary

The work described in this thesis focuses on the characterization of the mechanism(s) underlying the deregulated activation of the intrinsic or mitochondrial apoptotic pathway in non-small cell lung cancer (NSCLC) cells. Apoptosis suppression might contribute to the chemoresistance often observed in NSCLC patients, and elucidation of the molecular causes of this resistance may provide clues for the development of targeted apoptosis-based therapies. In this introduction, lung cancer and current treatment regimens are described, and mechanisms responsible for chemoresistance, in particular towards cisplatin, are summarized. Furthermore, the major apoptotic pathways are presented, with a detailed description of the mitochondrial apoptotic route and its regulation by Bcl-2 family members and inhibitors of apoptosis. Finally, known mechanisms of apoptosis-dependent chemoresistance and innovative apoptosis-targeting strategies for cancer therapy are discussed.

1. Lung cancer

Lung cancer is the most common cause of cancer death in the world (Jemal et al., 2006) with an overall 5-year survival of 15% (Brundage et al., 2002). In the United States the epidemiologic estimates for the year 2006 predict that 28 % of all cancer death will be due to lung cancer. In men, the incidence of lung cancer has decreased appreciably, but it is still the second most commonly diagnosed tumor type (92,700 new cases) only surpassed by prostate cancer. Among woman, lung cancer incidence continues to increase due to smoking habits (81,770 new cases), and is the most commonly diagnosed type of tumor after breast cancer (Wogan et a., 2004; Jemal et al., 2006). The most predominant cause of lung cancer is cigarette smoking, which accounts for over 90% of lung cancer cases (Bray et al., 2004). Other factors contributing to the onset of lung cancer are workplace agents, such as asbestos, arsenic, chromium, nickel or radon, and also other environmental factors, passive smoking, indoor radon and air pollution. The occurrence of lung cancer is related to race and socioeconomic status. For example, the mortality due to lung cancer among Afro-Americans is higher than among Caucasian Americans. This is likely related to a higher exposure to lung cancer

risk factors in the first group, such as smoking, diet and inhalation of workplace carcinogens (Alberg et al., 2005) and later stage at diagnosis (Schwartz et al., 2003). Studies carried out on the role of diet as a potential risk factor for lung cancer provided evidence that higher dietary intake of fruits or vegetables is correlated with a lower risk of lung cancer (Alberg et al., 1998).

Lung carcinomas are divided into non-small cell lung carcinomas (NSCLCs) and small cell lung carcinomas (SCLCs). NSCLCs are epithelial tumors that represent approximately 80% of lung carcinomas, and include three major histological types: squamous cell carcinoma, adenocarcinoma and large-cell carcinoma. The remaining 20% of lung tumors are small-cell carcinomas (SCLCs) that present neuroendocrine morphological features (Beasley et al., 2005). A worldwide tumor-node-metastasis (TNM) staging system is used in the management of NSCLCs (Mountain and Dresler, 1997). This system takes into account the tumor size, the presence of malignant cells in regional lymph-nodes, and the presence of distant metastases; stage I to IIA represent localized tumors in the absence of metastases, stage IIB to IIIB are locally advanced tumors with metastases in lymph nodes, and stage IV is advanced disease with distant metastases (see also Table 1). In NSCLC more than three quarters of all cases have locally advanced or widespread metastatic disease (stage IIIB and IV) at diagnosis.

SCLCs, on the other hand, are classified according to a simple two-stage system developed by the Veteran's Administration Lung Cancer Study Group (VALCS) (Zelen M, 1973). Limited disease is classified as a tumor that can be included in one radiation port; extensive disease is beyond these boundaries. Patients with a limited disease (LD) represent about 30-40% of SCLCs (Stahel et al., 1989).

1.1. Therapy for lung cancer

Treatment of cancer aims to remove or destroy the cancer cells while sparing normal cells. The most common treatment modalities applied in lung cancer are surgery for, and/or radiotherapy for localized tumors and chemotherapy for advanced or metastatic disease (Minna et al., 2002). Radiation therapy and chemotherapy can be applied as an initial treatment (neoadjuvant therapy) with the purpose to shrink the tumor for complete resection of borderline respectable tumors (stage III). Alternatively, radiation and chemotherapy can be used after surgery for eradication of micrometastases (adjuvant chemotherapy) or prevention of local relapse (radiation therapy).

Table 1. Lung tumor staging (acc. to Mountain, 1997).

Stage	Tumor Node Metastases	General Description	Survival Rate 1 Yr	5 Yr
Local				
IA	T1 N0 M0	T1 tumor: ≤ 3 cm, surrounded by lung or pleura; no tumor more proximal than lobe bronchus	94	67
IB	T2 N0 M0	T2 tumor: > 3 cm, involving main bronchus ≥ 2 cm distal to carina, invading pleura; atelectasis or invading pleura; or pneumonitis extending to hilum, but not entire lung	87	57
IIA	T1 N1 M0	N1: involvement of ipsilateral peribronchial or hilar nodes and intra- pulmonary nodes by direct extension	89	55
Locally advanced				
IIB	T2 N1 M0 T3 N0 M0	T3 tumor invasion of chest wall, diaphragm, mediastinal pleura, pericardium, main bronchus <2 cm distal to crania; atelectasis or pneumonitis of entire lung	73	39
IIIA	T1 N1 M0 T2 N2 M0	N2: involvement of ipsilateral mediastinal or subcranial nodes	64	23
	T3 N1 M0 T3 N2 M0	N3: involvement of contralateral (lung) nodes or any supraclavicular	32	3
IIIB	Any T N3 M0			
Advanced				
IIIB	T4 Any N M0	T4 tumor: invasion of mediastinum, heart, great vessels, trachea, esophagus, vertebral body, crania: separate tumor nodules; malignant pleural effusion	37	7
IV	Any T Any N M1	Distant metastasis	20	1

Tumor resection provides the best treatment for NSCLC. The best survival is obtained in patients who have pathologically confirmed stage I, and it progressively decreases depending on the staging. However, even with complete resection almost 50% of stage I NSCLC will relapse and die of the disease. The use of adjuvant chemotherapy has improved survival of about 5% in patients with stage II and III resectable NSCLC (Arriagada et al., 2004).

Patients with unresectable and metastatic disease may benefit from systemic chemotherapy, but the advantage of palliative chemotherapy is only approximately an increase of 2 months in median survival. NSCLC is a highly refractory cancer and major responses are only obtained in 20-30% of patients and they are short-lived. The backbone drugs of chemotherapy on NSCLC are the platinum compounds cisplatin and carboplatin (Jamieson and Lippard, 1999). Other chemotherapeutic

agents that are often used in doublet combinations with a platinum compound are taxanes (paclitaxel and docetaxel), vinca alkaloids (vinorelbine), and gemcitabine (Table 2) (Spira and Ettinger, 2004).

SCLC is a much more chemosensitive tumor than NSCLC, and chemotherapy represents the mainstay treatment for this disease. Approximately 20% of patients with limited disease can be cured by a concomitant combination of chemotherapy and radiotherapy. Surgery is rarely used in SCLC, because of its high propensity to develop early and wide spread metastatization, in comparison to NSCLC.

1.2. Chemoresistance of lung cancer

Despite an improved understanding of the molecular biology of lung cancer, and significant advances in the treatment, resistance to anticancer therapy is a major cause of treatment failure (Minna et al., 2002). Resistance to chemotherapy can be intrinsic or acquired. NSCLC is frequently resistant to first-line chemotherapy, and the initial response rate amounts to 20-30%. In contrast, SCLC is initially highly responsive to anticancer therapy, with 80-90% response rate, but upon relapse, the response rate to second-line chemotherapy is very low (Board et al., 2006). Furthermore, the problem of multidrug resistance (MDR) arises when tumors develop resistance not only towards formerly used drugs, but also towards other cytotoxic agents with different structure and mechanism of action (Gottesman et al., 2006). Several molecular mechanisms associated with clinical drug resistance have been recognized. These mechanisms include alterations in drug transport systems that decrease intracellular drug accumulation; metabolic resistance that alters activation and inactivation of drugs; alterations of drug-target complexes due to mutations or increased levels of drug targets; increased repair of drug induced damage; alterations in signalling pathways or drug-induced. Lung tumors may develop multidrug resistance and specific resistance to different anticancer agents conventionally used in the treatment.

Table 2. Current regiments treatment of lung cancer (acc. to Spira, 2004).

Type of Study and Group	Regimens	Conclusions
Adjuvant chemotherapy and radiotherapy after surgery	Etoposide, cisplatin, and radiotherapy vs. radiotherapy alone (Keller et al)	No advantage of chemotherapy
	Cisplatin-based adjuvant chemotherapy vs. observation 20 (IALCTCG)	4% absolute increase in overall survival with adjuvant chemo-therapy at 5 yr ($p<0.003$)
Addition of chemotherapy to radiotherapy in inoperable cancer	Cisplatin, vinblastine, and radiotherapy vs. radiotherapy alone (Dillman et al)	Increased survival rate at 1, 2, 3, and 7 yr with chemotherapy (13% vs. 6%)
	Cisplatin, vinblastine, and concurrent radiotherapy vs. cisplatin, vinblastine, and sequential radiotherapy (Curran et al.)	Increased survival with concurrent radiotherapy ($p=0.046$), but increased incidence of esophagitis (25% vs. 4%)
Neoadjuvant chemotherapy in stage IIIA diseases	Etoposide and cisplatin before and after surgery vs. surgery and radiotherapy (Roth et al.)	Increased survival with chemo-therapy (56% vs. 15% at 3 yr)
	Mitomycin, ifosfamide, and cisplatin before surgery and radiotherapy vs. surgery and radiotherapy (Rosell et al.)	Increased median survival with chemotherapy (26 mo vs. 8mo)
Neoadjuvant chemotherapy in stage I, II or III disease	Mitomycin, ifosfamide and cisplatin before surgery and radiotherapy vs. surgery and radiotherapy (Depierre et al.)	No benefits for patients with N2 disease; small survival advantage for patients with N0 or N1 disease at 1 and 4 yr
Chemotherapy for advanced diseases	Cisplatin and paclitaxel vs. cisplatin, cisplatin and gemcitabine, cisplatin and docetaxel, and carboplatin and paclitaxel (Schiller et al.)	Results approximately equivalent for all regimens; most adverse effect with cisplatin and gemcitabine, but also slightly higher survival rate; least adverse effects with carboplatin and paclitaxel
	Carboplatin, paclitaxel, and gefitinib vs. carboplatin and paclitaxel (Johnson et al.)	No advantage for gefitinib when given with standard chemotherapy

1.2.1. Resistance to cisplatin and carboplatin in NSCLC

Platinum drugs are the most widely used anticancer agents for the treatment of human malignances, including ovarian, testicular, bladder, cervical, head and neck

and lung carcinomas (Rozencweig et al., 1977). The antineoplastic activity of cisplatin relies on its ability to bind to DNA and form different types of adducts (Brabec and Kaspasrkova, 2005). The major type of adducts formed by cisplatin are 1, 2-intrastrand cross-links involving two adjacent bases (*cis*-GG and *cis*-AG) that comprise 50% and 28% of all formed adducts, respectively. Additionally, 10-13% of adducts are 1, 3-intrastrand cross-links involving non-adjacent guanines (*cis*-GNG) and interstrand adducts monofunctionally bound cisplatin to a guanine base (Jamieson and Lippard, 1999).

Cisplatin resistance is multifactorial. Mechanisms of resistance include reduced accumulation of the drug, increased intracellular detoxification, increased capability to repair cisplatin-DNA damage, and alterations in the induction of apoptosis.

The changes in intracellular accumulation of cisplatin may result from decreased cellular uptake or increased cellular efflux of the drug (Gorlik et al., 1997). Cisplatin enters the cell by passive diffusion or diffusion dependent on cellular membrane potential. Additionally, the ATP-dependent activation of efflux pump contributes to cisplatin resistance. Moreover, metallothioneins (MT) and glutathione (GSH)-related metabolism enzymes modulate cellular detoxification system. The increased expression of MT stress-response proteins that are involved in detoxification of heavy metals is associated with various cisplatin-resistant tumors. Cisplatin resistance might result from conjugation with GSH followed by inactivation of cisplatin or prevention of cisplatin-adducts formation. The level of GST-π isoenzyme expression was significantly associated with intrinsic resistance to cisplatin in lung cancer cell lines (reviewed in Seve and Dumontet, 2005).

The resistance to cisplatin/gemcitabine in lung cancer cells may also result from enhanced DNA repair (Lord et al., 2002) through .the nucleotide excision repair (NER), the transcription-coupled repair (TCR) or the global genomic repair (GGR) pathways. Moreover, there are evidences that cisplatin-DNA adducts kill cells by induction of programmed cell death, apoptosis (Eastman and Rigas, 1999).Many tumor cell types undergo apoptosis in response to DNA-damaging agents (Sedletska et al., 2005). For example, sensitive tumor cells treated with cisplatin have demonstrated the ability to activate the "caspase cascade" (Gonzalez et al., 2001). It has thus been proposed that alterations in the apoptotic program, such as the inhibition of caspases, may have a significant contribution to cisplatin resistance in tumor cells.

In this thesis the main focus was on defining the molecular mechanisms of cisplatin resistance in NSCLC cells that are related to the inhibition of apoptotic pathways.

2. Apoptosis

Apoptosis is a physiological form of cell death of eukaryotic cells that for the first time was described in the 19th century (Vogt, 1842). In 1972, Kerr and colleagues documented this phenomenon by describing the morphological changes that occur during apoptosis, such as chromatin condensation, cytoplasmic shrinkage and blebbing of the plasma membrane that leads to the formation of "apoptotic bodies" (Kerr et al., 1972). Several molecular hallmarks characterize apoptotic cell death including, hypercondensation of chromatin, cleavage of chromosomes into nucleosomal fragments, and externalization of phosphatidylserine (PS) residues (Strasser et al., 2000). Alterations in the process of apoptosis participate in the pathogenesis of a variety of human diseases. For example, enhanced apoptosis is involved in AIDS, neurodegradative disorders or ischemic stroke (Thompson, 1995). Furthermore, the downregulation of apoptosis has been related to tumor progression and the development of chemoresistance (Hanahan and Weinberg, 2000).

2.1. Apoptosis in tumorigenesis

The accumulation of neoplastic cells can result from enhanced proliferation and/or diminished cell death. Inefficient elimination of neoplastic cells might be a consequence of alterations in the apoptotic machinery (Kaufmann and Gores, 2000). The importance of apoptosis in tumor development was first established based on the observation that the Bcl-2 protein, a product of the *bcl-2* oncogene, did not enhance proliferation, as expected, but promoted cell survival by inhibiting apoptotic cell death (Hockenbery et al., 1990).

An impaired repair of DNA alterations may lead the accumulation of mutations, and result in malignant cell transformation (Hanahan and Weinberg, 2000). However, multicellular organisms have developed mechanisms that protect them against the development of cancer. A key player in this protection is the tumor suppressor p53 protein, "guardian of the genome" (Levine, 1997). Upon cellular stresses, such as DNA damage, hypoxia or aberrant proliferative signals, p53 is stabilized and induces either cell cycle arrest or apoptosis (Vousden and Lu, 2002).

Thus, p53 protects from the accumulation of oncogenic mutations (Brown and Attardi, 2005). The development of multiple cancers is associated with alterations in the p53 pathway (Wallace-Brodeur and Lowe, 1999). Loss or disabling of p53-dependent apoptosis accelerates brain tumorigenesis or promotes lymphomagenesis in mice (Symonds et al., 1994; Schmitt et al., 2002). The p53 protein regulates the expression of multiple genes. Among the p53-activated genes there are many that modulate the apoptotic machinery. Activation of several Bcl-2 family members is dependent on p53. Loss of *p53* gene results in loss of Bax expression and causes accelerated tumor growth (Yin et al., 1997). The BH3-only proapototic proteins, Noxa and PUMA are also activated in a p53-dependent manner. Upon DNA damage caused by hypoxia, DNA damaging agents or ER-stress, PUMA induces a very rapid apoptosis, but in *p53*-knockout mice apoptosis was abolished (Villunger et al., 2003). Additionally, the Apaf-1 gene is modestly activated by p53 in DNA damage-induced apoptosis (Kannan et al., 2001). The p53-dependent apoptosis can also be regulated independently on transcription. In this regard, p53 regulates apoptosis outside of the nucleus by activation of Bax, or by direct permeabilization of the outer mitochondrial membrane and cytochrome c release (Chipuk et al., 2004 and 2005).

2.2. Two major pathways of apoptosis

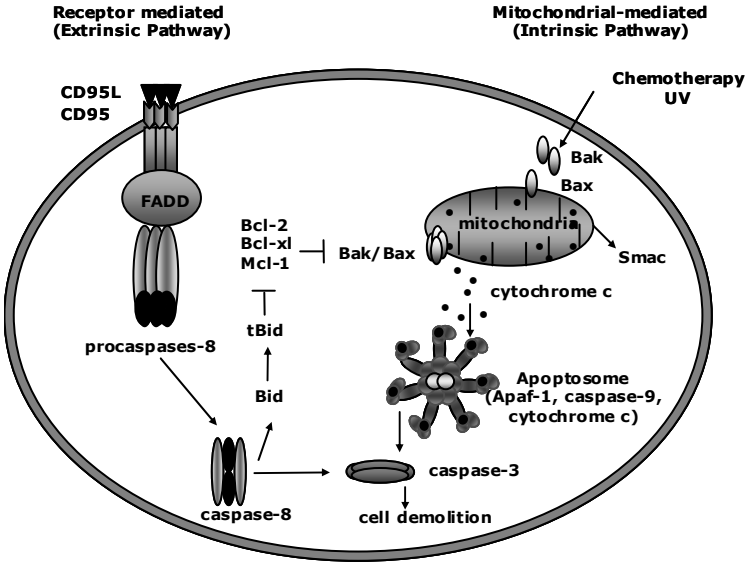
Activation of the apoptotic machinery can be triggered by either of two distinct pathways headed by initiator caspase-9 or caspase-8, respectively (Figure 1). The intrinsic pathway, which involves activation of caspase-9 in the apoptosome, is induced in a mitochondria-dependent manner in response to cellular insults, such as chemotherapeutic drugs or UV radiation (Kuida et al., 1998). Activation of the extrinsic pathway, led by caspase-8 is initiated by binding of a ligand, such as Fas, TNF or TRAIL to cell-surface receptors, termed "death receptors" (DRs). Members of the DR family comprise Fas/CD95, tumor necrosis factor-α (TNF-α) receptor-1, and the TRAIL receptors DR4 and DR5 (Ashkenazi and Dixit, 1999). Upon ligand binding, the adaptor molecule FADD or TRADD is recruited to the intarcellular region of the receptor, and binds multiple procaspase-8 zymogens, which results in the activation of caspase-8 (Salvesen, 1999). Once activated initiator caspases process and activate effector caspase-3 and caspase-7, initiating the demolition phase of apoptosis (Creagh and Martin, 2001). The effector caspase-3 can be activated in two different ways, and depending on the manner in which caspase-3 is activated, cells

can be classified into, type I and II cells. In type I cells activated caspase-8 directly cleaves and activates procaspase-3. In type II cells, activation of caspase-8 via death receptor is below the threshold that could enable the direct activation of procaspase-3 (Budihardjo et al., 1999). In these cells, caspase-8 cleaves Bid, a specific cytoplasmic proapoptotic member of the Bcl-2 family proteins. Cleaved Bid, called truncated Bid (tBid), leads to the disruption of mitochondria and the release of apoptogenic co-factors (see below). In this way, there is a crosstalk at the premitochondrial level between extrinsic and intrinsic pathways that amplifies the apoptotic response (Li et al., 1998).

2.2.1. The caspases

The caspases belong to a family of cysteine proteases that are synthesized as single-chain zymogens with an N-terminal prodomain and very low enzymatic activity (Nicholson and Thornberry, 1997). Upstream or initiator caspases, caspase-8 and –9, have large prodomains, and their activation involves both dimerization and stabilization. Downstream or effector caspases, such as caspases-3, -6 and –7, have short prodomains, and are activated by the initiator caspases through proteolytic cleavage (Earnshaw et al., 1999; Boatright and Salvesen, 2003).

Figure 1. Two major apoptotic pathways.



Initiator caspase-9

The apical protease of the intrinsic apoptotic pathway, caspase-9, is synthesized as a 46kDa zymogen (pro-caspase-9) that is composed of N-terminal prodomain, a large subunit (~20kDa/p20), a small subunit (~10kDa/p10), and a linker region (LR) between the large and small subunits (Thornberry et al., 1997). The long prodomain of caspase-9 contains a crucial protein-protein interaction motif, the Caspase Activation and Recruitment Domain (CARD), which is involved in formation of protein complexes with another CARD-containing protein, such as Apaf-1 (Hofmann et al., 1997). Like other caspases, the processing of caspase-9 occurs by cleavage at aspartate residues between the large and small subunits and the mature enzyme forms a heterotetramer containing two large and two small subunits. Procaspase-9 undergoes either an autocatalytic intrachain processing at Asp^{315} to yield a p35 subunit, or is cleaved at Asp^{330} by active caspase-3 to yield a p37 fragment (Figure 2). Caspase-9 activation is independent on cleavage, and processing of caspase-9 does not guarantee its full activation (Stennicke 1999; Srinivasula 1998). Only the association of caspase-9 monomers with the adaptor protein, Apaf-1 and the dimerization of the caspases ensure its full activation (Boatright et al., 2003).

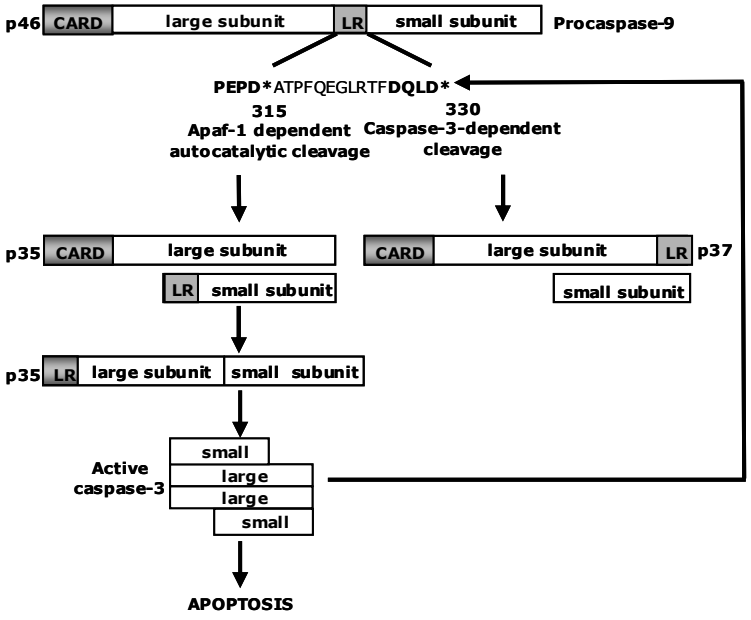
Effector caspases

The downstream caspase-3 and caspase-7 are usually more abundant and more active than the initiator caspases, and are thus considered the "workhorses of the apoptotic cascade" (Hengartner, 2000). Downstream caspases contain a relatively small prodomain, a large (~20kDa/p20) and a small (~10kDa/p10) subunits connected via interdomain linker. The role of the prodomain remains poorly characterized, but it may be involved in the caspase regulation by cellular compartmentalization or spatial sequestration (Meergans et al., 2000). Like initiator caspases, effector caspases are also synthesized as single-chain zymogens, but they exist in the cytosol as inactive homodimers, which are maintained in a latent state by steric hindrances imposed by the interdomain linker (Boatright and Salvesen, 2003). Active initiator caspases promote the activation of downstream caspases by cleavage of this interdomain linker, followed by translocation of activation loop and formation of an active site. The activated effector caspases, in turn, induce processing of many cellular substrates (Kaufmann and Hengartner, 2001).

2.2.2. Apoptosome

The mammalian apoptosome is a large oligomer containing Apaf-1, caspase-9, cytochrome c and dATP (Rodriguez and Lazebnik, 1999). An Apaf-1 heptamer forms the scaffold for the apoptosome (Bao and Shi, 2006). Apaf-1 is a 130kDa protein that contains a CARD-interacting domain on its N-terminal part that binds caspase-9, a nucleotide-binding domain (NBD) that binds dATP/ATP, and a long C-terminal extension composed of 13 WD40 repeats that are involved in the binding of cytochrome c (Hu et al., 1998). Within the apoptosome, Apaf-1 and cytochrome c coassemble to form a wheel-like structure with seven spokes, where the CARD and NBD domains form a central hub (Figure 3) (Acehan et al., 2002; Shi, 2002). The assembly of this polymeric platform is a crucial step in the activation of the intrinsic apoptotic pathway. The presence of extra dATP/ATP forces the exchange of dADP with dATP, and results in formation of an active apoptosome. Otherwise, an inactive aggregate of Apaf-1 is formed (Kim et al., 2005).

Figure 2. Proteolytic processing of caspase-9 and caspase-3 (acc. to Cain et al., 2002).



Two models describing the mechanism of caspase-9 activation on the apoptosome have been proposed. The "allosteric activation" model suggests that the activation of single molecule of caspase-9 occurs upon rearrangements of the active site induced by interactions with the apoptosome. The "proximity induced dimerization" model, on the other hand, proposes that the apoptosome provides a platform to recruit caspase-9 and promote its homodimerization due to the increased local concentration of the caspase 9 (Boatright et al., 2003, Pop et al., 2006). In the context of the dimer, contacts within the catalytic domain of the caspases causes activating rearrangements, such as translocation of the activation loop into the accepting pocket of the neighbouring monomer (Boatright et al., 2003).

In addition, the activation of an apical caspase may also be induced through its cleavage by effector caspases in a feedback loops. However, such activation may only occur when caspase-9 is already present on the apoptosome in a dimeric configuration (Cain et al., 2002; Zou et al., 2003).

2.3. Regulation of the intrinsic apoptotic pathway

A crucial step in the intrinsic apoptotic pathway is the permeabilization of mitochondria (Armstrong, 2006). This process, termed mitochondrial outer membrane permeabilization (MOMP), leads to the release of cytochrome c into the cytosol, and constitutes a "point of no return" in the pathway. The pathway can be regulated both upstream and downstream of MOMP (Verhagen and Vaux, 2002; Green 2003). Regulation upstream of MOMP involves interactions between members of the Bcl-2 family of proteins (Coultas and Strasser, 2003; Adams, 2003). Downstream of MOMP, another level of regulation is provided by the inhibition of active caspases by inhibitors of apoptosis. Finally, caspases are derepressed by Smac/DIABLO (Eckelman et al., 2006).

2.3.1. The Bcl-2 family of proteins and the regulation upstream of MOMP

The MOMP is regulated by interactions between Bcl-2 family proteins and the targeting of some of them to the mitochondria (Cory and Adams, 2002; Danial and Korsmeyer, 2004). This family of proteins is subdivided into three different groups based on their antiapoptotic or proapoptotic nature, and on the conservation of the Bcl-2 homology (BH1-4) domains (Figure 3). Multidomain antiapoptotic proteins include Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1/A1. Multidomain proapoptotic members

are Bax and Bak. Finally, the BH-3 only proapoptotic proteins are Noxa, Puma, Bid, Bim, Bad, Hrk, Bmf, and Bik. Bcl-2 family proteins are regulated at the transcriptional level and also through post-translational modifications (Puthalakath and Strasser, 2002; Kuwana and Newmeyer, 2003).

The antiapoptotic Bcl-2 proteins prevent the release of apoptogenic proteins, such as cytochrome c from mitochondria. On the contrary, the proapoptotic members, Bax and Bak induce MOMP and release of cytochrome c (Cheng et al., 2001; Scorrano and Korsmeyer, 2003). The BH3-only proteins are the most apical regulators of the apoptotic cascade, and are activated upon induction of cell death by various internal or external stimuli (Huang and Strasser, 2000).

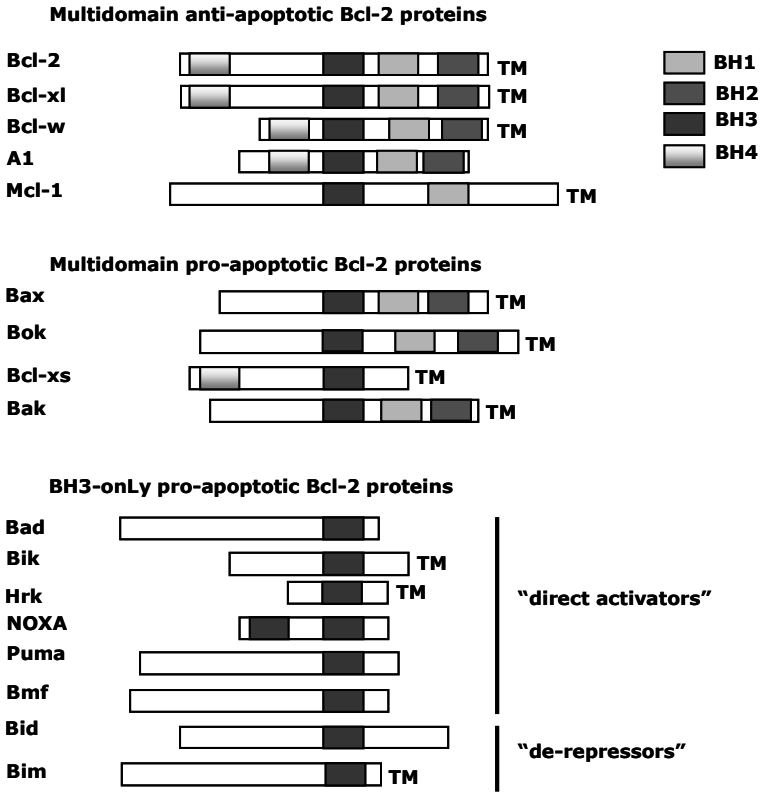
A critical mechanism that regulates the activity of Bcl-2 family proteins is their oligomerization or heterodimers formation. For example, two proapoptotic proteins, such as cytosolic Bax and Bak, a transmembrane protein of the outer mitochondrial membrane, undergo conformational changes upon induction by cytotoxic signals. Then, Bax translocates to the mitochondria and integrates into the outer mitochondrial membrane, where "pore-forming" oligomers of Bax and Bak are created, followed by membrane permeabilization (Lucken-Ardjomande and Martinou, 2005). BH3-only proteins that contain only one BH domain regulate the activation of Bax and Bak (Willis and Adams, 2005). Members of the BH3-only proteins are subdivided in two groups. The first group, termed "direct activators" includes Bid and Bim, which can directly activate Bax and Bak. The second one, termed "de-repressors" includes Noxa, PUMA, Bad, Bik, Bmf and Hrk (Letai et al, 2002). The "de-repressors" are unable to directly activate Bax and Bak, but can sequester the antiapoptotic proteins, such as Bcl-2, Bcl-xl and Mcl-1 and release previously inhibited proapoptotic Bim and Bid and lead to activation of Bax and Bak (Kuwana et al., 2005). This suggests that in the cytosol there are many checkpoints of cellular equilibrium, which upon receiving a signal to commit suicide decide whether to induce MOMP. In conclusion, MOMP depends on the regulation of Bcl-2 family proteins and the activation of Bax and Bak is a fundamental step initiating the mitochondria-dependent cell death.

2.3.2. The Inhibitors of apoptosis and the regulation downstream of MOMP

The intrinsic apoptosis pathway can be negatively regulated by direct interactions between processed caspases and inhibitory proteins (Deveraux et al., 1998; Salvesen and Duckett, 2002). The presence of a CARD domain on procaspase-9 enables interactions with other CARD-containing proteins, including TUCAN

(CARD8/CARDINAL) (Damiano and Reed, 2004). TUCAN has been proposed to selectively suppress apoptosis by interfering with binding of Apaf-1 to procaspase-9 (Pathan et al, 2001). However, other evidence

Figure 3. The Bcl-2 family proteins



suggests that there is no direct interaction between caspase-9 and TUCAN. In addition, downregulation of TUCAN did not sensitize some tumor cells to chemotherapeutic agents (Bouchier-Hayes et al, 2001; Checinska et al, 2006). TUCAN was also shown to regulate activation of caspase-1 in the THP-1 monocytic cell line and stable overexpression of TUCAN sensitized cells to apoptosis (Razmara et al., 2002). Studies conducted in lung and colon cancer specimens revealed that overexpression of TUCAN correlated with shorter patients' survival (Pathan et al, 2001; Krajewska et al., 2005; Checinska et al., 2006). In addition to its proposed antiapoptotic role, TUCAN has been reported to potently suppress NF-kB activation via interactions with IKK gamma and DRAL (Bouchier-Hayes et al, 2001; Stilo et al.,

2002). Thus, the regulation of NF-kB pathway by TUCAN points to a more complex, role of this protein in tumor biology than as a direct inhibitor of caspases.

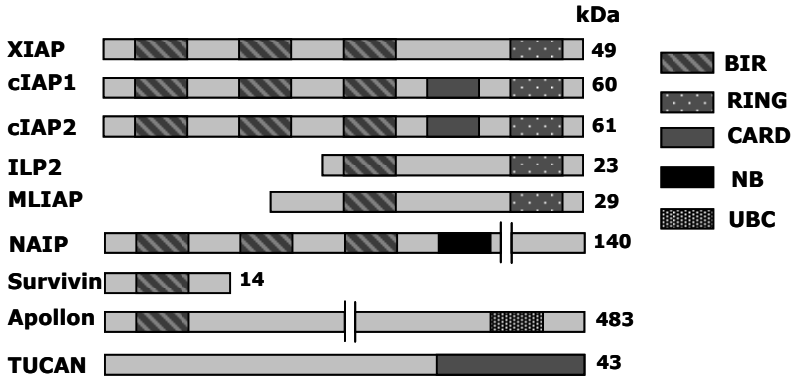
A better-characterized group of apoptotic regulators downstream of MOM is the inhibitor of apoptosis proteins (IAPs). IAPs were initially identified in baculoviruses and demonstrated the ability to inhibit insect-cell apoptosis after viral infection (Crook 1993). In mammalian cells, eight IAPs have been so far identified: XIAP, cIAP-1, cIAP-2, Survivin, ML-IAP/Livin, Apollon/BRUCE, ILP2, and NAIP (Deveraux and Reed, 1999). The IAPs family proteins members are characterized by the presence of one to three baculovirus IAP repeat (BIR) domains (Hinds et al., 1999). In addition to the BIR motifs, some IAPs contain a C-terminal zinc-binding RING domain, which might enable IAPs to polyubiquitylate caspases and target them for degradation by the proteasome (Yang et al., 2000). The cIAP-1 and -2 also contain a caspase activation recruitment domain (CARD), but the functional role of this domain remains unclear. Depending on the presence or absence of the BIR or RING domains the IAP proteins are divided into three classes (Figure 4) (Schimmer, 2004). The first class of IAPs includes X-linked IAP (XIAP), cIAP-1 and -2, ILP2 and ML-IAP/Livin. All these proteins contain one or three BIR domains, one RING motif, and in the case of cIAP-1 and -2 also a CARD motif. In the second-class, NAIP contains three BIR domains and a nucleotide binding motif (NB), but no RING finger (Roy et al., 1995; Maier et al., 2002). To the third class belong the largest and the smallest IAPs, Bruce/Apollon, and Survivin. Bruce/Apollon is an ubiquitin-conjugating enzyme (UBC) which ubiquitylates and facilitates proteasomal degradation of caspase-9 (Hao et al., 2004). The mechanism by which Survivin regulates apoptosis remains controversial (Banks, 2000).

The best characterized of mammalian IAPs is XIAP, which binds and inhibits processed caspases-3, -7 and –9 (Shiozaki et al., 2003; Scott et al., 2005). The processed caspase-9 monomer can be inhibited by XIAP via interactions between its BIR3 domain and the conserved IAP-binding tetrapeptide motif of the caspase. Other weaker interactions also seem to be essential for the efficient inhibition (Sun et al., 2000). XIAP-mediated inactivation of caspase-9 does not physically involve the active site of the caspase. Instead, the IAP traps the caspase and maintains it in an inactive state due to the absence of a supporting fragment that would be provided by caspase homodimerization (Shi, 2002).

On the other hand, the homodimers of caspases-3 and -7 once activated are vulnerable to inhibition by IAPs, particularly XIAP (Eckelman et al., 2006). The linker region preceding XIAP BIR2 interacts with these caspases. Inhibition of caspases-3

and -7 happens through steric hindrance, and has been described using a "hook, line and sinker" simile. The "hook", the N-terminal part of XIAP blocks the substrate-binding pocket of active caspases-3 or -7, the "line" connects the "hook" and the "sinker", which is responsible for the stabilization of the IAP-caspase interaction (Schimmer, 2004).

Figure 4. Inhibitors of apoptosis.



Besides XIAP, cIAP1 and cIAP2 have been reported to inhibit caspases. However, although cIAP-1 and -2 can bind to caspase-9, they lack the four residues necessary to keep the caspase in a catalytically inactive state (Shiozaki et al., 2003; Eckelman and Salvesen, 2006). On the other hand, although effector caspases can be blocked by direct interactions with cIAP-1 and -2, the inhibition is approximately 100-fold weaker than that caused by XIAP. Thus, these IAPs are considered as poor inhibitors of caspases (Deveraux and Reed 1999; Eckelman and Salvesen, 2006).

Besides direct caspase inhibition, IAPs may regulate apoptosis through different mechanisms. For example, some IAPs are able to polyubiquitylate proteins, which interact with them, e.g. caspases or IAPs themselves, and mediate their degradation by targeting to the 26S proteasome (Yang et al., 2000, Lorick et al., 1999). Finally, in addition to their role in apoptosis, certain IAPs can regulate cell cycle progression (Levkau et al., 2001), cell division (Uren et al., 1999) and signal transduction (Sanna et al., 2002).

2.3.3. Mitochondrial protein-Smac/DIABLO

Smac/DIABLO is an endogenous de-repressor of caspases, which counteracts the inhibitory effect of IAPs (Du et al., 2000). Smac is a 293 amino acid mitochondrial protein that is released into the cytosol along with cytochrome c upon MOMP (Chai et al., 2000). In the cytosol, Smac undergoes
maturation by proteolytic cleavage of its N-terminal part, which exposes an AVPI tetrapeptide motif. Through this motif Smac can interact with the BIR2 and BIR3 domains of XIAP (Liu et al., 2000). Smac binding to XIAP BIR3 prevents XIAP from binding to caspase-9 (Chai et al., 2000; Srinivasula et al., 2001). The AVPI motif of SMAC may also bind the BIR2 domain of XIAP, removing the inhibition of effector caspases. However, the affinity for BIR2 is ~10-fold lower than for BIR3.The molecular mechanism by which Smac disrupts the binding between XIAP and downstream caspases still remains elusive, but seems to involve steric hindrance rather that competitive binding (Chai et al., 2000; Fesik and Shi, 2001).

2.4. Apoptosis and chemoresistance

Resistance to anti-cancer agents is an important and unresolved clinical problem. Multidrug resistance may result from molecular alterations in drug efflux pumps and from inhibition of drug-target interactions. However, since drug-induced damage might initiate the activation of apoptosis as a secondary response, there are evidences for significant contribution of apoptotic defects in multidrug resistance (Johnstone et al., 2002; Siddik, 2003).

Apoptosis may be disrupted through the inhibition of the p53 pathway (Lowe et al., 1993). The p53 tumor suppressor gene is the most frequently mutated gene in human tumors and the loss of p53 function was shown to impair apoptosis in transgenic mice (Attardi and Jacks, 1999). However, the presence of wild-type p53 does not guarantee that cells undergo efficient apoptotic death (Schmitt et al., 1999). The disruption of mitochondria-dependent pathway is a frequent cause of drug resistance in various tumors. It can be caused by altered expression or mutations of Bcl-2 family members (Reed, 1995). Several clinical studies demonstrated that overexpression of Bcl-2 correlates with a poor prognosis (Catlett-Falcone et al., 1999). Moreover, overexpression of antiapoptotic Bcl-2 protein in an extensive panel of cancer cell lines exposed to different anticancer drugs was accompanied by inhibition of apoptosis (Yang and Korsmeyer 1996; Larochette et al., 1999; Johnstone et al., 2002). Another antiapototic protein, Bcl-xL, also seems to promote drug resistance by induction of cell survival (Simonian et al., 1997). Bcl-Xl

overexpression is associated with a poor outcome in patients with lymphomas, oropharynx carcinoma or soft-tissues sarcomas (Bairey et al., 1999; Aebersold et al., 2001; Kohler et al., 2002).

The alterations in the expression of proapoptotic Bcl-2 family members may also result in development of drug resistance. *In vitro* studies showed that Bax deletion blocked drug-induced apoptosis in a colon carcinoma cell line (Zhang et al., 2000). On the contrary, in fibroblasts the single deficiency for either Bax or Bak did not alter apoptosis response, but lack of both of them induced defective mitochondria stress-dependent apoptosis (Wei et al., 2001).

Apoptosis may also be disturbed downstream of the mitochondria, at the level of the apoptosome. The Apaf-1 protein seems to have a crucial implication in the response to drug-induced apoptosis in melanoma cell lines and clinical specimens. Apaf-1 deficient melanoma cells showed decreased apoptosis in response to doxorubicin (Soengas et al., 2001; Fernandez et al., 2005; Soengas et al, 2006). However, other studies have found that a lack of Apaf-1 expression does not necessarily correlate with inhibition of apoptosis, and may depend on the treatment regimens (Allen et al, 2005; Petlenburg et al., 2005). Additionally, the intrinsic apoptotic route might be altered by inhibition caused by endogenous suppressors of caspases, such as IAPs. The presence of excess levels of XIAP, cIAP-1 and cIAP-2 may cause blockage of initiator and effector caspases, and inhibit drug-induced apoptosis. IAPs are frequently overexpressed in cancer cells, and their elevated level is associated with chemoresistance (Ekedahl et al., 2002). Several studies have shown that XIAP downregulation by antisense oligonucleotide sensitizes cells to drug-induced apoptosis (Hu et al., 2003). However, cIAP-1 and cIAP-2 seem to play rather a minor role in apoptosis-dependent chemoresistance (Eckelman and Salvesen, 2006).

In summary, postmitochondrial alterations in cancer appear to be less common than defects upstream of mitochondria involving Bcl-2 family proteins. This would explain the fact that apoptosis can still occur in the presence of IAPs (Herr and Debatin 2001). Since alterations in the regulation of apoptosis contribute to cancer development, the restoration of the disturbed balance between cell proliferation and cell death may contribute to effective cancer treatment. Thus, proteins involved in apoptosis pathways are promising targets for cancer drug discovery, as described below.

2.5 Apoptosis-based therapy of cancer

The primary aim of targeted cancer therapy is to block the growth and spread of malignant tumor cells without damaging surrounding healthy cells. New generation drugs can recognize specific targets in or on tumor cells and act specifically against them. Targeted agents can be applied alone, or in combination with other targeted drugs, or with traditional chemotherapy. An increasing number of targeted agents are being approved for the treatment of several cancer types (Segota and Bukowski, 2004).

Malignant cells can be targeted by different approaches. Two major approaches are monoclonal antibodies and low molecular weight chemical molecules. Monoclonal antibodies can be conjugated to specifically deliver chemotherapeutic or radioactive agents, mark tumor cells for elimination by the immune system, and to inhibit membrane receptors. Small molecules might be used to modulate signaling pathways responsible for tumor cell proliferation and growth and they may target intracellular proteins more effectively than monoclonal antibodies (Fischer and Schulze-Osthoff, 2005).

Several components of the cell death machinery are attractive targets for direct promotion of apoptosis (Reed, 2006). These include the tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) (Buchsbaum et al, 2006), the anti-apoptotic or pro-apoptotic Bcl-2 family members (Papadopoulos, 2006), the caspases, the inhibitors of apoptosis (IAPs), and Smac/DIABLO (Table 3) (Reed, 2003; Fischer and Schulze-Osthoff, 2005).

2.5.1. Therapeutic targeting of Tumor Necrosis Factor (TNF) and TNF-Related Apoptosis-Inducing Ligand (TRAIL)

The extrinsic apoptotic pathway is activated via the tumor necrosis factor (TNF) receptor superfamily upon binding a ligand, such as TNF-α, CD95 ligand or TNF-related apoptosis-inducing ligand (TRAIL). Ligand binding is

Table 3. Apoptosis-based therapeutics (acc. to Fischer, 2005).

Death receptors TRAIL receptor	HGS-ERT 1	Agonistic TRAIL-R1 mAB	Apoptosis induction in tumor cell lines and mouse model	Phase 2
	HGS-ERT2	Agonistic TRAIL-R2 mAB	Apoptosis induction in tumor cell lines	Phase 1
	HGS-TR2J	Agonistic TRAIL-R2 mAB	Apoptosis induction in tumor cell lines	Phase 1
	PRO 1762	Soluble human Apo2L/TRAIL	Apoptosis induction in tumor cell lines	Phase 1
TNF	Recombinant TNF-α	Combination of TNF and chemotherapy	Apoptosis induction in tumor-associated blood vessels	Approved for melanoma therapy
Caspases Pan-caspase	Small-molecule compounds	Caspase activators	Activation of caspases solely in tumor cells	Preclinical
	RGD peptides	Caspase activators	Induction of apoptosis in cancer cells	Preclinical
Caspase-3	Immunocaspase -3	Cell-permeable HER2 mAb fused to caspase-3	Growth inhibition of HER2-positive tumors in a mouse xenograft model	Preclinical
	Ad-G/iCasp3	Adenoviral chemically inducible caspase-3	Reduction in tumor growth inducible caspase-3	Preclinical
	PEF-F8-CP3	Caspase-3 fusion construct with single-chain	Antigen-dependent induction apoptosis	Preclinical
Caspase-9	FKBP12/caspase -9 fusion protein	Chemically inducible dimerization of caspase-9	Anti-angiogenic in mouse model upon induction of caspase-9 dimerization	Preclinical
IAPs and Smac XIAP	BIR3 antagonists	Small-molecule antagonist of IAPs	Proapoptotic in tumor cell lines	Preclinical
IAPs and Smac	Capped tripeptide XIAP antagonist	BIR3 ligand of XIAP	Antitumor activity in breast cancer mouse model	Preclinical
	TWX024	Nonpeptidic small-molecule inhibitor of BIR2/caspase-3 interactions	Prevent XIAP/caspase-3 interactions in vitro, synergistic with anti-CD95 and TRAIL	Preclinical
	Polyphenylurea derivatives	BIR-2-specific nonpeptidic inhibitors100	Direct activation of caspases xenografts tumor growth suppression	Preclinical
	XIAP antisense and RNAi constructs	Inhibitor of XIAP expression	Induce apoptosis *in vitro* synergistic with chemotherapy in mouse model	Preclinical
	AEG35156	XIAP antisense oligonucleotide	Tumor growth suppression in combination with chemotherapy in mouse model	Phase1
Smac	Smac-mimetic compound	XIAP-binding Smac-mimetic compound	Enhanced cisplatin-induced apoptosis in prostate cancer cells	Preclinical
Bcl-2 proteins	Bcl-2 blocker	Small molecule inhibitors of Bcl-2/Bcl-xL	Apoptosis induction in tumor cells	Preclinical
	Antimycin A derivatives	Natural and synthetic Bcl-2/Bcl-xL inhibitors	Bind to BH3 pocket of Bcl-2 and Bcl-xL and induce apoptosis	Preclinical
	Synthetic Compound binding to the BH3 domain of Bcl-2	Bcl-2 18mer antisense oligonucleotide	Kills drug-resistant chronic lymphocytic leukemia cells delays development of fatal lymphoma in mice, increases dacarbazine effectiveness in melanoma model	Phase 3 for NSCLC

followed by formation of the death-inducing signaling complex (DISC), which recruits and leads to the activation of caspase-8 or –10, and the subsequent cleavage-mediated activation of downstream caspases (Fulda and Debatin, 2006). Despite the exciting perspectives resulting from preclinical studies with TNF and CD95 agonists, these compounds could not be used in clinical trials because of severe hepatic toxicity (Daniel et al., 2001). However, TNF was able to destroy blood vessels of the tumor, and improve their permeability to chemotherapeutics. In locally advanced melanoma and sarcoma the delivery of high-dose TNF with chemotherapy has significant activity when given by limb perfusion technique (Eggermont et al., 2001; Curnis et al., 2000).

Triggering the TRAIL receptor seems to be a more promising approach, given that TRAIL ligand specifically damages cancer cells, but spares healthy cells (Ichikawa et al., 2001). Agonistic TRAIL-R1 or TRAIL-R2 monoclonal antibodies and soluble human Apo2L/TRAIL are currently being evaluated (Menoret et al., 2006, Marini et al., 2006). The apoptosis induction in a broad range of cancer cell lines and xenografts models, the lack of side effects in non-human primates and mice, and synergism with conventional chemotherapeutics led to phase I and II clinical trials (Lawrence et al., 2001). Hints of activity have been shown by this therapeutics alone; however a higher efficacy is expected by combination with chemotherapy or other agents that stimulate apoptosis via different pathways (Fischer and Schulze-Osthoff, 2005).

2.5.2. Therapeutic targeting of Bcl-2 family members

Anti-apoptotic Bcl-2 proteins are overexpressed in many cancers, including melanoma, lung, pancreatic, ovarian, prostate cancer and are associated with resistance to conventional chemo-and radiotherapy. Therefore, the anti-apoptotic Bcl-2 members are attractive targets for anticancer therapy.

Two different approaches have been applied to target Bcl-2, Bcl-xl and Mcl-1. Antisense oligonucleotides or BH3 domain peptides are able to knockdown these proteins and, on the other hand, small molecule drugs will interfere with complex formation of anti- or proapoptotic Bcl-2 family members (Fischer, 2005).

Using antisense oligonucleotides, a reduction of the expression levels of Bcl-2 protein can be achieved. A 20-mer anti-Bcl2 oligonucleotide has been shown to induce or enhance apoptotic cell death in lymphoma or leukemia cells when applied alone or in combination with chemotherapeutic agents (Reed et al., 1990). Another

18-mer antisense oligonucleotide targeting Bcl-2 (oblimersen/genasense) (Frankel 2003) showed highly synergistic effects in combination with standard chemotherapy in a preclinical study performed in a xenograft breast cancer model (Chi et al., 2001; Klasa et al., 2002). Therefore, oblimersen was introduced in clinical trials, but it failed to meet a primary end-point of increasing survival in phase III trial in malignant melanoma and in refractory multiple myeloma. However, patients with chronic lymphocytic leukaemia (CLL) treated with oblimersen and traditional chemotherapy, e.g. fludarabine and cyclophosphamide showed improved overall response rate and thus, phase III trails with oblimersen were approved (Koziner, 2004). Similarly, phase III studies for non-small cell lung and phase II for hormone refractory prostate cancers have been initiated (Herbst and Frankel, 2004).

A different approach was used to regulate the level of Bcl-x proteins. Two forms of Bcl-x protein result from alternative splicing, pro-apoptotic Bcl-xs and antiapoptotic Bcl-xl. Making use of this property of Bcl-x, the splicing machinery was targeted to redirect Bcl-x mRNA processing into proapoptotic Bcl-xs, instead of using antisense oligonucleotides to knockdown Bcl-xl expression (Taylor et al., 1999).

Importantly, optimal results appear to be achieved by simultaneous downregulation of Bcl-2 and Bcl-xl proteins. Indeed, the bispecific Bcl-2/Bcl-xl antisense oligonucleotides showed to be effective in preclinical studies performed in many cancer cell lines (Zangemeister-Wittke et al., 2000; Millela et al., 2004).

Since the anti-apoptotic Bcl-2 proteins form complexes with proapoptotic BH3-only protein, their function can be inhibited for therapeutic purposes by employing peptides or small molecules mimicking the BH3 domain (Willis et al., 2005). A preclinical *in vitro* study demonstrated that BH3 peptides promote the disruption of Bax/Bcl-2 complexes, which was followed by the release of cytochrome c and apoptosis (Willis et al., 2005). Several strategies have been employed to improve the cellular permeability of BH3 peptides and direct them to the membrane. The first approach uses coupling of BH3 proteins to transduction domain, conjunction to fatty acids or delivery by cationic lipids. Encouraging results came from a study in human leukemic cell lines and mice with leukemia, where cell-permeable BID-based BH3-peptide mimetics, termed SAHBs (stabilized alfa-helix f Bcl-2domains), efficiently induced apoptosis (Walensky et al., 2004). Studies performed by Letai and co-workers showed that Bad- and Bik- based BH3 peptides bind to Bcl-2 and promote dislocation of bound proapoptotic proteins (Letai et al., 2002). The second approach involves non-peptidic organic compounds derived from Bid and Bim BH3 that promoted oligomerization of Bax and Bak. The structure-based designing of

these compounds guarantees efficient binding in micro- or nanomolar range to Bcl-2, Bcl-xl and Bcl-w proteins, and induction of apoptosis (Real et al., 2004; Wang et al., 2000). Some of these compounds, such as Gossypol, are being evaluated in phase I clinical trials in CLL patients (Mohammad et al., 2005). More recently, a Bad-based BH-3 compound, termed ABT-737, has demonstrated synergistic effect with chemo- and radiotherapy in a broad range of cancer cell lines. Furthermore, it, has shown apoptotic activity as a single agent in lymphoma and leukemia cell lines and primary cells, and induced complete regression of SCLC tumor xenografts in mouse (Oltersdorf et al., 2005).

In summary, antisense oligonucleotides against anti-apoptotic Bcl-2 family and BH3-mimic peptides or small molecules displayed impressive therapeutic potential in preclinical studies Therefore, the improvement of these strategies to efficiently induce apoptosis has an enormous potential in cancer therapy.

2.5.3. Therapeutic targeting of caspases

"Turning on" the death machinery by activation of initiator or effector caspases, may be a suitable approach for cancer therapy (Hengartner, 2000). Thus, adenoviral vectors expressing chimeric inducible caspase-3 and -9 were specifically targeted to prostate cancer cells and demonstrated rapid killing of cancer cells (MacCorkle et al., 1998; Shariat et al., 2001; Xie et al., 2001). On the other hand, since many tumors of different type, overexpress the erbB2/HER2 receptor novel a chimeric protein composed of anti-HER2-antibody linked to caspase-3 has been engineered and is currently being evaluated in the clinic (Jia et al., 2003). Another approach for the caspase-based therapy of cancer relies on the use of small cell-permeable drugs that are able to activate cellular caspases. For example, procaspase-3 contains the DDM tripeptide sequence near the active site, which keeps the enzyme in a dormant state. There is evidence that arginine-glycine-aspartate (RGD) synthetic peptides may interact with the DDM motif and promote catalytic activation of caspase-3 (Roy et al., 2001). RGD peptides are already in clinical trials for inhibition of tumor blood vessels formation, but their ability to induce caspase-3 activation could add to their therapeutic potential (Buckley et al., 1999).

2.5.4. Therapeutic targeting of Inhibitors of apoptosis (IAPs)

Given their function as negative regulators of apoptosis, the inhibition of IAPs, particularly XIAP, could provide substantial benefits for anti-cancer targeted therapy. Knockdown of XIAP protein using antisense oligonucleotides or RNA interference approach promoted apoptosis and synergistic killing effect of TRAIL or actimomycin D on cancer cells (Bilim et al., 2003; MacManus et al., 2004). The second-generation XIAP antisense oligonucleotide, AEG35156 /GEM® 640 demonstrated significant efficacy in preclinical studies and is currently evaluated in clinical trials (LaCasse et al., 2006). Additionally, several agents that target XIAP BIR domains are been testing in preclinical studies. Thus, a BIR3 antagonist showed proapototic and antitumor activity in tumor cells and breast cancer xenograft models (Oost et al., 2004). On the other hand, non-peptidic BIR2 inhibitors, e.g. polyphenylurea derivates, revealed proapototic activity and growth suppression of implanted tumors (Schimmer, 2004).

Encouraging results have been also obtained with Smac peptides or Smac-mimetic drugs that enhanced apoptotic effects of chemotherapeutic agents *in vitro* and *in vivo* (Arnt et al., 2002; Bockbrader et al., 2005). Smac peptidomimetics potently induced caspase activation, which was followed by apoptotic cell death in prostate cell lines, but also in xenografts mouse models of human glioma and NSCLC exposed to TRAIL or DNA-damaging agents, respectively (Fulda et al., 2002; Yang et al., 2003). Similarly, nonpeptidic Smac-mimetic compounds were capable of enhancing caspase-3 activation and potentiate cisplatin-induced cell death in human prostate cancer cells, or in glioblastoma cells exposed to TNF or TRAIL (Li et al., 2004; Sun et al., 2004).

3. Proteasome Inhibitors in Treatment of Lung Cancer

The proteasome inhibition is a novel promising therapeutic approach that has been evaluated in a number of cancers, including lung cancer (Lara et al., 2004). Proteasome inhibitors very efficiently disrupt degradation of intracellular proteins via the ubiquitin-proteasome pathway (Adams et al., 1999). A broad range of proteasome inhibitors has been analyzed, but only peptide aldehydes MG132 and CEP1612 have been screened *in vitro* and *in vivo* in preclinical studies in lung cancer

cell lines (Sun et al., 2001; Bang et al., 2004). The most effective, metabolically stable and more specific to the 26S proteasome is Bortezomib/PS341/Velcade a boronate peptide (Teicher et al., 1999). Bortezomib has been investigated as a single agent in many preclinical studies, and as a single-agent or in combination regimens in phase I and II clinical studies in lung cancer (Scagliotti, 2006). In preclinical studies, bortezomib proved to be a very potent apoptosis-inducing drug, which promotes a production of reactive oxygen species or regulates the balance between Bcl-2 family members (Ling et al., 2003). Bortezomib-mediated proteasome inhibition caused the blockage of the NF-kB pathway, which was followed by downregulation of Bcl-2 and promotion of apoptosis (Fahy et al., 2005; Mortenson et al., 2005). Studies conducted in a mouse model identified additional benefits form bortezomib treatment, such as a delay of tumor growth, and a reduction in the number of lung metastases (Teicher et al., 1999). Interestingly, positive results have been obtained from studies where bortezomib was used in combination regimens with anticancer agents, including carboplatin and gemcitabine (Mortenson et al., 2004). I*n vitro* and *in vivo* studies revealed massive induction of apoptotic cell death, and significant reduction of the tumor size. The successful preclinical results of bortezomib encouraged introducing this agent in clinical trials. So far, bortezomib is the only proteasome inhibitor evaluated in lung cancer patients (Aghajanian et al., 2002). Phase I and II clinical studies were performed in patients with advanced NSCLC using bortezomib in monotherapy and combination therapy with docetaxel or gemcitabine, and some patients showed partial response and stable disease (Davies et al., 2004; Fannucchi et al., 2005).

4. Outline of the thesis

In the introduction of this thesis, **Chapter 1,** general information about lung cancer and its resistance to currently used drug regimens are presented. Particular emphasis is put on the relations between the regulation of mitochondria-dependent apoptosis and tumorigenesis or chemoresistance. An outline of the conventional therapies against lung cancer is described, with an overview of recent advances in the field of preclinical and clinical apoptosis-based therapies. The principal aims of this thesis were to investigate the molecular mechanism(s) that regulate the suppression of caspases in the mitochondria-dependent apoptotic pathway in NSCLC cells, and to assess the benefits of apoptosis-based therapy in improving an efficacy of conventional chemotherapy.

Inhibition of caspase-9, which is involved in mitochondria-dependent pathway of apoptosis, contributes to the resistance to chemotherapy in NSCLC cells. In **Chapter 2** we have investigated whether the inhibition of caspase-9 is caused by the presence of the negative regulator of apoptosis TUCAN /CARDINAL /CARD8, which might block caspase-9 activation. In **Chapter 3** we determined the expression of TUCAN protein in specimens from patients with advanced NSCLC, and showed that differential localization of TUCAN seems to have a prognostic value for NSCLC. Since the preclinical study revealed that TUCAN do not play a role in inhibition of caspase-9 in NSCLC and the most potent inhibitor of apoptosis, XIAP that was overexpressed in this tumor type, we investigated in **Chapter 4** whether XIAP is involved in the suppressing of caspase-9-headed apoptosis. We attempted to explore the potential therapeutic benefits of Smac mimic molecules in combination treatment with DNA-damaging agents in NSCLC cells. Activation of caspase-9 and an increase of apoptotic cell death were observed upon exposure of NSCLC cells to bortezomib, a novel proteasome inhibitor. In **Chapter 5,** we focused on the molecular mechanism of action of bortezomib, which in contrast to cisplatin was able to efficiently promote a proapoptotic shift of the levels of Bcl-2 family proteins, which was followed by cytochrome c release.

Chapter 6 describes the results of a tandem mass spectrometry (MS)-based proteomics analysis of protein complexes associated with caspase-9 in NSCLC cells, under experimental conditions in which the caspase is free or recruited to the apoptosome. Our goal was to identify novel potential inhibitory proteins of the mitochondria-dependent apoptotic pathway.

Finally, the results and main findings of this thesis and their possible clinical implications are discussed in **Chapter 7**.

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