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Institut für Medizinische Mikrobiologie, Immunologie und Hygiene

Restoration of apoptosis sensitivity in human renal cell carcinoma by inhibition of B-cell lymphoma-2 proteins

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Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. Christian F.W. Becker
Prüfer der Dissertation:	1. Univ.-Prof. Dr. Michael Groll
	2. Univ.-Prof. Dr. Georg Alexander Häcker

Die Dissertation wurde am 03.03.2010 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 31.03.2010 angenommen.

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Abstract

Malignant transformation has been linked to pro-survival effects, and tumor cells accordingly often have defects in their cell death machinery. This has implications for both the development of a tumor and for the treatment resistance to chemotherapy. Renal cell carcinoma (RCC) is very resistant to chemotherapy clinically. The drug-response is at least in part determined by the ability of tumor cells to undergo apoptosis upon treatment. In most instances of drug-induced apoptosis the activation of the mitochondrial pathway is essential, where the release of cytochrome *c* into the cytosol has a pivotal role for the onset of apoptosis. The release of cytochrome *c* is regulated by the combined activity of pro- and anti-apoptotic Bcl-2 proteins. The expression of the pro-apoptotic Bcl-2 family member, the BH3-only protein Bim was here found to be reduced in the majority of primary RCC isolates compared to normal renal parenchyma and was varyingly expressed in patient-derived RCC cell lines (Zantl *et al.*, 2007). The reduction of Bim could be reversed by treatment of tumor samples with an inhibitor of histone deacetylation or chromatin methylation, suggesting that epigenetic regulation contributed to low expression of Bim. Importantly, expression of Bim appeared to correlate with apoptosis sensitivity in RCC cell lines. Expression analysis of other Bcl-2 proteins gave no indication that the differential expression of these proteins was the reason for the varying apoptosis sensitivity. In addition, a resistant cell line was able to undergo apoptosis upon over-expression of Bim and RNAi mediated down-regulation of Bim protected a susceptible cell line from apoptosis, supporting the hypothesis that Bim-loss was relevant in determining the apoptosis sensitivity in RCC cell lines (Zantl *et al.*, 2007). The data therefore suggested that Bim may have a tumor suppressive function in RCC.

In recent years it has become clear that tumor cells often over-express Bcl-2-like proteins to escape apoptosis and depend on these proteins for their survival. The targeting of anti-apoptotic proteins has therefore become an important anti-tumor strategy. The candidate substance developed furthest is ABT-737, generated by Abbot Laboratories (Oltersdorf *et al.*, 2005). ABT-737 inhibits the pro-survival function of Bcl-2 proteins selectively. ABT-737 on its own was here found to be almost inactive in RCC cell lines, but the combination of ABT-737 with several chemotherapeutic drugs demonstrated a strong synergistic pro-apoptotic effect. Consistent with the low affinity of ABT-737 for the Bcl-2-like proteins Mcl-1 and A1, reduction of these proteins by RNAi restored apoptosis sensitivity to single treatment with ABT-737, indicating that both proteins contributed to protection against ABT-737. The endogenous Mcl-1 and A1 antagonist, the BH3-only protein Noxa was induced by chemotherapeutic drugs, most strongly upon treatment with etoposide by a p53-independent mechanism. RNAi mediated knockdown of Noxa protected RCC cell lines against combination treatment, whereas knockdown of Bim or Puma had no effect, suggesting that only Noxa was required for complete apoptosis induced by combination therapy. During proteasome inhibition, Noxa was still able to neutralize Mcl-1 despite stabilization of Mcl-1 levels, thereby sensitizing RCC to ABT-737. Collectively, the data provided new insights in the molecular basis of apoptosis defects in human RCC and defined Bcl-2-inhibition as a promising future approach for treatment.

Index of Abbreviations

A1	Bcl-2 related protein A1
AMC	7-amino-4-methylcourmarin
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X
Bcl-2	B-cell lymphoma-2
Bcl-w	B-cell leukemia w
Bcl-x_L	B-cell leukemia XL
BH	B-cell lymphoma 2 (Bcl-2) homology
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting mediator of cell death
Bmf	Bcl-2 modifying factor
BSA	Bovine serum albumin
Bok	Bcl-2 ovarian killer
CAD	Caspase-activated DNase
CC	Clear cell
cDNA	Complementary DNA
CEBP	CCAAT-enhancer binding protein-α
CHOPα	CEBP homologous protein
CMV	Cytomegalovirus
COX IV	Cytochrome *c*-oxidase unit IV
Cy5	Cyanine dye
DEVD	Asp-Glu-Val-Asp
Diabolo	Direct IAP binding protein with low pI
DISC	Death inducing signalling complex
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGF	Endothelial growth factor
EGTA	Ethyleneglycol tetraacetic acid

eIF2α	eukaryotic initiation factor 2
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
Fadd	Fas associated death domain
Fas	Apoptosis stimulating fragment
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FoxO3a	Class O forkhead box transcription factor-3A
g	Acceleration of free fall (9.81 m/s^2)
GFP	Green Fluorescent Protein
GM-SCF	Granulocyte macrophage colony-stimulating factor
HDAC	Histone deacetylation
HDM2	Human double minute 2
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sufonic acid
HIF-1α	Hypoxia-inducible factor 1α
Hrk	(Japanese for suicide) Harakiri
HRP	Horse Radish Peroxidase
ICAD	Inhibitor of CAD
kDa	Kilodalton
LacZ	*β*-galactosidase
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Mouse double minute 2
MEF	Mouse embryonic fibroblasts
MOMP	Mitochondrial outer membrane permeabilization
Nbk	Natural born killer
Noxa	(Latin for damage) Noxa
OH	Hydroxyl group
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PP2A	Protein phosphatase 2A
Pro	Proline
Puma	p53 up-regulator of apoptosis
PVDF	Polyvinylidene fluoride
p53	Tumour protein 53
Ras	Rat sarcoma

RCC	Renal cell carcinoma
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT	Room temperature
rpm	Rounds per minute
RT-PCR	Reverse transcription PCR
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
tBid	Truncated BID
TBS	Tris Buffer Solution
TBS-T	Tris Buffer Solution 0.05% Tween-20
TEMED	N-N-N'-N'-tetra methylethylenediamide
TM	Trans-membrane domain
TNF	Tumor necrosis factor
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
TRAIL	TNF-related apoptosis-inducing ligand
Ub	Ubiquitin
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
v/v	Volume per volume
w/v	Volume per weight
WT	Wildtype
zVAD-fmk	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

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1 Introduction

1.1 Apoptosis

1.1.1 Physiological role of apoptosis

The term apoptosis is derived from two ancient Greek words meaning 'falling off' and suggesting a process similar to leaves falling off the tree, i.e. shedding of parts that are no longer needed. The term was introduced by Kerr *et al*. to describe a form of controlled cell death that is regulated in a cell-autonomous fashion (Kerr *et al.*, 1972). Apoptosis process is accompanied by a series of biochemical and morphological features, during which a cell and its nucleus shrink, inter-nucleosomal DNA fragmentation occurs, the cell breaks up into membrane vesicles that consecutively are phagocytosed or taken up by neighbouring cells in *vivo* (Adams and Cory, 2007b; Gross *et al.*, 1999; Hotchkiss *et al.*, 2009; Kerr *et al.*, 1972; Strasser *et al.*, 2000). Apoptosis is an evolutionarily conserved mechanism that is basically operative in flies, worms and mammals. Induction of apoptosis has a pivotal role in various physiological situations and allows for elimination of potentially dangerous cells, such as auto-reactive cells or tumor cells (Youle and Strasser, 2008). Apoptosis does not only play an integral part in these pathological situations but is also important during normal embryo development, in maintaining tissue homeostasis in adults, during lymphocyte development and in the defense against infectious agents, among other situations (Adams and Cory, 2007; Danial and Korsmeyer, 2004; Marsden *et al.*, 2002; Strasser, 2005). The observed morphological alterations are a consequence of activation of a group of cysteine proteases termed caspases, which are synthesized as zymogens and need to be activated by proteolytic processing. Upon activation they execute cell death by cleaving the carboxyl side of aspartate residues in several hundreds of substrates that are responsible for the morphological picture of apoptosis and cellular demolition (Thornberry and Lazebnik, 1998). Most of the substrates can be categorized in functional groups. Examples of identified substrates include *ß*-actin and vimentin (cytoskeletal and structural proteins), cyclin E and MDM2/HDM2 (cell cycle regulation), PARP-1 and DNA-PK subunit c (DNA repair), eIF2*α* and components of the ribosome (protein translation) or TNFR1 and EGFR (membrane-associated receptors). The cellular consequences for the cleavage of most of these substrates are uncertain, but some substrates can be linked to distinct morphological alterations. One important example is the cleavage of the inhibitor of caspase-activated DNases (ICAD) by activated caspase-3 (Enari *et al.*, 1998) that liberates the active CAD nucleases to mediate inter-nucleosomal DNA fragmentation (Fischer *et al.*, 2003).

1.1.2 Molecular pathways of apoptotic signaling

Apoptosis can be initiated in all mammalian cells through at least two pathways (Figure 1), which are known as the extrinsic and the intrinsic (also called the mitochondrial) apoptotic-signaling pathway. One distinguishing feature is whether they strictly depend on regulation by Bcl-2 proteins

(mitochondrial pathway), and they particularly differ in terms of activation of initiator caspases to start the process, but both pathways activate a similar set of effector caspases (Danial and Korsmeyer, 2004; Strasser, 2005; Strasser *et al.*, 1995; Youle and Strasser, 2008).

The extrinsic apoptotic pathway is typically involved in eliminating virus-infected cells by natural killer cells or cytotoxic T cells and is activated by the binding of extracellular death ligands to their membrane-localized death receptors (Strasser, 2005) (Figure 1). Death receptors are members of the tumor necrosis factor (TNF) receptor superfamily of proteins that contain a conserved protein-protein-interaction motif in their intracellular region termed the "death domain" (Danial and Korsmeyer, 2004; Strasser *et al.*, 1995); examples of death receptors include TNFR1, CD95/APO/FAS and the TRAIL-receptors DR4 and DR5. Upon activation and ligation of death receptors the adaptor protein Fadd (FAS-associated protein with death domain) binds to the death domain through homotypic interaction. The activation of effector caspases requires the recruitment of the initiator caspase-8 to the adaptor protein Fadd (Newton *et al.*, 1998; Peter and Krammer, 2003; Strasser, 2005; Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998; Zhang *et al.*, 1998). The assembled signaling complex is termed DISC (death-inducing signaling complex), and DISC-formation causes the activation of caspase-8 and the activation of the downstream effector caspases-3, -6 and -7 by caspase-8 (Shi, 2002). In so-called type I cells such as lymphocytes, the Bcl-2 family of proteins are dispensable for regulating death receptor induced apoptosis, but in certain cell types like hepatocytes (type II cells) engagement of the mitochondrial pathway via the caspase-8 mediated cleavage of the BH3-only protein Bid (BH3-interacting-domain death agonist) to its truncated form (tBid) is essential to induce apoptosis in these cells (Kaufmann *et al.*, 2007; Strasser, 2005; Wang, 2001; Yin *et al.*, 1999).

Cellular stress signals arising from a diversity of insults, like growth-factor deprivation, treatment with chemotherapeutic drugs and many other stimuli activate the intrinsic pathway (Danial and Korsmeyer, 2004; Strasser, 2005; Youle and Strasser, 2008) (Figure 1). This pathway is regulated by the balanced interplay of pro- and anti-apoptotic proteins of the Bcl-2 family, which dictates whether a cell undergoes apoptosis or not. Death stimuli activate BH3-only (Bcl-2-homology domain 3 only) proteins, a pro-apoptotic subgroup of the Bcl-2 family (Huang and Strasser, 2000; Strasser, 2005), which initiate apoptosis by a mechanism that probably involves the neutralization of anti-apoptotic Bcl-2-like proteins or interaction with Bax and/or Bak (Willis *et al.*, 2005; Willis *et al.*, 2007) and that involves the activation and homo-oligomerization of Bax (Bcl-2-associated X protein)- and Bak (Bcl-2-antagonist/killer) (Cheng *et al.*, 2001; Wei *et al.*, 2001; Zong *et al.*, 2001). These proteins control mitochondrial outer membrane permeabilization (MOMP) and subsequent release of cytochrome *c* (cyt *c*) and other pro-apoptotic factors like Smac/Diabolo from mitochondria (Newmeyer and Ferguson-Miller, 2003). After its release into the cytosol, cyt *c* promotes the formation of a complex that is termed apoptosome (Danial and Korsmeyer, 2004; Shi, 2006; Wang, 2001; Youle and Strasser, 2008), supporting Apaf-1 (apoptotic-protease-activating factor 1)-mediated

activation of caspase-9 (Hakem *et al.*, 1998), which in turn leads to activation of effector caspases-3, -6 and -7 (Adams and Cory, 2007a).

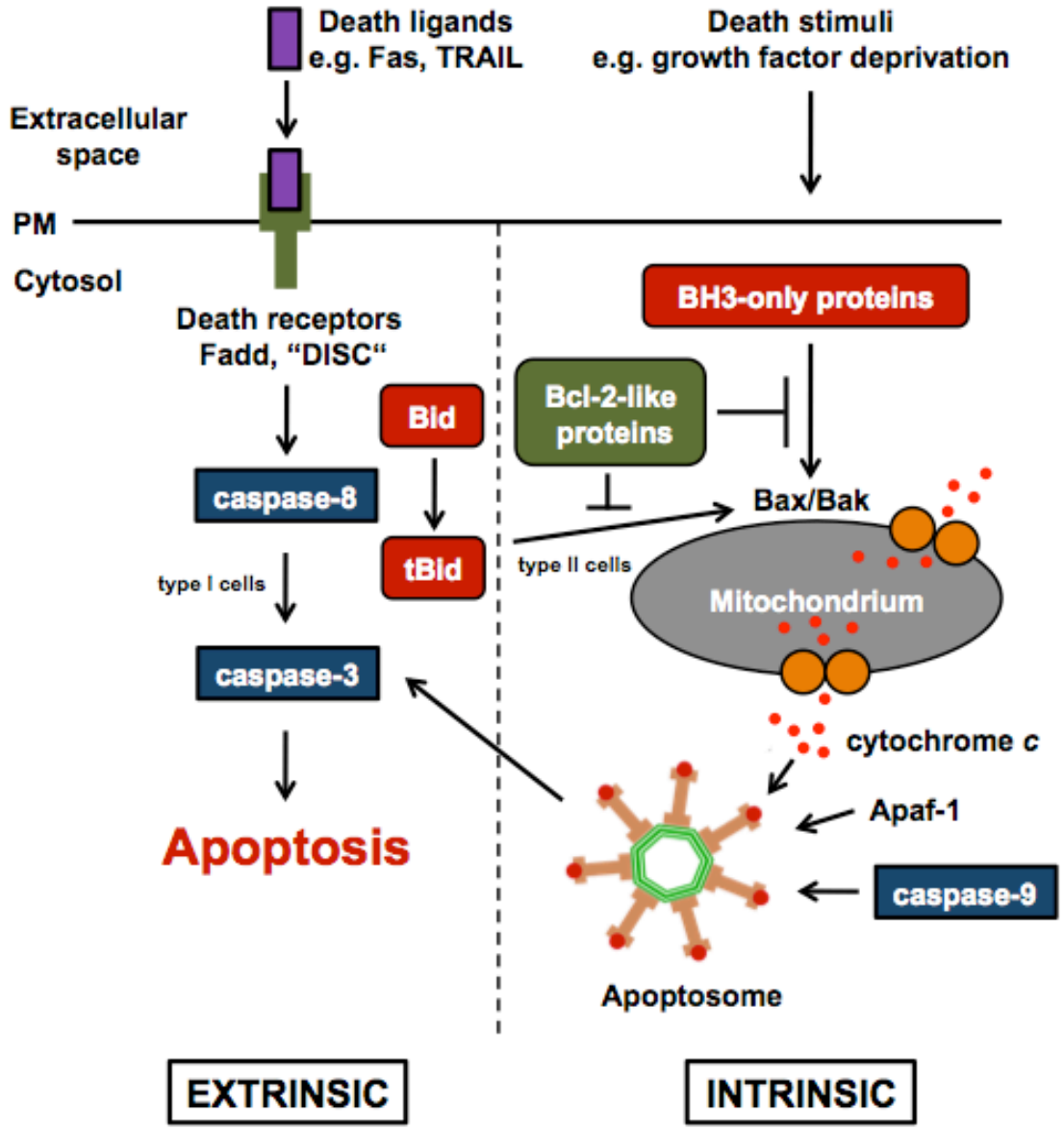

Figure 1 - Schematic representation of apoptotic signaling pathways

The extrinsic apoptotic pathway leads to activation of effector caspases (caspase-3 is shown here) and is activated when death ligands, i.e. Fas or TRAIL ligand bind to their specific membrane-localized death receptors (PM, plasma membrane). In type I cells the initiator caspase-8 is recruited to death receptors by the adaptor protein Fadd (FAS-associated via death domain), to induce DISC formation and activation of effector caspases. Activated caspases in turn cleave several substrates that are responsible for the picture of apoptosis. In type II cells caspase-8-mediated activation of the BH3-only protein Bid leads to engagement of the intrinsic pathway that is essential in these cells. The intrinsic pathway (also called the Bcl-2-regulated or mitochondrial pathway) is induced by growth factor deprivation and many other cytotoxic stimuli. Death stimuli activate the BH3-only proteins, the most up-stream pro-apoptotic subgroup of Bcl-2 proteins, which can induce apoptosis by a mechanism that requires Bax and Bak. These events can be blocked by anti-apoptotic Bcl-2-like proteins. Once activated, Bax and Bak promote cytochrome *c* release by inducing permeabilization of the outer mitochondrial membrane (MOMP), resulting in release of cytochrome *c*. The release leads to activation of Apaf-1 (apoptotic-protease-activating factor 1) and caspase-9 into a complex, termed apoptosome that can activate the effector caspase-3, resulting in apoptosis (adapted from Strasser, 2005; Youle and Strasser, 2008).

1.1.3 Structure-function relationship of Bcl-2 proteins

In mammals there exist around 20 proteins of the Bcl-2 family, including Bcl-2 itself and proteins that have conserved regions of sequence homology and secondary structural similarity to Bcl-2 (Figure 2) (Youle and Strasser, 2008). Members of the Bcl-2 family contain up to four distinct regions of sequence homology, known as Bcl-2 homology (BH) 1-4 domains. The nuclear magnetic resonance (NMR) and crystal structure of several Bcl-2 proteins has provided important insights into the structure-function relationship of Bcl-2 proteins (Day *et al.*, 2005; Hinds *et al.*, 2003; McDonnell *et al.*, 1999; Moldoveanu *et al.*, 2006; Muchmore *et al.*, 1996; Petros *et al.*, 2001; Suzuki *et al.*, 2000; Youle and Strasser, 2008). Knock-out studies in mice and extensive functional analysis in mammalian cells have helped to elucidate their molecular function. Bcl-2 proteins have been grouped into three classes according to their structure and pro- or anti-apoptotic function (Strasser, 2005; Youle, 2007; Youle and Strasser, 2008).

The pro-survival group of Bcl-2-like proteins inhibits apoptosis (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1). They possess up to four BH domains. The first three domains (BH1- 3) form a hydrophobic groove on the surface where binding to the BH3 domain of pro-apoptotic proteins can take place (Herman *et al.*, 2008; Liu *et al.*, 2003; Petros *et al.*, 2000; Sattler *et al.*, 1997). Over-expression of any of these proteins protects cells against apoptosis induced by many apoptotic stimuli, although the potency varies. Thus, they have at least some functional redundancy.

The second class of Bcl-2 proteins (with pro-apoptotic activity) is the group of so-called BH3-only proteins (Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa and Puma). They serve as initial sensors for apoptotic signals generated by various cellular stresses (Youle and Strasser, 2008). They are transcriptionally induced or activated by death signals and share only one conserved BH3 domain, a short α-helix. This domain allows interaction with the hydrophobic groove formed by the BH1-3 domains of pro-survival Bcl-2-like proteins. Over-expression of any BH3-only protein has in some systems been found to induce apoptosis that strictly depends on the presence of Bax or Bak although their potency greatly varies (Cheng *et al.*, 2001; Zong *et al.*, 2001). Thus, they clearly function upstream of Bax and Bak in the apoptotic signaling pathway. However, the same apoptotic stimulus may activate a different set of BH3-only proteins, depending on the cell type analyzed. The binding capacity of BH3-only proteins to other Bcl-2 has been analyzed by binding assays using peptides derived from the BH3 domains of BH3-only proteins or in cells upon over-expression (Certo *et al.*, 2006; Chen *et al.*, 2005; Kim *et al.*, 2006; Kuwana *et al.*, 2005). Based on these studies it has been suggested that once the BH3-only proteins become activated they insert into the hydrophobic groove of Bcl-2-like proteins to release Bax and Bak from inhibition (Willis *et al.*, 2007). In contrast an opposing model holds that certain BH3-only proteins (particularly Bim, tBid and Puma) directly activate Bax and Bak (Certo *et al.*, 2006; Kim *et al.*, 2006). Alternatively, the BH3-only proteins may require mitochondrial targeting and insertion into the outer mitochondrial membrane to allow for recruitment and activation of Bax/Bak (Lovell *et al.*, 2008; Weber *et al.*, 2007). Recombinant BH3-

only proteins (Bim, Bad and Bmf) are difficult to purify and have been found to remain largely unstructured in solution without an interaction partner and undergo a conformational change within their BH3-domain upon binding to Bcl-2-like proteins. Thus the observed structural plasticity may favor binding to multiple Bcl-2-like proteins (Hinds *et al.*, 2007).

The third class, the pro-apoptotic Bax, Bak and Bok proteins contain the BH1-3 domain and promote apoptosis by inducing mitochondrial outer membrane permeabilization (MOMP) and cyt *c*-release from mitochondria (Chipuk *et al.*, 2006; Newmeyer and Ferguson-Miller, 2003). Their structure shares similarity with Bcl-2-like proteins (Moldoveanu *et al.*, 2006; Sattler *et al.*, 1997; Suzuki *et al.*, 2000; Youle and Strasser, 2008). Cell lines derived from mice deficient for both *bax* and *bak* are still sensitive to apoptosis by the death receptor pathway, but are completely protected against apoptosis induced by over-expression of any of the BH3-only proteins (Lindsten *et al.*, 2000; Wei *et al.*, 2001). Thus, they are downstream effectors of BH3-only proteins. They differ in their cellular localizations as Bak is constitutively integrated into the outer mitochondrial membrane whereas Bax is a cytosolic protein that has its C-terminus membrane anchor buried within the hydrophobic BH3 binding pocket on the surface (Goping *et al.*, 1998; Hsu and Youle, 1998; Wolter *et al.*, 1997). This indicates that Bax needs an additional activation stimulus to expose its C-terminus and to translocate to mitochondria, thereby inducing MOMP.

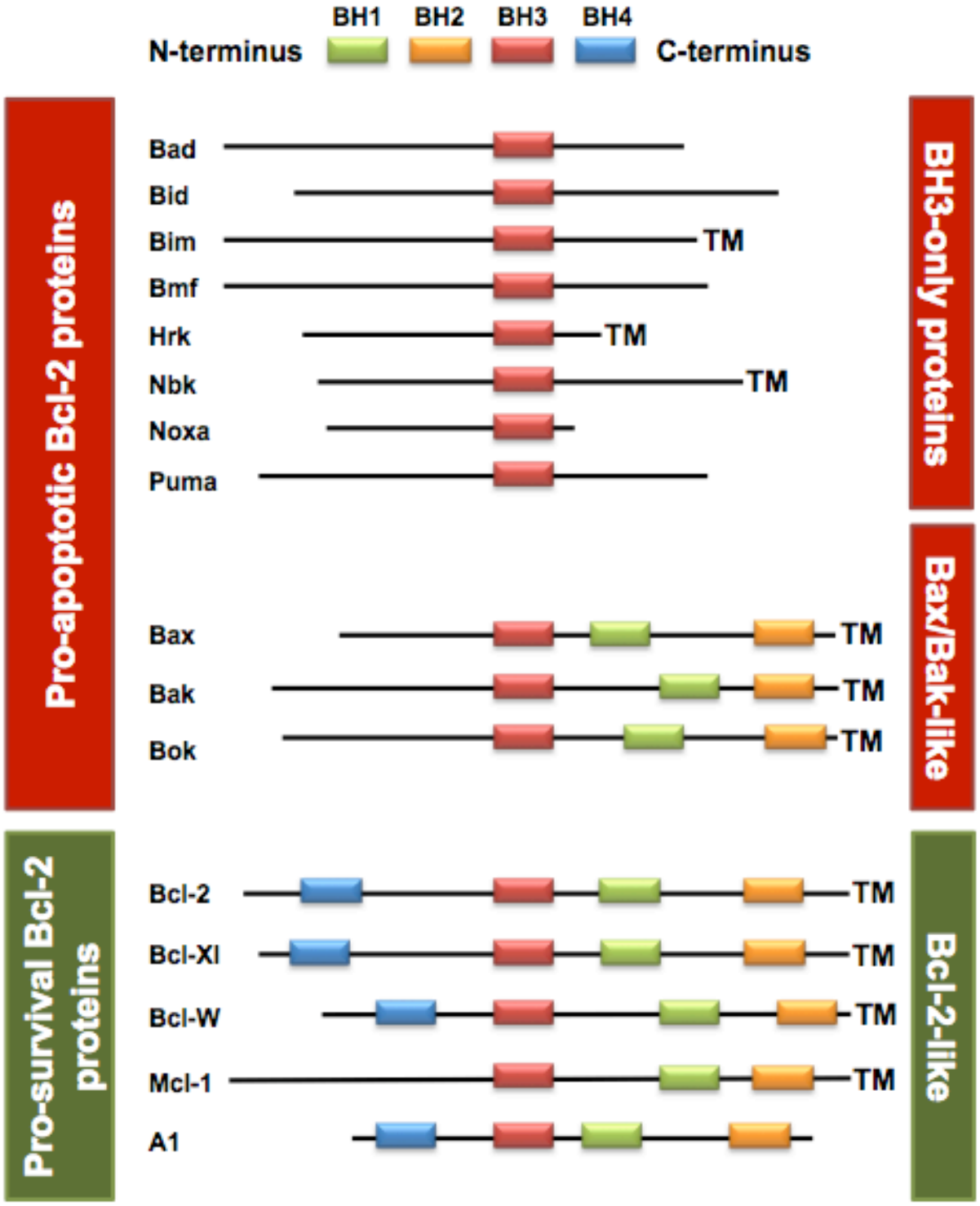

Figure 2 - Proteins of the Bcl-2 family, BH3 domains and functions

Sequence homologies of the BH1 to BH4 domains (colored boxes) and transmembrane (TM) domains of Bcl-2 proteins are shown, as derived from the determined structures or from secondary structure prediction. The BH3 domain (red) among the pro-apoptotic BH3-only proteins is responsible for interaction between the BH3-only proteins and the pro-survival Bcl-2-like proteins. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1 belong to the pro-survival group of Bcl-2-like proteins. Bax, Bak and Bok are considered to be pro-apoptotic as their activation induces permeabilization of the outer mitochondrial membrane (MOMP) and cyt *c*-release (Strasser, 2005; Youle and Strasser, 2008).

1.1.4 Activation of Bcl-2 proteins

The activation of BH3-only proteins can be achieved by both transcriptional activation and post-translational modification (Youle and Strasser, 2008). Gene targeting in mice revealed that in particular loss of the BH3-only protein Bim has important physiological roles (Bouillet *et al.*, 1999). The number of lymphoid and myeloid cells was increased in hematopoietic organs of Bim-deficient animals. T-cell development was impaired and older mice developed severe autoimmune kidney disease. Bim was therefore required for negative selection of auto-reactive thymocytes during development (Bouillet *et al.*, 2002). Bim-deficient lymphocytes were also protected from apoptosis induced by many stimuli; pre-T cells were refractory to dexomethasone or paclitaxel but not to FAS ligand or etoposide (Bouillet *et al.*, 1999). Thus, Bim is required for certain apoptotic stimuli. The expression of Bim can be induced by several transcription factors, for instance FoxO3a (class O forkhead box transcription factor-3A) that becomes activated upon cytokine withdrawal in hematopoietic cells (Dijkers et al., 2000; Stahl et al., 2002; You et al., 2006); or CEBPα (CCAAT-enhancer binding protein-α) and CHOP (CEBP homologous protein) that are induced by endoplasmatic reticulum stress (Puthalakath et al., 2007), translocate to the nucleus and can bind to the promoter region of *bim* gene to induce its transcription. *Bim* gene is regulated post-transcriptionally by producing three different isoforms via alternative splicing (Bim_S, Bim_L and Bim_{EL}) (O'Connor *et al.*, 1998; O'Reilly *et al.*, 2000). There are also examples of post-translational activation of Bim; The Bim_L and Bim_{EL} isoforms are probably sequestered to the microtubules-associated dynein light chain 1 (DLC1) in healthy cells and can be released from sequestration to the dynein motor complex by phosphorylation of JNK kinases (Lei and Davis, 2003; Puthalakath et al., 1999). Upon induction of apoptosis Bim can then translocate to mitochondria (Puthalakath *et al.*, 1999; Puthalakath and Strasser, 2002), but these results have not been reproduced by others. For instance in B and T lymphocytes Bim_L and Bim_{EL} can be found at mitochondria and this does not correlate with the induction of apoptosis (Bauer *et al.*, 2007; Gomez-Bougie *et al.*, 2004; Kirschnek *et al.*, 2005; Zhu *et al.*, 2004). Thus additional mechanisms exist that regulate the activity of Bim. The Bim_{EL} isoform is most strongly expressed in many cell types, whereas Bim_S lacks the DLC1 interaction motif and has a low expression (O'Reilly *et al.*, 2000) and is considered to be the most cytotoxic upon over-expression (O'Connor *et al.*, 1998), perhaps due to its activity is not subject to post-translational regulation. Bim_{EL} and Bim_L have been suggested to become phosphorylated at serine residues by the Erk kinase pathway, which usually targets Bim in healthy cells to ubiquitination and proteasome-dependent degradation (Ley et al., 2005), but mutations interfering with the phosphorylation sites do not alter the pro-apoptotic activity of Bim, as suggested by a knock-in approach in mice and *in vitro* (Hubner *et al.*, 2008; Kirschnek *et al.*, 2005; Ley *et al.*, 2003; Ley *et al.*, 2005; O'Connor *et al.*, 1998). In conclusion, the activation of Bim can be achieved by several mechanisms, depending on the cell type analyzed.

Noxa and *puma* are direct target genes of the tumor suppressor p53 in response to DNA damage and can also be up-regulated by p53-independent mechanisms (Nakano and Vousden, 2001;

Oda *et al.*, 2000; Villunger *et al.*, 2003a). Mice lacking either of the genes are phenotypically normal and do not develop spontaneous tumors. But lymphocytes deficient for Puma are protected from apoptosis induced by ionizing radiation and withdrawal of cytokines (Jeffers *et al.*, 2003). Also mice lacking Noxa showed resistance to genotoxic agents and ionizing radiation (Shibue *et al.*, 2003). Although both proteins are regulated by p53, it appeared that Puma was the main inducer of apoptosis in lymphocytes upon DNA damage, whereas Noxa was required in other cell types (Erlacher *et al.*, 2005). Puma does not appear to be mainly regulated by post-translational modifications but Noxa was found to become up-regulated by inhibiton of the proteasome in melanoma and other cell types, suggesting that its activity is mainly regulated by ubiquitination and proteasomal degradation (Fernandez *et al.*, 2005).

There are also examples of post-translational activation of other BH3-only proteins to highlight the complexity of this process (Youle and Strasser, 2008). For example, Bid is activated by caspase-8 mediated cleavage (Li et al., 1998; Luo et al., 1998), whereas Bad is released from its sequestration to 14-3-3 scaffold proteins by phosphorylation (Zha et al., 1996); Bmf is activated during anoikis (detaching of cells from the surrounding tissue) by release from action-myosin complexes, etc. (Puthalakath et al., 2001).

Another important mechanism how cells can regulate apoptosis is to alter the expression levels of pro-survival Bcl-2-like proteins. Mcl-1 is degraded by ubiquitination and proteasomal degradation in response to cytokine deprivation or ultraviolet (UV) radiation (Nijhawan *et al.*, 2003; Willis *et al.*, 2005) and can be up-regulated transcriptionally to prevent apoptosis by repressing its degradation (Warr *et al.*, 2005; Youle and Strasser, 2008; Zhong *et al.*, 2005). Bcl-x_L can be transcriptionally induced by growth factors through the JAK–STAT pathway to promote cell survival (Grad *et al.*, 2000). A1 is typically activated by the NFκB pathway to provide a survival advantage in myeloid cells (Karsan *et al.*, 1996). As described in the next sections the pro-apoptotic Bax and Bak proteins are expressed at constant levels and are primarily regulated by interaction with other members of the Bcl-2 family (Youle and Strasser, 2008).

1.1.5 Interaction of pro- and anti-apoptotic Bcl-2 proteins

Once the BH3-only proteins have been activated, they interact with anti-apoptotic Bcl-2-like proteins or may directly activate Bax and Bak to induce apoptosis (Certo *et al.*, 2006; Willis *et al.*, 2007). The binding of BH3-only proteins to specific anti- and pro-apoptotic Bcl-2 family members has been determined by using several binding assays or by following cyt *c* release from isolated cell-free mitochondria and artificial liposomes (Chen *et al.*, 2005; Kim *et al.*, 2006; Kuwana *et al.*, 2005; Letai *et al.*, 2002; Willis *et al.*, 2005; Youle and Strasser, 2008). Although often restricted to short peptides derived from the BH3 domains, these studies suggested that some BH3-only proteins, such as Bim and tBid, are able to bind to all pro-survival Bcl-2-like proteins, whereas others, such as Bad and Noxa, bind only to certain subsets of Bcl-2-like proteins (Figure 3). In addition to interaction with pro-

survival Bcl-2-like proteins, *in vitro* expressed full length proteins (Bim, tBid and Puma) were able to induce cyt *c* release from isolated mitochondria but only if Bax/Bak were present, suggesting that they can directly interact with Bax/Bak (Kim *et al.*, 2006). In addition to that, several groups reportedly demonstrated that recombinant caspase-8-cleaved tBid was able to enhance the translocation of active Bax to mitochondria or to artificial liposomes *in vitro*, where cellular components have been removed (Certo *et al.*, 2006; Kim *et al.*, 2004; Kuwana *et al.*, 2005; Lovell *et al.*, 2008), These data suggested that some BH3-only proteins may directly bind to and activate Bax and/or Bak. However, the mechanisms underlying this direct interaction are under debate and it is difficult if not impossible to detect such a binding of Bim, tBid or Puma to Bax and Bak under physiological conditions (Willis *et al.*, 2005; Youle and Strasser, 2008). By contrast, results from knockout mice double deficient for Bim and Bid suggested that these potential Bax/Bak direct activators are not required for apoptosis induced by the over-expression of Bax und Noxa (Willis *et al.*, 2007). Thus, it is unknown whether BH3-only proteins induce apoptosis primarily by inhibiting the pro-survival Bcl-2-like proteins or by activation of Bax and/or Bak directly, thereby causing MOMP, cyt *c*-release and the activation of apoptosis. Accordingly, two competing models have been proposed to explain the experimental observation (Figure 4), but the precise biochemical mechanisms leading to Bax and Bak activation are still not completely understood.

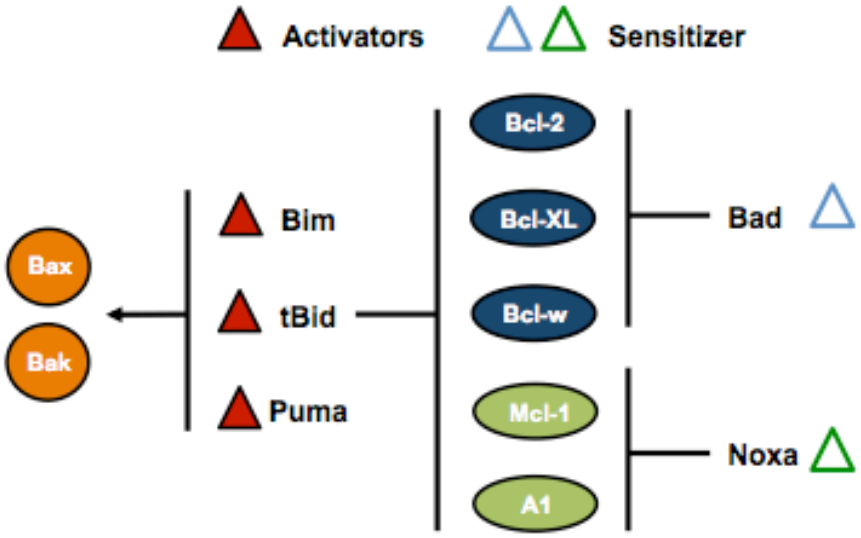

Figure 3 - Interaction of Bcl-2 proteins

Different binding assays established that BH3-only proteins bind either to all Bcl-2-like proteins or have a more restricted binding pattern as indicated. Certain BH3-only proteins like Bim, tBid and Puma can bind to all Bcl-2-like proteins and are thus considered to be more potent inducers of apoptosis; they have also been termed "activators" to indicate that they may also directly activate Bax and Bak (the status of Puma is uncertain in this respect). All other BH3-only proteins such as Bad and Noxa bind selectively to certain subsets of the Bcl-2 pro-survival proteins and thus are considered to be weaker killers and have been termed "sensitizer" to indicate that they can liberate "activators" from their sequestration to Bcl-2-like proteins (Certo *et al.*, 2006; Giam *et al.*, 2008; Willis *et al.*, 2005; Willis *et al.*, 2007).

1.1.5.1 The indirect activation model

The indirect activation model (also called displacement model) postulates that Bax and Bak have the ability to homo-oligomerize by an auto-activation mechanism, which importantly does not need any additional interaction of BH3-only proteins with Bax or Bak (Figure 4). Instead, BH3-only proteins function primarily by neutralizing their pro-survival Bcl-2-like relatives (Willis *et al.*, 2005). The role of pro-survival proteins is to repress Bax and Bak from auto-activation by direct inhibition. Bax and Bak are activated to release cyt *c* once they have been displaced from any of the pro-survival proteins by BH3-only proteins (Willis *et al.*, 2007). At least for the activation of Bak it could be shown that both Bcl-x_L and Mcl-1 sequester Bak in healthy cells to prevent damaging mitochondria. According to this model, cyt *c* is released when all of the pro-survival proteins have been neutralized, suggesting that Bad, which binds Bcl-2, Bcl-x_L and Bcl-w and Noxa, which, in contrast, binds Mcl-1 and A1 are weak inducers of apoptosis by themselves, but the combined action of Bad and Noxa is sufficient to induce apoptosis (Willis *et al.*, 2005; Willis *et al.*, 2007). Thus, Bak can be activated once Bcl-x_L and Mcl-1 have been neutralized for instance by the combination of Noxa and Bad, or by a single BH3-only protein which can bind to and inhibit all pro-survival members. The differences in the strength of apoptosis induction between BH3-only proteins are explained by the observation that Bim, tBid and Puma can bind to all pro-survival proteins, whereas the others only bind to a subset of these (Figure 3) (Chen *et al.*, 2005; Giam *et al.*, 2008). Evidence to support this model came from cells deficient for *bim* and *bid* and with additional Puma RNAi. Apoptosis induced by enforced co-expression of Bad and Noxa was not reduced in the absence of these putative direct activators, suggesting that direct activators were not required for induction of apoptosis. Moreover, Bak was able to mediate apoptosis without direct association with any of the BH3-only proteins, determined by co-immunoprecipitations (Willis *et al.*, 2007). Consistent with this model the BH3-mimetic ABT-737 which does not bind to Mcl-1 and A1 is more potent if combined with a Noxa-like partner (van Delft *et al.*, 2006).

1.1.5.2 The direct activation model

The truncated form of Bid was first found to induce a conformational change in either Bax or Bak, which established the idea that certain BH3-only proteins may interact with Bax and Bak directly to mediate cyt *c* release (Desagher *et al.*, 1999; Wei *et al.*, 2000). As mentioned above it was then demonstrated that oligopeptides (19-21 aminoacids) corresponding to the BH3 domain of BH3-only proteins have dual functions. Accordingly, the peptides that were able to activate Bax and Bak directly were termed "activators" (Bim, Bid and sometimes Puma), whereas others failed to do so. The other peptides were termed "sensitizers" (Bad, Bmf, Hrk, Noxa etc.) to indicate that they bind to pro-survival proteins and liberate the "activators" from them, but do not have this function by themselves (Figure 4). Contrary to other models, the direct activation model thus holds that in addition to interaction with pro-survival Bcl-2-like proteins a subgroup of BH3-only proteins activates Bax and Bak directly. Therefore, the "activator" BH3-only proteins are prevented from direct activation by forming a complex with pro-survival proteins in healthy cells. (Cartron *et al.*, 2004; Certo *et al.*, 2006; Kim *et al.*, 2006; Kuwana *et al.*, 2005; Letai *et al.*, 2002; Oh *et al.*, 2006; Walensky *et al.*, 2006). Upon reception of an apoptotic stimulus, one or several "sensitizer" proteins become activated and bind to pro-survival proteins to displace the "activator" proteins, which are then able to activate Bax/Bak directly to mediate apoptosis (Giam *et al.*, 2008; Letai *et al.*, 2002). Kuwana *et al* found similar results based on a liposome permeabilization assay. In this system the simple inactivation of pro-survival members was not sufficient to induce permeabilization unless a direct activator of Bax and Bak was present (Kuwana *et al.*, 2005). To overcome using the short BH3 peptides, Kim *et al* used in vitro transcribed and translated full length proteins to induce cyt *c*-release from isolated mitochondria. They found that Bim, tBid and Puma were able to activate Bax and Bak, and these "activators" were even required for apoptosis induced by co-expression of Bad and Noxa. Mutants of Bax and Bak that were unable to bind Bcl-2, Bcl-xL and Mcl-1 were not constitutively activate, suggesting that additional steps are required to activate Bax and Bak (Giam *et al.*, 2008; Kim *et al.*, 2006). The recent determination of the NMR structure of a Bim BH3 peptide in complex with Bax confirmed that this interaction occurs at a site different from the hydrophobic groove, where usually binding to Bcl-2 proteins takes place (Gavathiotis *et al.*, 2008). Evidence for these two proposed groups of BH3-only proteins has further been strengthened by demonstrating that mutants of Bim_S were able to induce apoptosis without detectable binding to anti-apoptotic Bcl-2-like proteins (Weber *et al.*, 2007), suggesting that Bim_S can induce the activation of Bax and/or Bak.

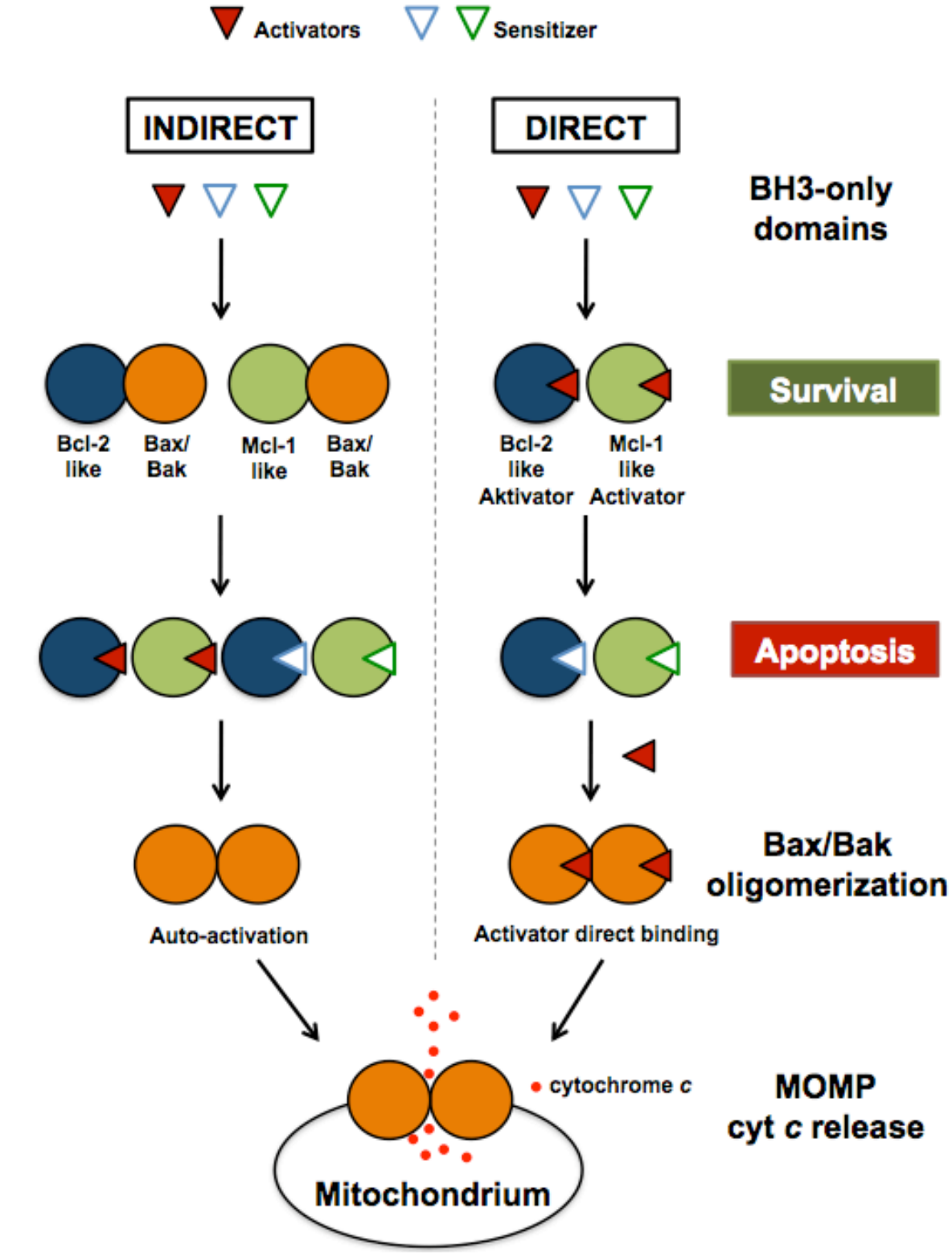

Figure 4 - Competing models for Bax/Bak activation by BH3-only proteins.

In the indirect model, Bax and Bak are prevented from auto-activation and oligomerization by interaction with pro-survival Bcl-2-like proteins, until BH3-only proteins bind to and neutralize the Bcl-2-like proteins to release Bax and Bak that can then induce permeabilization of the mitochondrial outer membrane (MOMP) and cytochrome *c* release. In the direct activation model, activator BH3-only proteins, such as Bim, are *de novo* induced or bound to pro-survival Bcl-2 proteins until displaced by "sensitizer " BH3-only proteins such as Bad and Noxa. Liberated "activators" can then bind to and activate Bax and Bak proteins and trigger their oligomerization, MOMP and cyt *c* release (Giam *et al.*, 2008).

1.2 Renal Cell Carcinoma (RCC)

1.2.1 The biology of renal cell carcinoma

All RCC types develop from the renal tubule epithelium. The most common form of RCC is the clear cell subtype, which occurs in about 85% of RCC (Kopper and Timar, 2006). The 2-year survival is less than 20 % in patients with metastatic clear cell RCC disease, and more than 50 % of all patients will develop metastases during their disease, despite surgical resection of the kidney (Mickisch, 2002; Mickisch and Mattes, 2005). For many years radical nephrectomy was the standard surgical treatment for patients with RCC. There are not many treatment options available and RCC is resistant to chemotherapy, with no single agent showing significant antitumor activity. Some trials explored immunotherapy as treatment for metastatic RCC. Interferon alpha (IFN α) and interleukin-2 (IL-2) have been widely applied, but the majority of patients do not benefit from this therapy (Reeves and Liu, 2009). This tumor has a highly vascular structure and patients present with abnormally elevated serum levels of vascular endothelial growth factor (VEGF), suggesting that angiogenesis is involved in the pathophysiology of RCC disease (Dosquet *et al.*, 1997; Edgren *et al.*, 2001; Jacobsen *et al.*, 2000; Sato *et al.*, 1999). Most cases of clear cell RCC are characterized by inactivation of the tumor suppressor von Hippel-Lindau (*VHL*) gene, which is connected to sustained angiogenesis (Latif *et al.*, 1993). Patients with the inherited von Hippel-Lindau disease typically develop RCC by inactivation of the *VHL* gene but this inactivation can also be seen in more than 60% of sporadic RCC cases (Gnarra *et al.*, 1995; Kondo *et al.*, 2002). Usually tumors of the either inherited or sporadic RCC disease have one allele of the *VHL* gene mutated (Kim and Kaelin, 2004; Rini, 2009b) and the second copy is lost by deletion, hypermethylation (Clifford *et al.*, 1998; Herman *et al.*, 1994) or somatic mutation, making it a good example of the Knudson two hit hypothesis, meaning that this tumor is the result of accumulated mutations (Knudson *et al.*, 1973). Xenograft experiments of RCC cell lines in mice confirmed the tumor suppressive function of the *VHL* gene. RCC cell lines with homozygous loss of *VHL* function induced tumor formation, whereas restoration of *VHL* function abrogated or resulted in minimal tumor formation (Iliopoulos *et al.*, 1995).

Under normoxic conditions the VHL protein forms an ubiquitin ligase complex targeting the hypoxia-inducible factor 1α protein (HIF-1α) for proteasome-dependent degradation (Maxwell *et al.*, 1999). The enzyme HIF-α-prolyl hydroxylase hydroxylates a proline residue on HIF-1α, which is then ubiquitinated and proteasomally degraded (Figure 5). Under conditions of hypoxia HIF-1α is stabilized and can translocate to the nucleus, where it binds to hypoxia response elements in the promoter region of several target genes. Therefore, deactivation of the *VHL* gene leads to constitutive transcriptional activation of a set of genes involved in vascular proliferation and glycolytic metabolism, thereby stimulating tumor progression and angiogenesis. Examples of these genes include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-α, (TGF-α) and basic fibroblast growth factor (FGF) (Bardos

and Ashcroft, 2004; Longo *et al.*, 2007). Some recent clinical approaches used to target the VEGF pathway for therapy of RCC (Longo *et al.*, 2007). Further, angiogenesis can also be activated by the phosphatidylinositol-3 kinase PI3K-AKT-mTOR signal transduction pathway, which is upstream of HIF-1α (Rini, 2009a). Inhibitors of this pathway may also be expected to demonstrate anti-tumor activity in RCC. Based on better understanding of the molecular pathways promoting clear cell RCC disease, several inhibitors have been developed and are being tested and applied to patients with metastatic clear cell RCC (Reeves and Liu, 2009). These agents target VEGF, its receptor VEGFR and the mTOR signaling pathway. Sunitinib, Sorafenib and Temsirolismus showed a slight improvement of clinical responses in randomized trials (Patel *et al.*, 2009; Thompson Coon *et al.*, 2009), resulting in the first drug approvals for treatment of RCC for 20 years. There is still a strong need of better therapeutic agents to further improve overall clinical responses or to induce stable tumor regression.

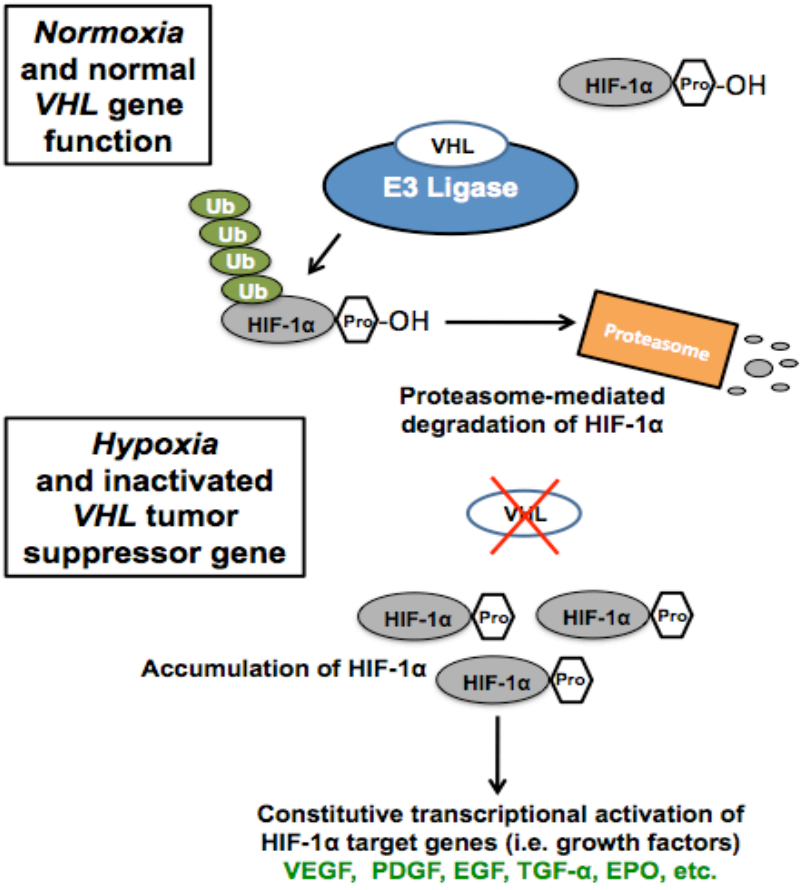

Figure 5 - Activity of the von Hippel-Lindau (VHL) tumor suppressor

Normal and inactivated von Hippel-Lindau (VHL) pathway in renal cell carcinoma, leading to increased production of vascular endothelial growth factor (VEGF) and other factors. Under conditions of *normoxia* and normal von Hippel-Lindau (*VHL*) gene function, VHL protein is part of an E3 ubiquitin ligase complex that targets hypoxia-inducible factor-1α (HIF-1α) for proteasomal degradation. Under *hypoxia* and with an inactivated *VHL* gene, the proline residue on HIF-1α is not hydroxylated (OH) and HIF-1α is not degraded by the proteasome, leading to accumulation of HIF-1α transcription factors. Activated HIF-1α translocates into the nucleus and leads to transcription of a large repertoire of hypoxia-inducible genes, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). These ligands bind to their receptors on endothelial cells, leading to increased angiogenesis, proliferation, and cellular metabolisation, etc.; Pro, proline; Ub, ubiquitin; OH, hydroxy group (Rini, 2009b).

1.2.2 The link between cancer and apoptosis

Non-transformed cells have checkpoints that would normally eliminate any form of deregulated cells by apoptosis, i.e. the entry from the G_1 in the S phase will not occur until DNA damage has been repaired. Tumor cells have thus to acquire the ability to overcome these checkpoints (Adams and Cory, 2007; Chonghaile and Letai, 2008). The cells achieve this by obtaining mutations in key proteins that regulate not only cellular proliferation and senescence but also apoptosis. For instance, to maintain a high proliferation rate, cancer cells create their own growth signal thereby becoming independent to negative growth regulation and apoptosis (Hanahan and Weinberg, 2000). Abnormal activation of oncogenes, DNA damage and cell cycle regulation usually causes the activation of the mitochondrial (intrinsic) apoptotic pathway, where the activation of BH3-only proteins is essential. Thus, cancer cells have to find mechanism to overcome apoptotic signaling induced by these proteins. Evasion of the normal apoptotic program is central for tumor initiation, progression and resistance to chemotherapy. Therefore, targeting the mechanisms tumor cells use to escape apoptosis may have great therapeutic benefit (Certo *et al.*, 2006; Chonghaile and Letai, 2008; Letai, 2008).

One mechanism is to increase the expression of pro-survival Bcl-2 proteins at the level of mitochondria. Bcl-2 was initially cloned from the breakpoint of the chromosomal translocation t(14; 18) in patients with follicular lymphoma (Adams and Cory, 2001; Cory *et al.*, 2003; Tsujimoto *et al.*, 1985). The translocation places the *bcl-2* gene next to enhancer elements of the immunoglobulin promoter causing an increased expression of Bcl-2. The translocation can be seen in 90% of follicular lymphomas and in 30% of diffuse large B cell lymphomas. Bcl-2 over-expression has also been linked to gene amplification (Rao *et al.*, 1998), hypermethylation of the *bcl-2* gene (Hanada *et al.*, 1993) or chromosomal deletions causing the loss of micro-RNAs involved in the silencing of Bcl-2 (Cimmino *et al.*, 2005). A series of genetic studies in mice demonstrated the oncogenic potential of Bcl-2 (Chonghaile and Letai, 2008; Strasser *et al.*, 1990; Vaux *et al.*, 1988). The co-expression of *c-myc* and *bcl-2* transgenes in mice showed that Bcl-2 was required for apoptosis inhibition, rather than further promoting c-myc dependent proliferation. Therefore, deregulated cellular proliferation can be coupled with a compensatory inhibition of apoptosis to achieve a synergistic effect on tumorigenesis (Chonghaile and Letai, 2008).

Alternatively, tumor cells can promote cell survival by inactivation of pro-apoptotic proteins. A very common defect of cancers is a deactivation of the tumor suppressor gene p53, which is seen in more than 50 % of all tumors. This has (among other effects) a negative effect on the activation of apoptosis, as the BH3-only proteins Noxa and Puma are direct transcriptional targets of p53, leading to abrogation of a DNA damage response pathway (Greenblatt *et al.*, 1994; Jeffers *et al.*, 2003; Nakano and Vousden, 2001; Oda *et al.*, 2000; Shibue *et al.*, 2003; Villunger *et al.*, 2003a). Recently, loss of Bim expression has been implicated as a tumor suppressor in epithelial tumors and renal cell carcinoma (Egle *et al.*, 2004; Tan *et al.*, 2005; Zantl *et al.*, 2007). Also mutations of the pro-apoptotic multi-domain member Bax have been observed in some solid tumors and hematopoietic malignancies

(Chonghaile and Letai, 2008; Meijerink *et al.*, 1998; Meijerink *et al.*, 1995; Rampino *et al.*, 1997; Villunger *et al.*, 2003b). Thus, inactivation of pro-apoptotic proteins may further promote tumor survival.

It has been accepted that increased expression of Bcl-2 can provide protection against chemotherapy (Martinou *et al.*, 1994; Miyashita and Reed, 1993; Takahashi *et al.*, 2003; Zhang and Insel, 2001). As mentioned previously, the abnormal phenotype of oncogene activation required for deregulated tumor growth initiates apoptotic signals in the form of activated BH3-only proteins (Chonghaile and Letai, 2008). Tumor cells may be selected for increased levels of Bcl-2-like proteins to compensate for the pro-apoptotic signals generated. Therefore, the Bcl-2-like proteins are presumably bound to BH3-only proteins, and tumor cells acquire a state where they depend to the anti-apoptotic protein for survival (Certo *et al.*, 2006; Chonghaile and Letai, 2008; Letai, 2008).

1.2.3 Resistance to apoptosis in renal cell carcinoma

The acquired resistance towards apoptosis is considered a universal feature in the development of cancer cells (Hanahan and Weinberg, 2000). Defects in apoptosis are not only linked to survival advantages in tumor development but may also confer inherent resistance to chemotherapeutic drugs (Johnstone *et al.*, 2002). Human renal cell carcinoma (RCC) cell lines are very resistant towards apoptosis. The molecular basis of this resistance is not fully understood but might be achieved by deregulation of pro- and anti-apoptotic Bcl-2 proteins. Accordingly, Bcl-2 was found to be over-expressed in some cases of RCC (Huang *et al.*, 1999), which was linked to progression in patients (Kallio *et al.*, 2004). It has been assumed that caspase activation following the release of cytochrome *c* remains intact in RCC (Gerhard *et al.*, 2003), suggesting that resistance to apoptosis arises from upstream defects in the apoptotic signal transduction. Loss of the pro-apoptotic BH3-only protein Nbk/Bik appeared to be a common feature of RCC but its contribution to apoptosis sensitivity is not known (Sturm *et al.*, 2006). The BH3-only protein Bim has a strong pro-apoptotic capacity, as it is able to interact with all pro-survival Bcl-2 proteins and may also directly activate Bax and Bak to promote apoptosis (see above; (Certo *et al.*, 2006; Chen *et al.*, 2005)). The tumor suppressive function of Bim became first apparent under oncogenic expression of c-myc in B cell lymphoma (Egle *et al.*, 2004). Bim was required for apoptosis induced by c-myc and additional loss of Bim has been shown to accelerate lymphomagenesis. Moreover, the *bim* gene was found to be deleted on both alleles in mantle cell lymphoma and inactivated by promoter hypermethylation in Burkitt lymphoma (Mestre-Escorihuela *et al.*, 2007). Expression of Bim was also described to be frequently reduced in renal cell carcinoma and was relevant in determining the apoptosis sensitivity in these cells, where restoration of Bim function in RCC correlated with increased drug response and tumor cell apoptosis (Zantl *et al.*, 2007). Thus, expression of Bim in non-transformed normal cells may be critical for counteracting oncogenesis (Pinon *et al.*, 2008), and could have implications for suppression of RCC development and sensitivity to chemotherapeutic drugs.

1.2.4 Inhibition of anti-apoptotic Bcl-2 proteins

An important therapeutic intervention was to develop inhibitors that mimic the BH3 domain to antagonize the pro-survival Bcl-2-like proteins directly (Chonghaile and Letai, 2008; Cragg *et al.*, 2009; Fesik, 2005). The BH3 mimetic ABT-737 was developed by Abbott Laboratories, using high throughput NMR-based screening of chemical libraries to identify small molecules that fit into the hydrophobic pocket of Bcl-2-like proteins. ABT-737 was then derived as a chemically linked combination of two of these ligands that bound to proximal sites in the hydrophobic pocket of Bcl-x_L (p2 and p4, Figure 6). This compound binds with high affinity in the nanomolar range to several Bcl-2-like proteins and inhibits their pro-survival function. ABT-737 interacts with Bcl-2, Bcl-x_L and Bcl-w, but not with Mcl-1 and A1 (Lee *et al.*, 2007; Oltersdorf *et al.*, 2005). ABT-737 was ineffective at activating apoptosis in cells doubly deficient of *bax* and *bak*, showing that the mechanism of action depends on the Bcl-2 family of proteins (van Delft *et al.*, 2006). In most tumors ABT-737 as a single agent was less effective to mediate apoptosis, but was able to synergize with conventional chemotherapy in various human tumors and preclinical mouse models (Cragg *et al.*, 2009). Thus, it seems that chemotherapy can provide the additional priming event that is required to enable the Bcl-2 antagonist to kill intrinsically resistant tumor cells (Chonghaile and Letai, 2008). Consistent with the low affinity of ABT-737 for Mcl-1, multiple reports have shown that high basal levels of Mcl-1 expression are associated with resistance to ABT-737, while knockdown or blockade of Mcl-1 re-sensitized tumor cells to single treatment with ABT-737 (Chen *et al.*, 2005; Del Gaizo Moore *et al.*, 2007; Konopleva *et al.*, 2006; Lin *et al.*, 2007; Nguyen *et al.*, 2007; van Delft *et al.*, 2006). Thus, ABT-737 sensitivity appeared to correlate with high expression of Bcl-2, Bcl-x_L and Bcl-w (Cragg *et al.*, 2009), along with often low expression of Mcl-1 or A1 (Vogler *et al.*, 2009) and with a greater amount of Bim bound to Bcl-2, which can be liberated by the BH3 mimetic to mediate apoptosis (Del Gaizo Moore *et al.*, 2007; Deng *et al.*, 2007). A slightly modified compound termed ABT-263 with identical function and improved bioavailability in animals has now entered clinical trials phase I and II (Tse *et al.*, 2008).

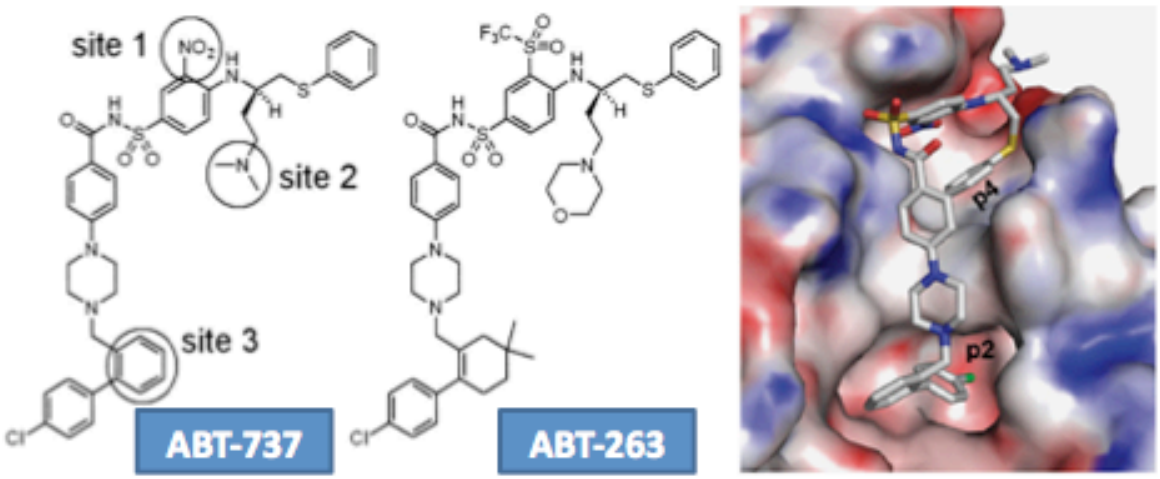

Figure 6 - Chemical structures of the Bcl-2 inhibitors ABT-737 and ABT-263

The chemical features and the three structural sites in ABT-737 (site 1 to site 3), that impair the binding to serum albumin are shown. To improve the bioavailability of this compound, the three sites along the backbone of ABT-737 were modified to maximize its oral activity in animals. These improvements resulted in the identification of ABT-263 (Tse *et al.*, 2008). Crystal structure of ABT-737 in complex with Bcl-x_L is shown on the right. Structure analysis of the Bcl-x_L:ABT-737 complex confirms that the hydrophobic binding pocket (p2 and p4) is occupied by ABT-737, where usually binding to BH3 domains takes place. Surface is colour-coded: blue, positive potential, red, negative potential, grey, neutral potential (Lee *et al.*, 2007).

1.3 Aims

The strong apoptosis resistance to chemotherapy in RCC is well-documented. This project aimed at the characterization of defects in mitochondrial signal transduction in RCC and at the delineation of novel approaches of restoring apoptosis sensitivity in resistant RCC tumor cells.

Previous work had indicated that caspase activation, following release of cyt *c* into the cytosol, is intact in RCC (Gerhard *et al.*, 2003). Death signals emanating from various cellular origins require the activation of BH3-only proteins, which are instrumental in activating Bax/Bak and causing the release of cyt *c*. Although a loss of BH3-only expression would be expected to promote tumorigenesis, only few data are available on the role of this class of proteins in human tumors. The first part of this work thus aimed at analyzing the relationship between expression levels of BH3-only proteins and the ability of RCC cells to undergo apoptosis on a quantitative level and at examining functional consequences of a potential BH3-only loss for determining apoptosis sensitivity.

The second part of this work was focused on exploring the ability of the novel Bcl-2 inhibitor ABT-737 to overcome apoptosis resistance in RCC. A number of leads pointed to the possibility that RCC may rely on anti-apoptotic Bcl-2 proteins for survival. The second aim of this work was thus to understand this balance and in particular to understand the relative importance of the two different subsets of Bcl-2 proteins, that can be either targeted by ABT-737 or not. A more detailed analysis of the mitochondrial signaling pathway is important to understand tumorigenesis and to identify vulnerable points in RCC's ability to support growth and treatment resistance.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemical product	*Manufacturer*
ABT-737	Abbot Laboratories (USA)
Acetic acid (100%)	Roth (Karlsruhe)
Acrylamide/bisacrylamide solution	Bio-Rad (Munich)
All Blue Protein Standard	Bio-Rad (Munich)
Ammonium persulfat (APS)	Sigma-Aldrich (Steinheim)
Ampicillin	Sigma-Aldrich (Steinheim)
Annexin-V (human; fluorochrome conjugate FITC)	BD Pharmingen (Hamburg)
Biocoll	Biochrome (Belin)
Blasticidin (stock: 5 mg/ml)	Invitrogen (Karlsruhe)
Bovine serum albumine (BSA)	Sigma-Aldrich (Munich)
Bromphenolblue	Serva (Heidelberg)
Calcium chloride	Sigma-Aldrich (Steinheim)
Calcium phosphate	Roth (Karlsruhe)
Chloroform	Sigma-Aldrich (Steinheim)
Collagenase	Sigma-Aldrich (Steinheim)
Cycloheximid	Roth (Karlsruhe)
Deoxynukleotide (dATP, dCTP, dGTP,dTTP)	Roche (Mannheim)
DEVD-AMC	Bachem (Weil am Rhein)
Digitonin	Sigma-Aldrich (Steinheim)
Dithiothreitol (DTT)	Merck (Darmstadt)
Dimethyl sulfoxide (DMSO)	Merck (Darmstadt)
DMEM	PAA (Pasching, Austria)
DNase I Amplification Grade	Invitrogen (USA)
Doxycycline	Fluka (Ulm)
EDTA	Roth (Karlsruhe)
EGTA	Roth (Karlsruhe)
Ethanol (99%, 70%)	Pharmacy, TUM, (Munich)
Ethidium bromide	Roth (Karlsruhe)
Etoposide	Sigma-Aldrich (Steinheim)
Fetal bovine serum (FCS) tetracycline-negative	PAA (Pasching, Austria)
Fetal bovine serum (FCS) ultra-low endotoxin	PAA (Pasching, Austria)
Formaldehyde (37%)	Roth (Karlsruhe)
Glycine	Sigma-Aldrich (Steinheim)
Glycerol	Roth (Karlsruhe)
Glutamine	Sigma-Aldrich (Steinheim)
HEPES	Fluka (Ulm)
Hoechst	Sigma-Aldrich (Steinheim)
Hydrochloric acid (HCl)	Pharmacy, TUM (Munich)
Isopropanol	Merk (Darmstadt)
K3[Fe(CN)]	Sigma-Aldrich (Steinheim)
K4[Fe(CN)]	Sigma-Aldrich (Steinheim)
Lipofectamine 2000	Invitrogen (Karlsruhe)
Lipofectamine RNAi Max	Invitrogen (Karlsruhe)
Low-fat dry milk	Roth (Karlsruhe)
Magnesium chloride ($MgCl_2$)	Roth (Karlsruhe)
Methanol (MeOH) (99%)	Roth (Karlsruhe)

MG-132	Sigma-Aldrich (Steinheim)
Nonidet P-40 (NP-40)	Sigma-Aldrich (Steinheim)
Opti-MEM	Invitrogen (Karlsruhe)
Paclitaxel	Sigma-Aldrich (Steinheim)
PAGE ruler protein standard	Fermentas (St. Leon-Rot)
Paraformaldehyde (PFA)	Sigma-Aldrich (Munich)
peqGOLD TriFast	Peq-Lab (Erlangen)
Penicillin/Streptomycin 100x concentrate	PAA (Pasching, Austria)
Phosphate buffered saline (PBS)	PAA (Pasching, Austria)
Polybrene	Sigma-Aldrich (Munich)
Ponceau S	Roth (Karlsruhe)
Potassium chloride (KCl)	Roth (Karlsruhe)
Propidium iodide	Sigma-Aldrich (Munich)
Protease inhibitor cocktail	Roche (Mannheim)
RPMI 1640	PAA (Pasching, Austria)
Saponin	Sigma-Aldrich (Munich)
Sodium hydroxide (NaOH) (0,2 N, 10 N)	Pharmacy, TUM, (Munich)
Sodium chloride (NaCl)	Merck (Darmstadt)
Sodium dodecyl sulfate (SDS)	Roche (Basel, Switzerland)
Sucrose	Sigma-Aldrich (Steinheim)
Staurosporine (STS)	Sigma-Aldrich (Steinheim)
SuperScript II reverse transcriptase	Invitrogen (USA)
Synthetic oligopeptide Bim-BH3	Biosyntan (Berlin)
TEMED (N,N,N',N'-tetramehylendiamine)	Bio-Rad (Munich)
Tetracycline	Fluka (Ulm)
Trichostatin A	Roth (Karlsruhe)
Tris(hydroxymethyl)-aminomethan	Roth (Karlsruhe)
Triton-X-100	Bio-Rad (München)
Trypsin-EDTA	PAA (Pasching, Austria)
Tween 20	Sigma-Aldrich (Steinheim)
Vinblastine	Sigma-Aldrich (Steinheim)
Zeocin	Invitrogen (Karlsruhe)
zVAD-fmk	Bachem (Weil am Rhein)
2-mercaptoethanol	Sigma-Aldrich (Steinheim)
5-aza-2`-deoxycytidine	Sigma-Aldrich (Steinheim)
5-Fluoruracil	Sigma-Aldrich (Steinheim)

2.1.2 Laboratory equipment

Product	*Supplier*
Bakteria incubator WB300	Mytron (Heiligenstadt)
Cell culture plates (6-, 12-, 24- and 96-well)	Falcon (USA)
Cell culture plates (10 cm, 15 cm)	Falcon (USA)
Centrifuges Megafuge 1.0 R, 2.0 R	Haraeus (Hanau)
Centrifuge Biofuge 15 R	Haraeus (Hanau)
Centrifuge 5415C bench-top	Eppendorf (Hanau)
Centrifuge Sorvall RC 26 Plus	Haraeus (Hanau)
Centrifuge-ultra	Du Pont (USA)
CO_2 incubator Hera Cell 240	Haraeus (Hanau)
Eppendorf tubes (1.5 ml)	Eppendorf (Hanau)
Electrophoresis chamber Mighty Small SE260	Hoefer (USA)

FACS tubes	Peske (Aindling/Arnhofen)
Falcon tubes (15 ml, 50 ml)	Greiner (Nürtingen)
Petri dishes (50 mm, 100 mm)	Peske (Aindling/Arnhofen)
Photographic film	Kodak (Stuttgart)
Pipettes (1-25 ml)	Greiner (Nürtingen)
Pipette tips	Peske (Aindling/Arnhofen)
Parafilm	Roth (Karlsruhe)
Sterile filters (0,45µm)	Zefa (Harthausen)
Syringes (5 ml, 10 ml, 20 ml)	Braun (Melsungen)
Syringes (Omnifix, 27G)	Braun (Melsungen)
FACS Aria	Becton Dickinson (USA)
Film developer Curix 60	Agfa-Gaevert (Cologne)
Flowcytometer FACS Calibur	Becton Dickinson (USA)
Laminar flow hood HERAsafe HSP 18	Haraeus (Hanau)
Light microscope Axiovert 40 C Leica	Carl Zeiss (Göttingen)
Spectrophotometer NanoDrop	Thermo-Fischer (Schwerte)
Thermocycler TRIO-Thermoblock	Biometra (Göttingen)
pH meter MultiCal pH526	WTW (Weilheim)
Power supply unit EPS 1001	Amersham (Sweden)
PVDF membrane	GE (UK)
UV visualization Eagle Eye-Still Video System	Statagene (USA)
Western blot chamber Mini-Trans-Blot Cell	Bio-Rad (Munich)
Whatman protran nitrocellulose membrane 0.2 µm, 0.45µM	Schleicher (Dassel)

2.1.3 Buffers

Buffer	*Composition*
Annexin V-binding buffer	10 mM HEPES, pH 7.4 140 mM NaCl, 2.5 mM $CaCl_2$
DEVD assay buffer	10 mM HEPES, pH 7.0 50 mM NaCl 2 mM $MgCl_2$ 5 mM EGTA 0.1 % (w/v) CHAPS 0.1 g/l BSA 100 µM DEVD-AMC
Digitonin permeabilization buffer	20 mM HEPES, pH 7.2 100 mM KCl 5 mM $MgCl_2$ 1 mM EDTA 1 mM EGTA 250 mM Sucrose 1x Protease inhibitors cocktail (Roche) 200 µg/ml Digitonin
FACS buffer	PBS, 0.5% BSA (w/v)
FACS buffer for fixation	PBS, 4 % (v/v) Paraformaldehyde
FACS buffer for permeabilization	PBS, (w/v) 0.5% BSA, 0.75% Saponin

Gel running buffer (SDS-PAGE)	50 mM Tris 384 mM Glycine 3.5 mM SDS
HEPES buffered saline (2x) ($CaCl_2$ transfection)	40 mM HEPES, pH 7.8 250 mM NaCl 10 mM KCl 1.5 mM Na_2HPO_4, 0.2% (w/v) Glucose
LacZ fixation buffer	PBS 0.2 % (v/v) gluteralaldehyde 5 mM EGTA 5 mM $MgCl_2$
LacZ staining buffer	PBS 0.2 % (v/v) gluteralaldehyde 5 mM EGTA 5 mM $MgCl_2$ 10 mM K3[Fe(CN)] 10 mM K4[Fe(CN)] 0.5 mg X-Gal
MB buffer (Sub-cellular fractionation)	10 mM HEPES, pH 7.5 210 mM Mannitol 70 mM Sucrose 1 mM EDTA 1x Protease inhibitors cocktail (Roche)
MB-EGTA buffer (Sub-cellular fractionation)	10 mM HEPES, pH 7.5 210 mM Mannitol 70 mM Sucrose 1 mM EGTA 1x Protease inhibitors cocktail (Roche)
MB-EGTA-Triton-X-100 buffer (Sub-cellular fractionation)	10 mM HEPES, pH 7.5 210 mM Mannitol 70 mM Sucrose 1 mM EGTA 1% (v/v) Triton-X-100 1x Protease inhibitors cocktail (Roche)
NP-40 lysis buffer (DEVD assay)	20 mM Tris/HCl pH 8.8 10 mM NaCl 1 mM EDTA 0.5% (v/v) NP-40 1x Protease inhibitor cocktail
Propidium iodide staining buffer (PI)	PBS, 10% (v/v) FBS, 5 µg/ml PI
Resolving gel (5 gels) (SDS-PAGE)	7.5 ml Resolving gel buffer 12.6 ml Bis-acrylamide 9.9 ml H_2O 180 µl Ammonium persulfat 10% 30µl TEMED

Resolving gel buffer	1.5 M Tris/HCl, pH 6.8 14 mM SDS
Stacking gel (5 gels) (SDS-PAGE)	3.75 ml Stacking gel buffer 2.4 ml Bis-acrylamide 13.8 ml H_2O 70 µl Ammonium persulfat 10% 30 µl TEMED
Stacking gel buffer (SDS-PAGE)	0.5 M Tris/HCl pH 6.8 14 mM SDS
1x TBS	20 mM Tris/HCl pH 7.6 137 mM NaCl
1x TBS-T	20 mM Tris/HCl pH 7.6 137 mM NaCl 0.1% (v/v) Tween 20
Triton-X-100 lysis buffer (Total cell lysates)	50 mM Tris/HCl pH 7.4 150 mM NaCl 1 mM EDTA 1% (v/v) Triton-X-100 1x Protease inhibitor cocktail
Western blot (WB) blocking buffer	1x TBS-T, 5 % (w/v) low-fat dry milk
WB blocking buffer	1x TBS-T, 5 % (w/v) BSA
WB transfer buffer	20 mM Tris, 154 mM Glycine 20% (v/v) MeOH
4x Laemmli buffer	62.5 mM Tris/HCl pH 6.8 70 mM SDS 5% (v/v) 1 M DTT 10% (v/v) Glycerol 1 mg/ml bromphenolblue

2.1.4 Antibodies

Specifity	*Clone*	*Source*	*Company*
A1	Polyclonal	Rabbit	Cell Signaling (USA)
A1	Polyclonal	Rabbit	Dr. J. Borst (Netherland)
Active caspase-3	C92-605	Rabbit	BD Pharmingen (USA)
Bad	Polyclonal	Rabbit	Cell Signaling (USA)
Bak NT	Polyclonal	Rabbit	Upstate (USA)
Bax NT	Polyclonal	Rabbit	Upstate (USA)

Bcl-2	Bcl-2/100	Mouse	BD Pharmingen (USA)
Bcl-x_L	polyclonal	Rabbit	Cell Signaling (USA)
Bcl-w	Polyclonal	Rabbit	Cell Signaling (USA)
Bid	Polyclonal	Rabbit	Cell Signaling (USA)
Bim	Polyclonal	Rabbit	Sigma-Aldrich (Steinheim)
Caspase-8	1C12	Mouse	Cell Signaling (USA)
COX IV	20E8	Mouse	Molecular Probes (USA)
Cytochrome *c*	7H8.2C12	Mouse	BD Pharmingen (USA)
Mcl-1	22	Mouse	BD Pharmingen (USA)
Noxa	114C307.1	Mouse	Alexis (USA)
Puma	Polyclonal	Rabbit	Cell Signaling (USA)
p53	1C12	Rabbit	Cell Signaling (USA)
Tubulin	DM 18	Mouse	Sigma-Aldrich (Steinheim)

2.1.5 Secondary antibodies

Specifity/Conjugate	*Clone*	*Source*	*Company*
Anti-mouse IgG HRP	Polyclonal	Goat	Sigma-Aldrich (Steinheim)
Anti-rabbit IgG HRP	Polyclonal	Goat	Sigma-Aldrich (Steinheim)
Anti-rabbit IgG FITC	Polyclonal	Donkey	Dianova (Hamburg)
Anti-rabbit IgG Cy5	Polyclonal	Donkey	Dianova (Hamburg)

2.1.6 Plasmids

Name	*Application*	*Resistance*	*Supplier*
pcDNA4/TO/*myc*-HisA	Cloning	Ampicillin	Invitrogen
pcDNA4/TO-Bim_S	PCR, Transfection	Ampicillin	G. Häcker
pcDNA4/TO-Bim_L	PCR	Ampicillin	G. Häcker
pcDNA4/TO-Bim_{EL}	PCR	Ampicillin	G. Häcker
pcDNA4/TO-Puma	Transfection	Ampicillin	G. Häcker
pcDNA4/TO-LacZ	Transfection	Ampicillin	G. Häcker
pLVTHM	Cloning of shRNA	Ampicillin	Addgene
pLVTHM-shLuciferase	Transduction	Ampicillin	own
pLVTHM-shMcl-1	Transduction	Ampicillin	own
pPAX	Packaging	Ampicillin	Addgene
pMD.2G	Envelope	Ampicillin	Addgene

2.1.7 RNAi

Oligonucleotides for cloning of shRNA were synthesized at Metabion (Munich). siRNAs were ordered as duplexes from MWG Biotech (Munich). The 3`overhangs were dTdT. For some genes two non-overlapping sequences were ordered.

Name	*Application*	*Sequence*
shLuc_fwd	shRNA	5'-GCGGTGCCCGTGCGCTGCTGGTGCCAACTTCAA GAGAGTTGGCACCAGCGCACTTTTTGGAAAT-3'
shLuc_rev	shRNA	5'-CGATTTCCAAAAAGTGCGCTGCTGGTGCCAACT CTCTTGAAGTTGGCACCAGCACACGGGGA-3'
shMcl1_fwd	shRNA	5`-CGCGTCCCCACGCGGTAATCGACTCAATTCAA GAGATTGAGTCCGATTACCGCGTTTTTTGGAAAT-3`
shMcl1_rev	shRNA	5`-CGATTTCCAAAAAACGCGGTAATCGGACTCAA TCTCTTGAATTGAGTCCGATTACCGCGTGGGGA-3`
siA1-1 (441)	siRNA	5`-GGAAGAAUUGUAACCAUAU-3`
siA1-2 (511)	siRNA	5`-CGGAUGUGGAUACCUAUAA-3`
siBim-1	siRNA	5`-GGAAGAAUUGUAACCAUAU-3`
siBim-2	siRNA	5`-CCACUAUCUCAGUGCAAUG-3`
siControl	siRNA	5`-GCGCAUUCCAGCUUACGUA-3`
siMcl-1	siRNA	5`-GGUUUGGCAUAUCUAAUAA-3`
siNoxa	siRNA	5`-AGUCGAGUGUGCUACUCAA-3`
siPuma -1	siRNA	5`-CCGAGAUGGAGCCCAAUUA-3`
siPuma -2	siRNA	5´-CCUGGAGGGUCCUGUACAA-3`
sip53	siRNA	5`-GUACCACCAUCCACUACAA-3`

2.1.8 Oligonucleotides

Oligonucleotides for PCR were synthesized at Metabion (Munich).

Name	*Application*	*Sequence*
Bim_fwd	PCR	5'-CGGGATCCAGACCAAATGGCAAAGCAACCTTCT-3'
Bim_rev	PCR	5'-GGAATTCAATGCATTCTCCACAC-3'
LVTHM_seq	Sequencing	5'-TGTCGCTATGTGTTCTGG-3'
CMV_seq	Sequencing	5'-CGCAAATGGGCGGTAGGCGTG-3'

2.1.9 Media

Medium	*Composition*
RCC growth medium (RPMI)	RPMI 1640 (PAA, Pasching, Austria) 10% FBS (ultra-low endotoxin, PAA) 100 U/ml penicillin (PAA) 100 µg/ml streptomycin (PAA)
RCC growth medium for primary cells (Click´s RPMI)	Click´s RPMI 1640 (Biochrome, Berlin) 10% FBS (PAA) 5 mM glutamine (Sigma, Steinheim) 100 U/ml penicillin (PAA) 100 µg/ml streptomycin (PAA) 10 µg/ml gentamicin (PAA) 50 µg/ml vancomycin (Sigma)
HeLa-Trex Noxa K9 growth medium (DMEM)	DMEM (PAA) 10% tetracycline negative FBS (PAA) 10 µg/ml gentamicin (PAA) 50 µg/ml vancomycin (Sigma) 5 µg/ml blasticidin (Invitrogen, USA) 125 µg/ml zeocin (Invitrogen)
293FT growth medium (DMEM)	DMEM (PAA) 10% FBS (PAA) 100 U/ml penicillin (PAA) 100 µg/ml streptomycin (PAA)
Freezing medium	90% growth medium 10% DMSO
Lysogeny broth (LB) medium	10 g/l tryptone 5 g/l yeast extract 10 g/l sodium chloride
S.O.C. Medium	2% tryptone 0.5% yeast extract 10 mM NaCL 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose

2.1.10 Bacteria

Bacteria were grown at 37 °C in LB or S.O.C medium.

Strain	*Application*	*Genotype*	*Supplier*
E. coli TOP10	Cloning, Amplification	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-	Invitrogen, USA

2.1.11 Cell lines

Cell lines	*Medium*	*Description, tumor grade*	*Supplier*
RCC-21	RPMI	kidney clear cell carcinoma cell line, pT2, NO, MX/GII	DKFC, Germany
RCC-26A	RPMI	kidney clear cell carcinoma cell line, pT2a, Nx, M1/ GII	DKFC Germany
RCC-30	RPMI	kidney clear cell carcinoma cell line, pT1, No, Mx/GI	DKFC Germany
RCC-1M	RPMI	kidney clear cell carcinoma cell line, pT2, N1, Mx/ GII-III (lung-metastasis)	DKFC Germany
RCC-KP	RPMI	kidney clear cell carcinoma cell line, pT3b, M1/GIII	DKFC Germany
Caci-2	RPMI	kidney clear cell carcinoma cell line	ATCC, USA
HeLa-Noxa	DMEM	cervical adenocarcinoma cell line expressing human Noxa under control of tet-repressor	Invitrogen USA
293FT	DMEM	293 embryonal human kidney cell expressing SV40 large T antigen	Invitrogen, USA

2.1.12 Drugs and inhibitors

Name, stock (DMSO)	*Activity*	*Supplier*
ABT-737, 5 mM	Inhibition of Bcl-2 proteins	Abbott Laboratories (USA)
Cycloheximid, 10 mM	Inhibition of protein synthesis	Sigma-Aldrich
Etoposide, 50 mM	DNA alkylating agent	Sigma-Aldrich
Paclitaxel, 2 mM	Stabilisation of microtubules	Sigma-Aldrich
Trichostatin A, 1 mM	Inhibition of histone deacetylation	Sigma-Aldrich
Vinblastine, 1 mM	Stabilisation of microtubules	Sigma-Aldrich
Aza-deoxycytidine, 10 mM	Inhibition of chromatin methylation	Sigma-Aldrich
5-Fluorouracil, 100 mM	Inhibition of thymidylate synthase	Sigma-Aldrich

2.2 Methods

2.2.1 Molecularbiology

2.2.1.1 Cloning of shRNA containing lentiviral vector

To generate lentiviral vectors that contain the selection marker GFP and Mcl-1 or Luciferase specific shRNA, 2 µg of the lentiviral vector pLVTHM were digested with the restriction enzymes ClaI (5U) and MluI (10U) for 2 hours at 37°C in 2x Tango buffer (Fermentas) in a final volume of 50 µl. The restriction enzymes were heat-inactivated at 65 °C for 20 min. The generated phosphorylated overhangs were dephosphorylated by addition of shrimp alkaline phosphatase (2U) directly to the restriction samples for 1 h at 37 °C (Fermentas). The samples were heat-inactivated for 1 h at 65 °C. The cut vector was run on a 0.75 % agarose gel, and then excised using a sterile scalpel. Linearized vector DNA was extracted with the Promega Gelextraction Kit (Promega) according to the manufacturer's instructions. DNA was eluted into nuclease-free water (30 µl) and its concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

To create the insert, synthetic 5' phosphorylated oligonucleotides were annealed, specific for a target-sequence in human Mcl-1 or firefly Luciferase (Metabion). 10 µM of the sense and antisense oligonucleotides were mixed with water to a final volume of 40 µl. The samples were heated in a thermo block to 98 °C for 5 min and then slowly cooled down to room temperature by switching of the thermo block (around 1 h). Annealing was confirmed by running the samples on a 3 % agarose gel.

Ligation was performed using a molar ratio of 1:3 of cutted vector DNA and insert DNA in 1x ligation buffer with 10U T4 ligase (Fermentas) in a final volume of 20 µl. The solution was incubated at room temperature for 1 h. Two µl of the ligation products were diluted in 50 µl of TOP10 competent *E. coli* (Invitrogen) and cooled on ice for 1 h. Bacteria were heat-shocked at 42°C for 2 minutes in a water bath, cooled on ice shortly, and incubated with 250 µl S.O.C. medium at 37 °C for 1 h while horizontally shaking at 300 rounds/min. An aliquot of 200 µl and 50 µl of the bacteria suspensions were spread out on LB-ampicillin (100 µg/ml) plates and incubated overnight at 37°C.

The following day, colonies were picked and were cultured in 4 ml LB medium with 100 µg/ml ampicillin overnight. Vector DNA was purified using the Wizard Plus SV Miniprep Kit (Promega) according to the manufacturer's instructions. The correct insert sequence of the cloned products (pLVTHM-shMcl-1 or pLVTHM-shLuciferase) was verified by sequencing (Medigenomix) with the pLVTHM-seq primer (Metabion), and the verified vector was re-transformed into *E. coli*. Picked colonies were re-cultured in 4 ml LB medium with ampicillin overday, followed by an overnight culture in 200 ml LB medium with ampicillin, and vector DNA was purified with the Maxi Prep Kit (Promega), according to the manufacturer's protocol and eluted into 800 µl nuclease-free water. The concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

2.2.1.2 Generation of shRNA-containing lentivirus

Lentiviral particles containing shRNA vectors were generated in 239FT cells (Invitrogen). The cells were transiently transfected by calcium phosphate precipitation with plasmids encoding the viral packaging and envelope vectors, and the lentiviral vectors encoding shRNA against Mcl-1 or Luciferase. 293FT cells were seeded the day before transfection at 2.5 x 10^6 cells per 10 cm culture plate, and medium (DMEM, 10 % fetal bovine serum) was changed the next morning. 10 ml/plate fresh medium was added. 20 µg pLVHTM-shMcl-1 or shLuciferase vector, combined with 15 µg PAX2 (packaging vector) and 6 µg MD2.G (envelope vector) were mixed and diluted to 500 µl with H_2O, and 500 µl of 2x HBS were added. Upon addition of 50 µl 2.5 M $CaCl_2$, and after 20 minutes incubation at room temperature the complete solution was added drop-wise to the medium. The precipitate was removed after 6 hours, and 6 ml/plate of fresh medium was added. Cells were left to produce virus for further 48 hours, then the supernatant was cleared of cells and debris by centrifugation at 500 x g for 5 minutes at room temperature, followed by filtration through a 0.45 µm disposable filter. The virus containing medium was aliquoted and stored at – 80 °C.

2.2.1.3 Lentiviral transduction of RCC-26A cells

The RCC cell line 26A was harvested by trypsination with Trypsin-EDTA (PAA, Pasching, Austria) and 5 x 10^4 cells were centrifuged at 1500 rpm for 5 min at RT in a bench top centrifuge. The cell pellet was resuspendend in 1 ml of lentivirus containing medium in the presence of 6 µg/ml polybrene (Sigma) and seeded in a 6-well culture plate. The cells were incubated overnight at 37 °C and 5 % CO_2 and the next day 1 ml of fresh RCC medium was added to the cells and then cultured for further two days. The third day cells were harvested by trypsination and transferred to a 10 cm culture plate and cultured for further 5 days by replacing medium every second day. After one week cells were selected for GFP expression (indicative for constitutive expression of shRNAs) using high speed cell sorting (FACS Aria, Becton Dickinson, USA). Flow cytometry analysis confirmed that over 95 % of cells were GFP-positive after sorting.

2.2.1.4 Semi-quantitative RT- PCR

The reverse transcription of mRNA generates in the presence of a poly (dT) oligonucleotide single-stranded cDNA complementary to mRNA. Total RNA from RCC cell lines was extracted using the PeqGOLD Trifast Isolation kit (PeqLab, Erlangen). RNA was digested with (2U) DNase I (Invitrogen) for 15 min at room temperature to eliminate genomic DNA. The DNase I was inactivated by addition of 1 µl of 25 mM EDTA solution to the reaction mixture and by incubation at 65 °C for 10 min. 2 µg of RNA was then mixed with an oligo (dT) primer and reverse transcribed to cDNA with the SuperScript II first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. The cDNA products were amplified by PCR (GoTaq DNA Polymerase, Promega) with oligonucleotides complementary to the target sequence of human Bim (Metabion). Porphobilinogen deaminase was amplified as a control of cDNA integrity. Amplification PCR conditions were as follows: initial denaturation at 94 °C for 4 min; 25 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min and a final extension step at 72 °C for 10 min. Amplification of a mixture of cDNAs encoding for Bim_S, Bim_L and Bim_{EL} was used as a positive control for the Bim PCR. PCR products were separated by electrophoresis on a 2 % agarose gels and visualized under UV light after ethidium bromide staining.

2.2.1.5 Quantitative RT-PCR

Total RNA was extracted from cells 48 h after siRNA knockdown using RNeasy mini kit (Qiagen, Hilden) as described by the manufacturer and analyzed by quantitative RT-PCR. 1 µg of RNA was reverse transcribed using Expand Reverse Transcriptase (Roche Diagnostics, Mannheim) and poly (dT) oligonucleotide (Roche) according to the manufacturer's protocol. Quantitative PCR was performed using the LightCycler TaqMan Master Kit (Roche) together with the Universal Probe Library system (Roche). Relative gene expression was expressed as a ratio of the expression level of the gene of interest to that of Hypoxanthine-phosphoribosyl-transferase (HPRT) RNA determined in the same sample. The mean values of not transfected controls were normalized to 100 %.

2.2.2 Proteinbiochemistry

2.2.2.1 Preparation of total cell lysates

To obtain total cell lysates, cells were harvested, washed twice with PBS, and the pellet was resuspended in lysis buffer (approx. 100 µl per 10^6 cells) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100 and protease inhibitor cocktail (Roche, Mannheim) and incubated for 1 h on a rotary wheel in the cold room (approx. 2 – 3 °C). After centrifugation at 13000 rpm for 10 min at 4°C in a bench top centrifuge, the supernatant was transferred into a fresh plastic cup and the protein concentration was determined by Bradford reaction (section 2.2.2.3) (Biorad, Munich). The solution was either frozen at -80 °C or 4 x Lämmli buffer added at a ratio of 4:1. The protein extract samples were incubated at 96 °C for 5 min to denature proteins and to disrupt disulfide bonds and subjected to SDS-PAGE, followed by Western blotting (section 2.2.2.4) with specific antibodies (table 2.1.4).

2.2.2.2 Subcellular fractionation

To isolate mitochondria a subcellular fractionation protocol was performed, adapted from a previously published protocol (Eskes *et al.* 2000). Approx. 10 x 10^6 cells were harvested by trypsination, washed twice with PBS and centrifuged at 500 x g for 5 min at RT. The pellet was resuspended in 300 µl isotonic mitochondrial buffer (MB-buffer) and incubated on ice for 15 min. The cells were mechanically disrupted by 25 passages through a 27G needle on ice. The lysed cells were than centrifuged twice at 2000 x g for 5 min at 4°C, and after each centrifugation step the pellet was discarded. The supernatant was centrifuged at 13000 x g for 10 min at 4°C and the insoluble pellet containing mitochondria was resuspended in 50 µl of MB-EGTA buffer. The supernatant containing cytosol was ultra-centrifuged at 120000 x g for 1h at 4 °C and the pellet was discarded. The protein concentration of the supernatant was measured by Bradford reaction and stored at -80 °C. The mitochondria solution was centrifuged at 500 x g for 3 min at 4°C. The generated pellet was discarded and the supernatant centrifuged again at 10000 x g for 10 min at 4°C. The generated pellet was resuspended in 50 µl in MB-EGTA buffer and the protein concentration was determined by Bradford reaction. The supernatant containing mitochondria was than centrifuged again at 13000 x g for 10 min at 4°C and the pellet was finally resuspended in 50 µl MB-EGTA-Triton-X-100 buffer. The solutions were either frozen at -80 °C or immediately subjected to SDS-PAGE and Western blotting (section 2.2.2.4).

2.2.2.3 Determination of protein concentrations by Bradford

Protein concentrations were determined by Bradford reaction, using the Bio-Rad Bradford kit. Bovine serum albumin (Sigma) dissolved in water was used to generate a standard curve (0.02 µg/ml – 0.8 µg/ml). 1 µl of the lysates was added to 250 µl Bradford reagent in a 96-well plate and after 10 minutes incubation at RT in the dark, protein concentrations were measured in duplicates by absorption at 595 nm in a Tecan Sunrise spectrophotometer (Tecan, Männedorf).

2.2.2.4 SDS-PAGE and Western blotting

Proteins can be separated according to their molecular weight in an electric field by SDS-polyacrylamid gel electrophoresis (SDS-PAGE). The resolving gel was prepared in resolving gel buffer containing 12.5 % bis-polyacrylamide (Sigma). The composition of the gels is shown in table 2.1.3. Polymerisation was initiated by addition of ammonium persulfate and TEMED. The solution was mixed and poured into a gel chamber, and immediately overlayed with isopropanol. After polymerization for 1 h, the alcohol was washed away. The stacking gel was prepared in stacking gel buffer containing 3.6 % bis-polyacrylamide. Polymerisation was initiated by addition of ammonium persulfate and TEMED as before. The solution was mixed and poured on top of the resolving gel. After polymerization over night, denatured protein extracts were loaded on the gel along with an appropriate molecular weight protein marker (All blue, Bio-Rad; PAGE ruler, Fermentas). The samples were run through the stacking gel at 130 V until the bands entered into the lower resolving gel (15 min), and through the resolving gel at 180 V, followed by horizontally transfer of the separated proteins onto a nitrocellulose or PVDF membrane (Whattman, Dassel; GE Healthcare, Buckinghamshire, UK) by blotting in transfer buffer (Tris-buffered saline (TBS)/20% methanol) at 100 V for 60 min at 4°C. After transfer, membranes were blocked for 1 h at RT with TBS + 0.05 % Tween-20 (TBS-T) containing either 5% (w/v) low-fat dry milk or 5% (w/v) BSA (Sigma). After rinsing with TBS-T the membranes were incubated with human antibodies diluted in the corresponding blocking buffer either over night at 4 °C or for 2 – 3 h at RT with gentle agitation. Unbound primary antibodies were removed by washing three times for 5 min at RT in TBST. After blocking again in 5% milk-TBS-T for 30 min the membranes were incubated with horseradish-conjugated (HRP) secondary antibodies diluted in 5% milk-TBS-T for 1 hour at RT while shaking. Membranes were washed three times for 5 minutes in TBS-T. For detection of membrane-bound antibodies the membranes were incubated with enhanced chemiluminescence substrate (ECL-Plus, GE Healthcare). For visualization of protein bands the membranes were exposed to a photographic film for varying time periods and subjected to film exposure. Exposed films were developed using a Curix 60 machine (Agfa, Cologne).

2.2.2.5 Cytochrome *c* release from digitonin permeabilized cells

Following the treatment with indicated chemotherapeutic drugs for 24 h, 2 x 10^5 RCC-26A cells per sample were harvested by trypsinization, washed twice with PBS and resuspended in 50 µl permeabilization buffer (20 mM HEPES pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose and protease inhibitor cocktail (Roche)) containing 200 µg/ml digitonin (Sigma). The cells were incubated for 60 min at 30°C in the presence of dimethyl sulfoxide (DMSO), BH3-only oligopeptide Bim (100 µM) or ABT-737 (1 µM, 5 µM and 50 µM). The Bim-peptide (sequence MRPEIWIAQERRRIGDEFNA) was synthesized at Biosynthan GmbH (Berlin, Germany). Cells were then centrifuged for 10 min at 13000 rpm in a bench top centrifuge to separate insoluble pellets containing mitochondria from supernatants containing cytosolic fractions with released cyt *c*. The cell pellets and supernatants were solubilised in equivalent volumes by adding 4x Lämmli buffer. Finally the samples were incubated at 96 °C for 10 min to denature proteins and subjected to separation by SDS-PAGE and Western blotting (section 2.2.2.4) with a specific antibody for human cyt *c*. The membranes were re-probed with an anti-Bak as a mitochondrial marker.

2.2.3 Cell biology

2.2.3.1 Cell culture

RCC cell lines were cultured in growth medium (RPMI-1640, 10 % fetal bovine serum (FBS, ultra-low endotoxin), 100 U/ml penicillin and 0.1 µg/ml streptomycin) (PAA, Pasching, Austria) and maintained in a humidified incubator (Thermo) at 37 °C with 5 % CO2. Trypsin-EDTA (PAA) was used for detaching the cells upon reaching 80 % confluency (3 to 4 days). Trypsinization was stopped after 5 min by adding an excess of FBS containing growth medium and cells were thoroughly resuspended. 10 - 30 % of cells were transferred into a fresh cell culture plate and further cultured in fresh RCC growth medium. For long-term storage, the same trypsinization procedure was performed to harvest cells from cell culture plates. Cells were then pelleted and resuspended in freezing medium (90 % growth medium, 10 % dimethyl sulfoxide), and stored either in liquid nitrogen or in a freezer at -80 °C. Frozen cells were rapidly thawed at 37 °C in a water bath and added immediately into pre-warmed (37 °C) medium for re-culture. Cells were usually seeded into plates one day before performing experiments and medium was replaced the next day.

2.2.3.2 Isolation of primary cells

Primary cells were isolated from affected kidneys of patients with local clear cell RCC undergoing nephrectomy for tumor treatment in the Klinikum rechts der Isar (Technische Universität München). Both macroscopically normal and malignant tissue samples (1-2 g each) were excised within 1 h of nephrectomy and digested in complete Click´s RPMI medium (Click´s RPMI, 10 % FBS, 5 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 µg/ml gentamicin and 50 µg/ml vancomycin (Sigma)) at a weight/volume ratio of three, containing 3 mg/ml collagenase (Sigma) for 1-3 h at 37 °C on a rotary shaker. The resulting cell suspension was passed through a metal mesh (200 µm width) and washed three times in complete medium. Cells were layered in medium onto a discontinuous Biocoll gradient (Biochrome, Berlin; two phases: 75 or 100% in PBS) and centrifuged for 30 min at 400 x g. The fraction at the medium - 75% interface was transferred into a fresh plastic cup. The cells were recovered by centrifugation for 5 min at 400 x g, washed with PBS and used for experiments or preparation of total cell lysates.

2.2.3.3 Transient transfection of DNA into RCC cell lines

Cells from RCC cell lines were seeded at 90 % confluency in 6-well culture plates in RCC growth medium without antibiotics one day before transfection. For liposomal transfection of plasmids encoding human Bim_S or Puma, 1 µg DNA of pcDNA-BimS or pcDNA-Puma was combined with 0.1

µg DNA of pcDNA-ß-galactosidase (for determination of transfection efficiencies) and mixed with Lipofectamine 2000 (Invitrogen) in a ratio of 1:3 and incubated in FBS-free RPMI medium for 25 min at room temperature (according to the manufacturer the complex is stable up to 6 hours at room temperature). The complexes were added to the cells, and the 6-well plates were gently shaken to mix the medium thoroughly. Medium was replaced after 6 hours. 24 h later cell were used for determination of transfection efficiency by staining for *ß*-galactosidase (LacZ) in 6-well plates. Apoptosis was quantified by morphological scoring of LacZ-positive cells (section 2.2.3.6.6).

2.2.3.4 Transient transfection of siRNA into RCC cell lines

siRNAs were designed according to published guidelines (Reynolds *et al.*, 2004; Ui-Tei *et al.*, 2004) and synthesized from Metabion (Munich, Germany). 3' overhangs were carried out as dTdT. The control siRNA sequence was designed as a non-silencing control containing a random sequence that does not match within the human and murine genome. Transient knockdown of A1, Bim, Mcl-1, Noxa, Puma, and p53 was achieved using gene specific siRNA. Sequences of specific siRNAs are shown in section 2.1.7. 8 x 10^4 RCC cells/well were seeded into a 6-well plate in antibiotics-free growth media. 24 h later 20 nM of siRNA was incubated with 1.25 µl of RNAi MAX (Invitrogen) in a total volume of 250 µl in OptiMEM (Invitrogen). After 20 min of incubation at room temperature the siRNA and RNAi MAX mixture was completely added to the wells in a drop-wise manner giving a total volume of 1.5 ml. At 24 h or 48 h post transfection with siRNA, cells were assayed for gene knockdown by quantitative RT-PCR (section 2.2.1.5) and Western blotting (section 2.2.2.4) or were taken for further experiments and treated with chemotherapeutic drugs for 24 h as indicated.

2.2.3.5 Induction of apoptosis by chemotherapeutic drugs

Cells from RCC cell lines were seeded overnight in 6-well plates at 60 % – 70 % confluency (approx. 1.5 x 10^5 cells/well). The following day, the medium was removed and 1.5 ml of fresh growth medium was added. The stock solutions of the chemotherapeutic drugs were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C or -80 °C in a freezer. The concentrations of the drugs are shown in table 2.1.12. The cells were treated with the chemotherapeutic drugs by addition of stock solutions to the given final concentrations for various time periods (in most instances incubation time was 24 h). The solvent DMSO was included in the experiments as an untreated control. The following sections describe the staining methods for quantification of cell viability and apoptosis. The mean values were calculated from at least three independent experiments. To determine statistical significance student`s t-test was applied and p values below 0.04 were considered as statistical relevant.

2.2.3.6 Assays for quantification of cell viability and apoptosis

2.2.3.6.1 Annexin V-binding assay

After induction of apoptosis by treatment with chemotherapeutic drugs (section 2.2.3.5), the medium containing detached dead cells was collected and transferred into a 15 ml conical tube (Greiner, Germany). The adherent RCC cells were rinsed with PBS, which was again added to the collection tube. The remaining cells were harvested by trypsination for 5 min at 37 °C/5 % CO_2. The reaction was stopped by addition of FBS-containing growth medium and cells were transferred into the collection tube. The cells were centrifuged at 500 x g for 5 minutes and washed once in PBS and were then resuspended in 500 µl cold annexin V buffer. Two aliquots of 200 µl were transferred into 96-well plates (V-shape) and cells were pelleted at 4°C. Cells were then resuspended in 50 µl Annexin-V buffer containing annexin V-FITC (1:50, BD Pharmingen) and incubated at 4°C in the dark for 10 minutes. After addition of 200 µl annexin V buffer, cells were analyzed by flow cytometry (FACS Calibur, Becton Dockinson).

2.2.3.6.2 Propidium iodide staining

After treatment with drugs RCC cells were harvested (section 2.2.3.6.1) and centrifuged at 500 x g for 5 minutes at 4 °C and resuspended in 200 µl cold PBS (PAA). The cells were splitted and duplicates of 100 µl were transferred into 96-well plates with V-shaped bottom and cells were pelleted again at 4 °C. Cells were washed twice with PBS/10 % FBS and then resuspended in 200 µl cold PBS/10 % FBS containing propidium iodide (5 µg/ml, Sigma) and put on ice. The cells were immediately analyzed by flow cytometry within 5 min (FACS Calibur, Becton Dickinson). To block caspase activation 100 µM zVAD-fmk was added in some experiments 1 h prior treatment with drugs.

2.2.3.6.3 Active caspase-3 staining

Cells from RCC cell lines were treated with the indicated drugs (section 2.2.3.5), harvested and washed twice in PBS (as described in section 2.2.3.6.1), following fixation in 4 % paraformaldehyde in PBS for 10 min at RT. The cells were centrifuged at 500 x g for 5 minutes at RT, washed twice in PBS and then resuspendend in 200 µl permebilization buffer (0.5 % BSA and 0.75 % (w/v) saponin (Sigma) in PBS). After re-pelleting the cells were stained with monoclonal anti-active-caspase-3 antibody (1:500 in a volume of 25 µl, BD Pharmingen) in permeabilisation buffer for 1 h at RT. After addition of 150 µl permeabilization buffer and one washing step in 200 µl permeabilization buffer, the cells were stained with polyclonal anti-rabbit-FITC antibody (1:200 in a volume of 25 µl, Dianova, Hamburg) in permeabilization buffer. After two washing steps in permeabilization buffer and one step in PBS/0.5 % BSA, the cells were resuspended in 200 µl PBS/0.5 % BSA and put in the dark at 4 °C until flow cytometry analysis (FACS Calibur, Becton Dickinson).

2.2.3.6.4 HOECHST staining of nuclei

To determine morphology of apoptotic nuclei, RCC cells were seeded in 6-well plates at 1.5 x 10^5 cells/well. The following day, medium was changed and cells were treated with chemotherapeutic drugs as described in section 2.2.3.5. 30 min prior to the end of the time period, cellular nuclei were stained by addition of 1 µg/ml Hoechst dye (Sigma) and further incubated at 37 °C/5 % CO_2 for 30 minutes. Cells were harvested by trypsination, washed twice with PBS, centrifuged at 500 x g for 5 minutes at 4 °C and resuspended in 10 µl PBS. The sample was applied to glass slides, and the number of "healthy" and apoptotic nuclei present in randomly chosen fields of vision were scored under a fluorescence microscope. A total of 300 - 400 cells were examined per sample from at least three different fields.

2.2.3.6.5 Effector caspase activity

Application of DEVD assay allows determination of caspase-3 activity in apoptotic cells. More precisely the effector caspase activity, which features the proteolytic activity that is able to cleave after the sequence Asp-Glu-Val-Asp (DEVD), is measured. Following treatment with drugs (2.2.3.5) the RCC cells were harvested by trypsinization, and washed twice with PBS. Cells were lysed in NP-40 lysis buffer for 10 minutes on ice. Cell lysates were collected after centrifugation at maximum speed (13000 rpm) in a bench top centrifuge at 4 °C (Eppendorf, Hamburg). Triplicates of aliquots (10 µl) were added to 90 µl of DEVD assay buffer containing 100 µM DEVD-7-amino-4-methyl-coumarin (DEVD-AMC) fluorimetric substrate (Bachem). Reactions were incubated in 96-well flat-bottom plates at 37 °C for 30 minutes, before fluorescence intensity of free AMC was measured.

2.2.3.6.6 LacZ staining of cells

To determine transfection effienciences in RCC cells transfected with plasmids encoding for Bim_S and Puma (section 2.2.3.3), cells were stained for ß-galactosidase (LacZ) 24 h upon transfection. The medium was carefully removed and cells were washed with PBS, following fixation for 5 min at RT in PBS containing 0.2 % gluteralaldehyde, 5 mM EGTA and 5 mM $MgCl_2$ and washed with PBS. The cells were stained by adding ß-galactosidase substrate in staining buffer (PBS, containing, 5 mM EGTA, 5 mM $MgCl_2$, 10 mM K3[Fe(CN)], 10 mM K4[Fe(CN)] and 0.5 mg X-Gal substrate) and incubated at 37 °C/5 % CO_2 for 12 to 24 h. After staining the number of blue LacZ-positive cells present with normal and apoptotic morphology was determined under a light microscope. At least 300 cells per well were counted from random fields.

3 Results

3.1 Bcl-2 proteins and resistance to apoptosis in RCC cell lines

The link between deregulated expression of pro- and anti-apoptotic Bcl-2 proteins and RCC´s progression and resistance to chemotherapy is poorly understood (section 1.2.3). Over-expression of anti-apoptotic Bcl-2-like proteins is a prominent mechanism how tumor cells can achieve a survival advantage. A consistent down-regulation of pro-apoptotic proteins may also reduce the sensitivity to apoptotic stimuli. The molecular events following the release of cytochrome *c* (cyt *c*) appeared to be intact in RCC (Gerhard *et al.*, 2003) and therefore this project focused on the upstream components regulating the mitochondrial release, where the activation of BH3-only proteins is instrumental to induce this process. Here, the group of pro-apoptotic BH3-only proteins was investigated, in particular the role of Bim and its contribution to RCC growth and to resistance against chemotherapeutic drugs. Investigation of primary tumor cells for loss of Bim expression and functional analysis of the underlying mitochondrial pathway in patient-derived RCC cell lines may be helpful in establishing whether the loss of Bim expression is functionally relevant in determining the apoptosis sensitivity of RCC cells. The results may suggest that Bim has a tumor suppressive function in RCC and this may have implications for developing new strategies to improve RCC´s drug response.

3.1.1 Loss of Bim expression in renal cell carcinoma

To determine the expression of Bim in RCC, cells from the clear cell subtype were investigated, which is the most common form of RCC (80 % of all cases). Primary cells were isolated from tumor samples and adjacent normal tissue from the affected kidney of three patients and total cell lysates were analyzed by Western blotting (Figure 7A). A clear reduction of Bim expression in the tumor samples compared to normal kidney parenchyma was observed. Detection of tubulin was used to confirm equal loading of the samples. The low expression of Bim in tumor samples may suggest that Bim-loss contributes to RCC´s development. Expression of Bim was then compared in three patient derived RCC cell lines (Figure 7B). Of these, the cell line 26A showed low expression of Bim, while Bim was easily detected in the 1M cell line and had an intermediate expression level in the cell line 30. Zantl *et al* analyzed a broader panel of tumor isolates and RCC cell lines by Western blotting and immunohistochemistry, including the patients shown here (9 cell lines and 43 patients). Collectively the data suggested that loss of Bim expression is a very common event in clear cell RCC, both in primary tumor cells and in patient-derived cell lines (Zantl *et al.*, 2007). The low expression of Bim may thus have implications for both growth and treatment resistance of RCC.

To determine the sub-cellular localization of Bim, cells from the RCC cell lines were separated into mitochondrial and cytosolic fractions using centrifugation protocols and subjected to

Bim detection by Western blot analysis (Figure 7C). Detection of cytochrome *c* oxidase complex 4 (COX-4) or caspase-8 served as a mitochondrial and a cytosolic marker respectively. The strength of cytosolic Bim expression confirmed the results obtained in total cell lysates. Bim was not only localized in the cytosol, but could also be detected at mitochondria in the high expressing RCC cell line 1M, suggesting that regulation of Bim activity requires post-translational modification or activation, rather than Bim-translocation to mitochondria during apoptosis.

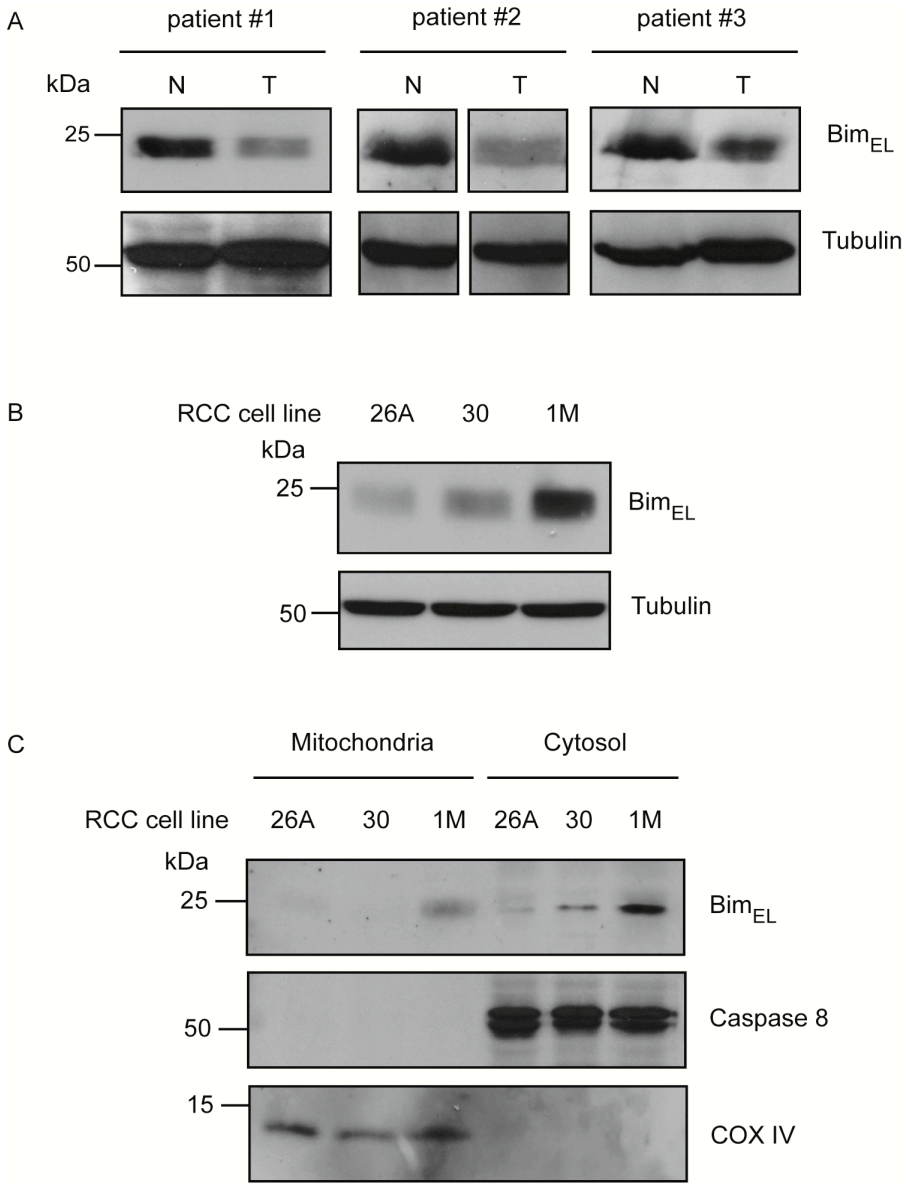

Figure 7 - Low expression of Bim in renal cell carcinoma

A, Primary epithelial cells were isolated from three patients with clear cell RCC and expression of Bim was analyzed in pairs of normal kidney (N) and tumor (T) by Western blot analysis. Expression of the Bim_{EL} isoform could be detected in RCC cells. The white line between the bands of patient #2 indicates that samples were on the same blot, but were not consecutive. B, Bim_{EL} was also assessed by Western blot analysis in three patient-derived RCC cell lines (26A, 30 and 1M). Detection of tubulin served as a loading control. C, Expression and sub-cellular localization of Bim_{EL} in RCC cell lines. Untreated cells were subjected to isolation of mitochondria and detection of caspase-8 and cytochrome *c* oxidase complex four (COX IV) served as a mitochondrial and cytosolic marker. Shown are representative blots from three separate experiments.

3.1.2 Epigenetic regulation of Bim

Several lines of evidence point to the possibility that epigenetic mechanisms are associated with initiation and progression of tumors. Transcriptional silencing of tumor suppressor genes can be achieved through epigenetic modification like histone deacetylation or chromatin methylation. The acetylation status of histones and the methylation pattern of genes can influence the access of transcription factors and determines the strength of transcriptional gene activation. The function of histone deacetylases (HDACs) is to decrease acetylation of histones and to inhibit gene transcription (Lane and Chabner, 2009). The observed loss of Bim expression in RCC may occur during tumor development through epigenetic regulation. To test for this possibility, primary cell isolates from tumors of two RCC patients were treated with the broad-spectrum HDAC inhibitor trichostatin A (TSA, Figure 8). Inhibition of histone deacetylation clearly demonstrated an induction of Bim expression in cells from the two tumor samples analyzed, suggesting that transcriptional inactivation of the *bim* gene may occur through histone deacetylation during tumor development. One tumor sample was also treated with the methylation inhibitor 5-aza-2`-deoxycytidine (AZA) and gave a similar induction of Bim as inhibition of histone deacetylation. A similar induction by TSA and AZA was observed in the three RCC cell lines 26A, 30 and 1M (Zantl et al., 2007).

Transcriptional activation of genes by inhibition of histone deacetylation may require additional synthesis of transcription factors, i.e. the induction of Bim by TSA could depend on the activation of a *bim* gene-specific trans-activator. To determine if Bim mRNA is directly induced by treatment with TSA, the Bim low-expressing cell line 26A was analyzed by reverse transcription PCR in a time-course experiment. TSA induced the expression of all three major splice forms of Bim mRNA with increasing strength over time (3-9h, Figure 4B). Adding of *de novo* protein synthesis inhibitor cycloheximide failed to block the induction of Bim mRNA, suggesting that TSA acted directly and does not depend on synthesis of other protein factors. Therefore epigenetic mechanisms appeared to contribute to low expression of Bim during development of RCC and in RCC cell lines. This may be relevant for increased tumor growth and treatment resistance of RCC.

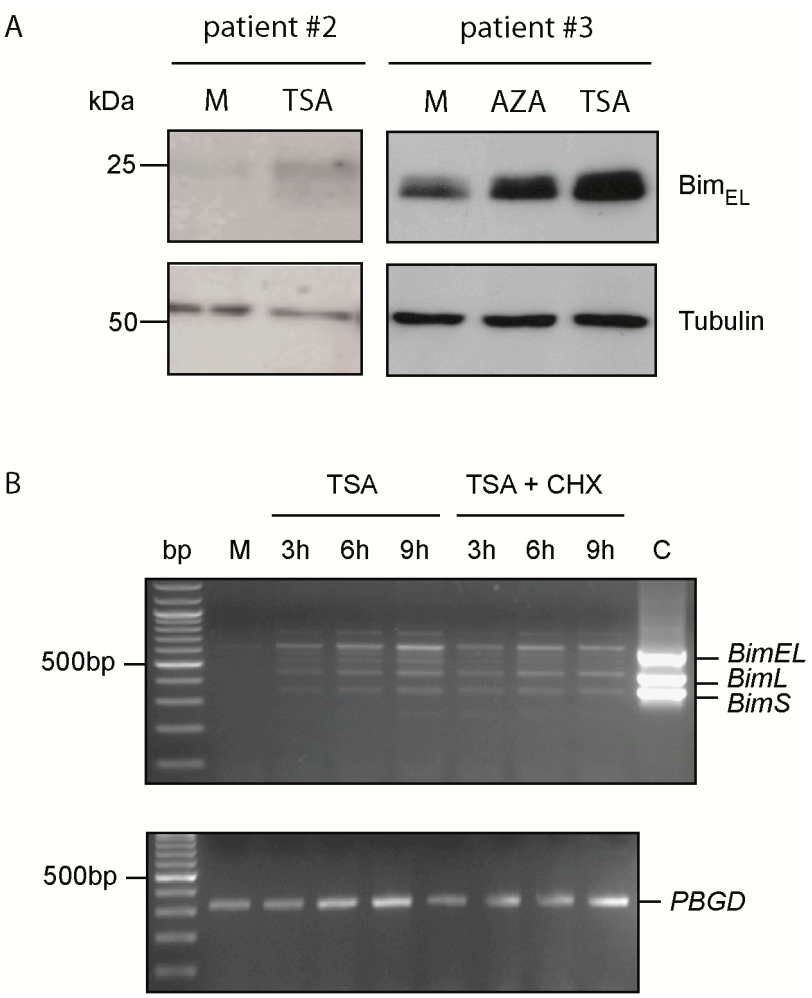

Figure 8 - Inhibition of histone deacetylation induces the expression of Bim

A, Primary cell isolates from tumors of two patients with clear cell RCC were seeded in 6-well plates overnight and treated the next day with 800 nM trichostatin A (TSA) and 3 µM 5-aza-2`-deoxycytidine (AZA) for 24 h or were left untreated (M). Expression of Bim_{EL} was analyzed by Western blotting and tubulin served as a loading control. B, cells from the cell line RCC-26A were treated in 6-well plates with 800 nM trichostatin A (TSA) or with the combination of trichostatin A and 10 µM cycloheximid (CHX) for the indicated time periods. Total cellular RNA was isolated and subjected to semi-quantitative reverse transcription PCR. Amplification PCR was done using Bim-specific oligonucleotides. The positive control (C) was a mixture of plasmids coding for Bim_S, Bim_L and Bim_{EL}. Porphobilinogen deaminase (PBGD) was amplified as a control of cDNA integrity. Similar results were obtained in three independent experiments of RT-PCR analysis.

3.1.3 Bim expression correlates with apoptosis sensitivity of RCC cell lines

The results above described a low expression of pro-apoptotic Bim in RCC. This could be taken to imply that the more Bim is expressed in RCC cell lines, the more apoptosis is induced. However, the contribution of Bim to apoptosis sensitivity of RCC cell lines is not known. Besides expression of Bim, differential expression of other Bcl-2 family proteins could determine the apoptosis sensitivity of RCC. Although basic expression differences may be expected in different RCC cell lines, any over-expression of pro-survival or down-regulation of pro-apoptotic proteins may contribute to increasing apoptosis resistance. To exclude an involvement of other Bcl-2 proteins than Bim to apoptosis sensitivity, two experimental lines were followed. The RCC cell lines 26A (low) and 1M (high expression of Bim) were treated with the genotoxic drug etoposide, and apoptosis was quantified by staining for active caspase-3 and flow cytometry analysis (Figure 9). The observed pattern was then compared with the expression of Bim and other Bcl-2 family members (Figure 10).

Only little apoptosis was induced by etoposide, but the RCC cell line 26A exhibited a more resistant apoptotic phenotype compared to 1M, which was the most sensitive cell line when treated with etoposide (Figure 9). Similar differences in apoptosis sensitivity had been described by previous studies in our laboratory (Zantl *et al.*, 2007), observed by quantification of apoptotic nuclei or by measurement of caspase activity upon treatment with adriamycin, etoposide, staurosporine and ultraviolet radiation. Differential expression of other Bcl-2 proteins followed by Western blot analysis, like the anti-apoptotic members Bcl-2, Bcl-x_L, Bcl-w and Mcl-1 or the pro-apoptotic Bax and Bak proteins, gave no indication that the differential expression of these proteins was the reason for the varying apoptosis sensitivity (Figure 10). Within the group of pro-apoptotic BH3-only proteins Bim was low expressed in the RCC cell line 26A and had a high expression level in the cell line 1M (Figure 10, left panel and Figure 7B). Noxa was nearly undetectable in both RCC cell lines and therefore a HeLa clone is shown that stably expresses human Noxa as a positive control, whereas Bad, Bid and Puma were easily detected but did not show a differential expression between the RCC cell lines that could account for the difference in apoptosis. Thus, apoptosis sensitivity in RCC appeared to correlate with the expression of Bim and not with the expression of other Bcl-2 proteins.

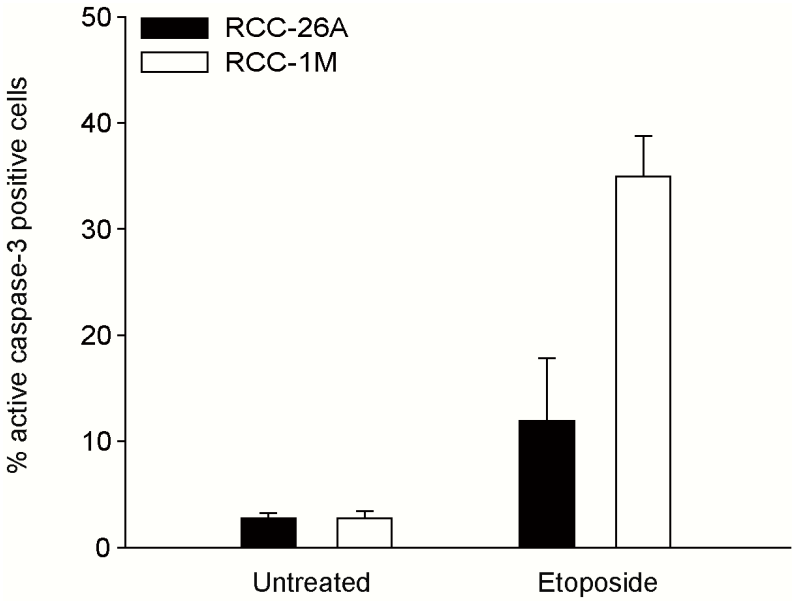

Figure 9 - Apoptosis sensitivity of RCC cell lines correlates with the expression of Bim

Cells from RCC cell lines 26A (low) and 1M (high expression of Bim) were left untreated or treated with 200 µM etoposide for 24 h. Apoptosis was quantified by active caspase-3 staining. The values represent the means/SEM of three independent experiments.

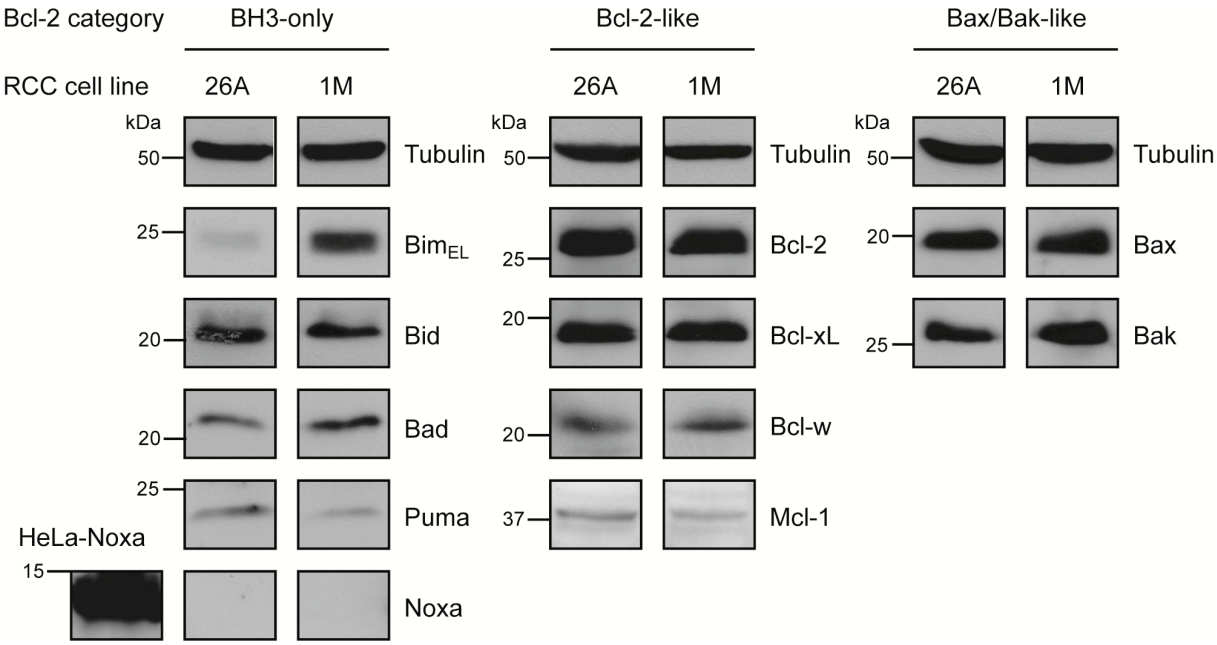

Figure 10 - Expression of Bcl-2 proteins in the RCC cell lines 26A and 1M

Expression levels of pro-apoptotic (left and right panel) and anti-apoptotic (middle panel) Bcl-2 proteins in untreated cells from the RCC cell lines 26A and 1M. As a positive control for Noxa expression a HeLa clone was used that stably over-expresses human Noxa. The white line between the bands indicates that samples were on the same blot, but were not consecutive. Detection of tubulin served as a loading control. The immunoblots shown are representatives of three separate experiments.

3.1.4 Experimental over-expression of Bim in an apoptosis-resistant RCC cell line

These data showed that apoptosis sensitivity of RCC cell lines correlated with the expression of Bim, suggesting that Bim-loss could be functionally relevant in determining the apoptotic response to treatment with drugs. RCC cells may thus require the presence of Bim to undergo apoptosis, which would suggest that restoring Bim function in a resistant RCC cell line could increase tumor cell apoptosis to an extent similar to that of a sensitive cell line. This hypothesis can be tested experimentally.

To evaluate the capacity of Bim to induce apoptosis in a Bim low-expressing RCC cell line, transient transfection experiments were carried out. $Bim_{S,}$ the most active isoform of Bim, whose activity is not dependent on post-translational modifications, was over-expressed in the apoptosis-resistant RCC cell line 26A and in the most sensitive cell line 1M (Figure 11). Based on *β*-galactosidase expression (co-transfected with Bim_S), transfection efficiency was comparable between the two cell lines. Over-expression of Bim_S induced a similar level of apoptosis in both cell lines. Thus, restoring Bim function in a resistant RCC cell line induced apoptosis. Since the sensitivity of the resistant cell line 26A to Bim_S-induced apoptosis was not reduced compared to the sensitive cell line 1M, one explanation would be that differential expression of other Bcl-2 proteins like the pro-apoptotic Bax and Bak and the pro-survival Bcl-2 proteins, downstream of Bim activation are not a responsible factor for the strong apoptosis resistance observed in RCC-26A. Furthermore, transfection of Puma, which has a similar binding capacity to Bcl-2-like proteins as Bim, gave no differences in apoptosis sensitivity between 26A and 1M, further confirming that differences in downstream effectors are not the reason for the differences in apoptosis resistance between the cell lines.

The results above suggested that expression of Bim could be relevant in determining apoptosis sensitivity of RCC cells. Zantl *et al* tested this hypothesis in a direct approach. They down-regulated Bim by RNAi-mediated knockdown in the high expression RCC cell line 1M, which was the cell line most sensitive to apoptosis induced by various stimuli (Zantl *et al.*, 2007). Transient transfections of Bim-specific siRNA showed that reduced expression of Bim correlated with a decrease in apoptosis sensitivity to external apoptotic stimuli. Thus, expression of Bim was relevant in determining the apoptosis sensitivity of 1M cells, suggesting that loss of Bim expression in the resistant RCC cell lines contributes to their drug resistance. Therefore, the presence of Bim in developing RCC may have a tumor suppressive function and loss of Bim expression may contribute to increased tumor growth and resistance to drug treatment.

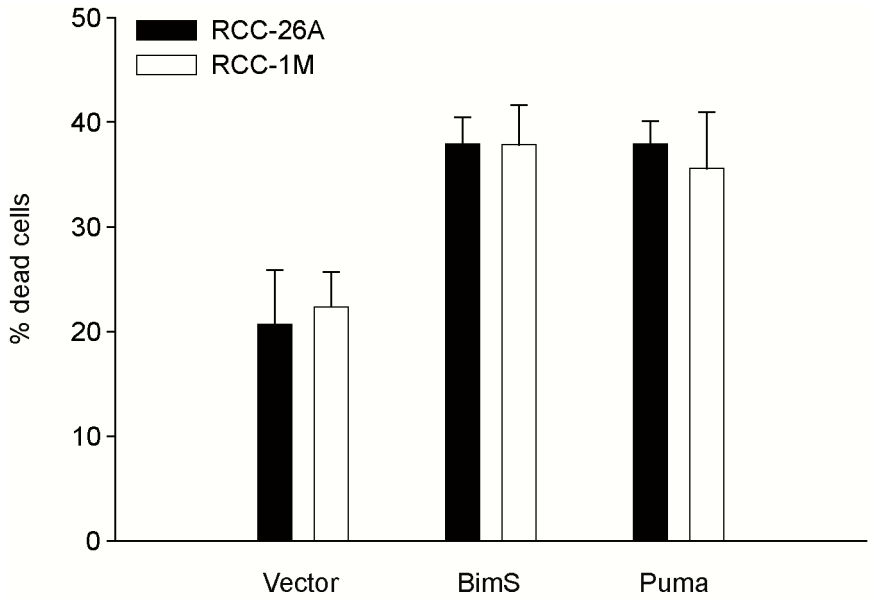

Figure 11 - Over-expression of BH3-only proteins in RCC cell lines induces apoptosis

Cells from the RCC cell lines 26A (black) and 1M (open bars) were seeded in 6-well plates at 80 % confluency in triplicates and were transiently transfected the next day using Lipofectamine 2000 (Invitrogen) with empty vector and plasmids coding for human Bim_S and Puma. A β-galactosidase expression vector was co-transfected to determine transfection efficiencies. 24 h post transfection cells were stained in wells for β-galactosidase (LacZ) and the morphology of LacZ-positive cells (dark blue staining) were scored dead or alive blind-probed. Per well approx. 300 cells were counted from random fields. The values represent the means (of triplicates)/SEM of three independent experiments.

3.2 Targeting the Bcl-2 proteins in RCC cell lines

3.2.1 ABT-737 inhibits the pro-survival function of Bcl-2 proteins

Previous work has shown that low expression of the pro-apoptotic Bim protein correlated with reduced apoptosis sensitivity of RCC cell lines, suggesting that RCC cell lines may require the presence and activation of Bim to undergo apoptosis (Zantl et al., 2007). It has been accepted that BH3-only proteins can bind to Bcl-2-like proteins to prevent apoptosis. Thus, the Bcl-2-like proteins may further support survival of RCC cells with low expression of Bim. Here, we aimed to overcome RCC´s Bim-dependency by inhibiting Bcl-2-like proteins to increase apoptosis responses. Targeting the mechanism RCC uses to escape apoptosis could be important to increase apoptosis sensitivity and may thus have implications to improve RCC´s drug response.

Enormous progress in understanding the molecular mechanism of apoptosis led to the development of inhibitors targeting the apoptotic machinery directly. The BH3 mimetic ABT-737 showed good activity as an inhibitor of Bcl-2-like proteins in various tumor cells, but has not been tested in RCC cells yet (section 1.2.4). In many tumor cells ABT-737 on its own does not induce significant apoptosis possibly because it does not antagonize the anti-apoptotic function of Mcl-1 and A1 (Chen *et al.*, 2005; Oltersdorf *et al.*, 2005). This could be taken to explain why ABT-737 has a low single-agent activity, but may synergize with chemotherapeutic drugs, for instance, by a mechanism involving the induction of BH3-only proteins that bind to and limit the anti-apoptotic function of Mcl-1 and A1. Conventional chemotherapy was able to provide the additional Mcl-1 and A1 neutralization event that would be required for enabling ABT-737 to induce apoptosis in resistant tumor cells (Chonghaile and Letai, 2008; Cragg *et al.*, 2009). Thus, this project aimed at determining RCC´s sensitivity to ABT-737, either as a single agent or in combination with chemotherapeutic drugs. Functional analysis of Bcl-2 proteins in RCC cell lines may be helpful to elucidate the molecular mechanisms underlying the cooperation between ABT-737 and conventional drugs. This could be important to determine which BH3-only proteins become activated by treatment with drugs and if they have the ability to overcome RCC´s treatment resistance by neutralizing the subgroup of Bcl-2-like proteins that is not targeted by the BH3 mimetic.

3.2.2 ABT-737 enhances apoptosis induced by vinblastine, paclitaxel and etoposide

To evaluate the sensitivity of RCC cells to ABT-737, apoptosis was quantified in a panel of four patient-derived RCC cell lines by active caspase-3 staining (Figure 12). To compare the differences between the RCC cell lines, all experimental parameter including culture conditions, cell number, incubation times and concentrations of the drugs were standardized. Annexin V-binding as a measure

of apoptosis was also tested in one cell line and this assay confirmed the results obtained by active caspase-3 staining (RCC-26A, Figure 13).

Consistent with the low affinity of ABT-737 to anti-apoptotic Mcl-1 and A1, ABT-737 was found to be almost inactive, suggesting that Mcl-1 and A1 support the survival of RCC cells (Figure 12). However, there was a slight induction of apoptosis in the Caci-2 cell line treated with 1 μM ABT-737. A possible explanation for this observation may be that the expression levels of Mcl-1 and A1 are not sufficient to inhibit the pro-apoptotic proteins that have been displaced from the Bcl-2-like proteins by the BH3 mimetic. These factors may include activator BH3-only proteins or monomeric Bax and Bak. Several chemotherapeutic drugs were tested in a combination treatment with ABT-737. The drugs have been used for many years to treat RCC clinically but demonstrated only low activity *in vitro* and response rates *in vivo* in most instances. Since RCC is thus resistant to conventional chemotherapy, the majority of patients do not benefit from single agent treatment. In accordance to clinical data, any of the four chemotherapeutic drugs tested induced only little apoptosis of RCC cell lines, suggesting that the Bcl-2-like proteins, which are not targeted by the drugs protect against apoptosis for instance by sequestration of either activator BH3-only proteins or Bax and Bak. In sharp contrast, the combination of ABT-737 with three of the four drugs tested increased apoptosis and demonstrated a strong synergistic pro-apoptotic activity in RCC cell lines. This effect was strongest for etoposide but also significant for vinblastine and paclitaxel, indicating that the combination with these drugs had the activity of neutralizing Mcl-1 and A1, which in turn led to activation of effector caspases.

No such synergistic effect was seen for the combination with 5-FU in any of the lines tested, even at later time points where 5-FU on its own induced significant apoptosis in the RCC cell line 26A (48 h, Figure 14). The cell line Caci-2 may be most informative in terms of 5-FU treatment, since ABT-737 induced considerable apoptosis on its own in these cells. However, there was still no synergistic effect with 5-FU even in Caci-2 cells, suggesting that 5-FU failed to provide the additional Mcl-1 and A1 neutralization event, which is required to undergo synergistic apoptosis. Detection of cell death, determined by propidium iodide staining gave similar results as staining for apoptosis. To determine if cell death depended solely on activation of caspases, a caspase family inhibitor was used. Importantly, cell death induced by combination treatment depended on caspases and could be completely blocked by adding the pan-caspase inhibitor zVAD-fmk, confirming that combination treatment induced specific apoptosis and not another form of caspase-independent cell death (Figure 15). Thus, ABT-737 can prime or sensitize resistant RCC cell lines for apoptosis induced by vinblastine, paclitaxel and etoposide but not by 5-FU, determined by measurement of cell viability and of a specific apoptotic marker.

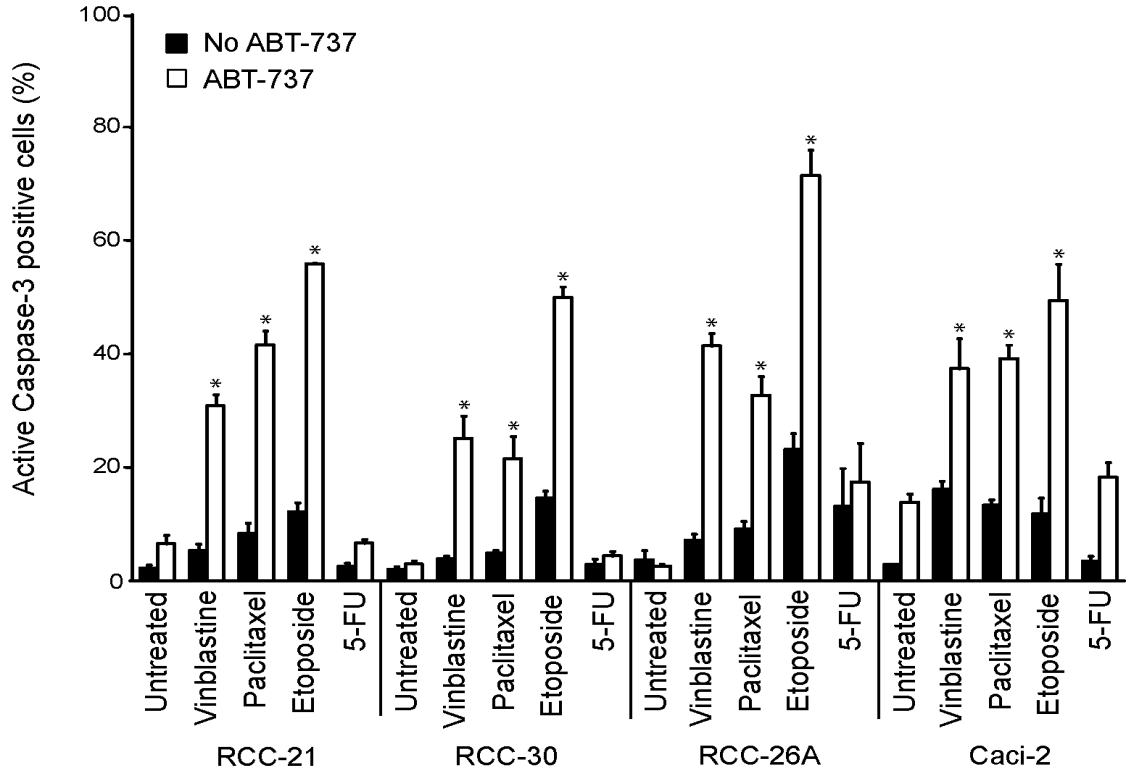

Figure 12 - ABT-737 potentiates apoptosis induced by chemotherapeutic drugs

Cells from RCC cell lines were left untreated or treated with 1 µM ABT-737, 100 nM vinblastine, 200 nM paclitaxel, 200 µM etoposide, 1 mM 5-FU or with the combination of ABT-737 plus chemotherapeutic drugs. Apoptosis was quantified by active caspase-3 staining upon 24 h treatment with drugs. Shown are the means/SEM of three independent experiments; * $P < 0.04$, combination treatment versus either ABT-737 or chemotherapeutic drugs

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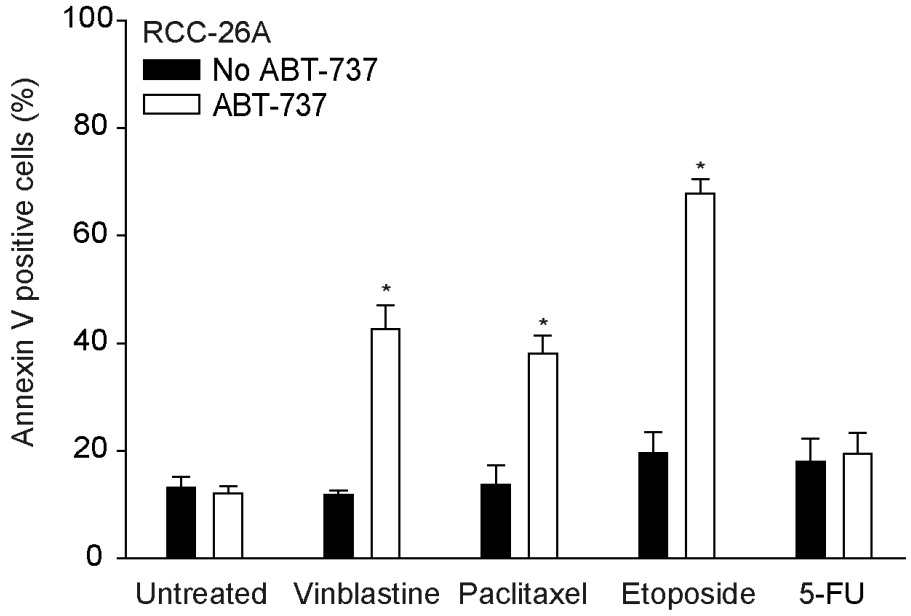

Figure 13 - Apoptosis sensitivity of RCC-26A cells determined by annexin V-binding

RCC-26A cells were treated as described in Figure 12 and apoptosis was quantified by assessing annexin V-binding. The values represent the means/SEM of three separate experiments. * $P < 0.04$, combination treatment versus either ABT-737 or chemotherapeutic drugs.

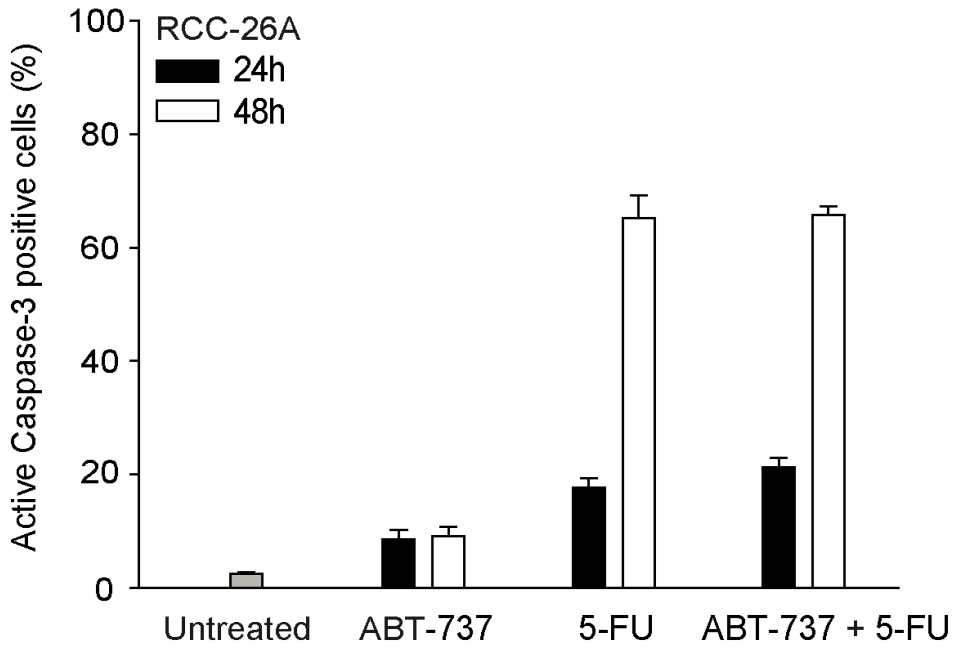

Figure 14 - No pro-apoptotic synergism between 5-FU and ABT-737

RCC-26A cells were left untreated (gray bar) or treated with 1 μM ABT-737, 1 mM 5-FU or with the combination of ABT-737 plus 5-FU. Apoptosis was quantified by active caspase-3 staining upon 24 h (black bars) and 48 h (open bars) treatment with drugs. Shown are the means/SEM of three independent experiments.

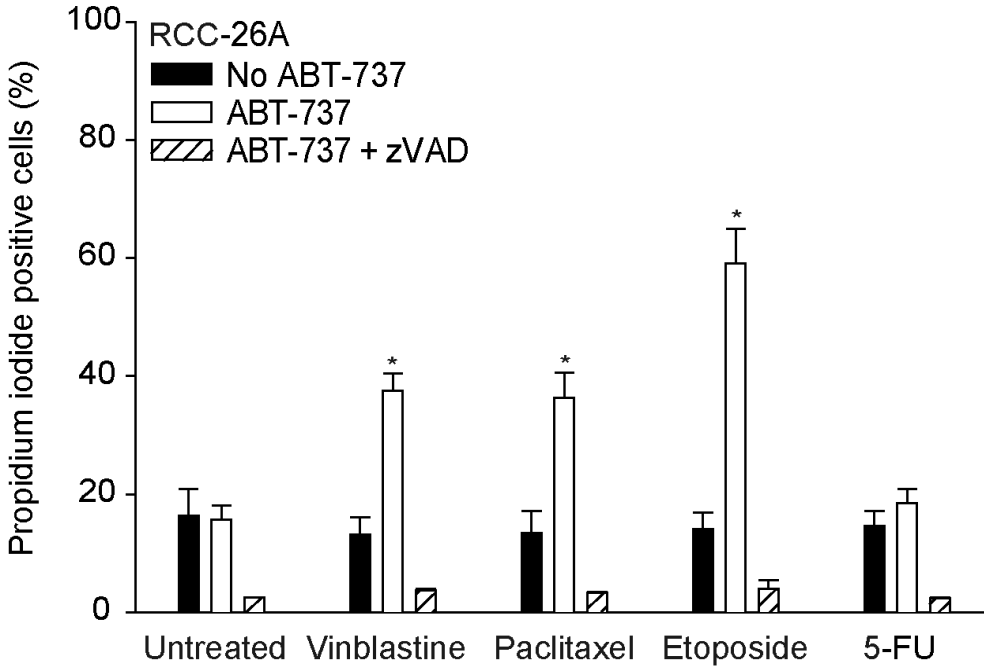

Figure 15 - Cell death induced by combination treatment requires caspase activity

RCC-26A cells were treated as described in Figure 12 and cell death was quantified by propidium iodide staining upon treatment with drugs for 24 h. 100 μM zVAD-fmk was added to block caspases 1 h prior to treatment with ABT-737 and drugs. The values represent the means/SEM of five separate experiments. * $P < 0.04$, combination treatment versus either ABT-737 or chemotherapeutic drugs.

3.2.3 Etoposide sensitizes for ABT-737 at the level of mitochondria

Current models of mitochochondrial apoptosis focus on the role of Bax and Bak activation, leading to MOMP (mitochondrial outer membrane permeabilization) and subsequently to cyt *c* release from mitochondria. The release can be achieved once (all of) the anti-apoptotic Bcl-2 proteins have been neutralized for instance by the pro-apoptotic Bim protein (Willis et al., 2007); or when certain activator BH3-only proteins have been displaced from Bcl-2-like proteins to activate Bax and/or Bak directly (Certo et al., 2006). However, both models require the activation of BH3-only proteins for the release of cyt *c*.

Previous experiments demonstrated that treatment with etoposide most strongly sensitized for ABT-737-mediated apoptosis. This would support a mechanism where the drugs induce the inactivation of Mcl-1 and/or A1, whereas ABT-737 inhibits the other Bcl-2-like proteins, leaving complete inhibition of anti-apoptotic proteins. Here, we aimed to follow these molecular decisions leading to cyt *c* release from mitochondria *in vitro* by analyzing permeabilized RCC cells upon treatment with drugs, where cytosolic components had been removed. An oligopeptide encompassing the Bim BH3 domain has been shown to initiate cyt *c* release from permeabilized cells (Kuwana et al., 2005). The existing models postulate that the release can occur once the peptide interacts with Bcl-2-like proteins or may directly activate Bax and/or Bak. The Bim-peptide therefore served as a positive control in our experiments and was found to induce cyt *c*-release from mitochondria in untreated and pre-treated RCC cells as found in other cellular models (Figure 16A).

To further analyze the collaboration of ABT-737 and chemotherapeutic drugs at the level of mitochondria, RCC cells were first treated with etoposide or 5-FU for 24 h and then permeabilized in the presence of ABT-737 or solvent (DMSO), followed by seperation into supernatants containing cytosolic components and insoluble pellets containing permeabilized cells. The fractions were subjected to cyt *c* detection by Western Blot analysis (Figure 16). Previous results suggested that cyt *c* is not released into the cytosol by treatment with drugs since there was only little apoptosis induced. In accordance with results obtained in intact cells, treatment with etoposide or 5-FU failed to induce the cyt *c* release, suggesting that the amount of Bcl-2-like proteins that is not targeted by the drugs is sufficient to block the release. ABT-737 was also inactive on its own, very likely because it lacks binding to Mcl-1 and A1. However, in cells that had been pre-treated with etoposide and then permeabilized in the presence of ABT-737, there was a substantial release of cyt *c* on a scale similar to the Bim peptide. This suggests that etoposide-treatment had the effect of neutralizing Mcl-1 and/or A1. It may be expected that 5-FU has no such a neutralizing effect, since there was no synergistic apoptosis in combination with ABT-737 detectable. In accordance with results in intact cells 5-FU failed to sensitize permeabilized cells for ABT-737 induced cyt *c* release, although a 50-fold higher ABT-737 concentration was used here (Figure 16A). Similar to previous results, even a concentration of 1 µM ABT-737 was sufficient to induce cytochrome *c*-release in etoposide pre-treated cells (Figure

16B). The data thus suggest that only treatment with etoposide but not 5-FU is able to neutralize Mcl-1 and/or A1, thereby leaving mitochondria sensitive for ABT-737.

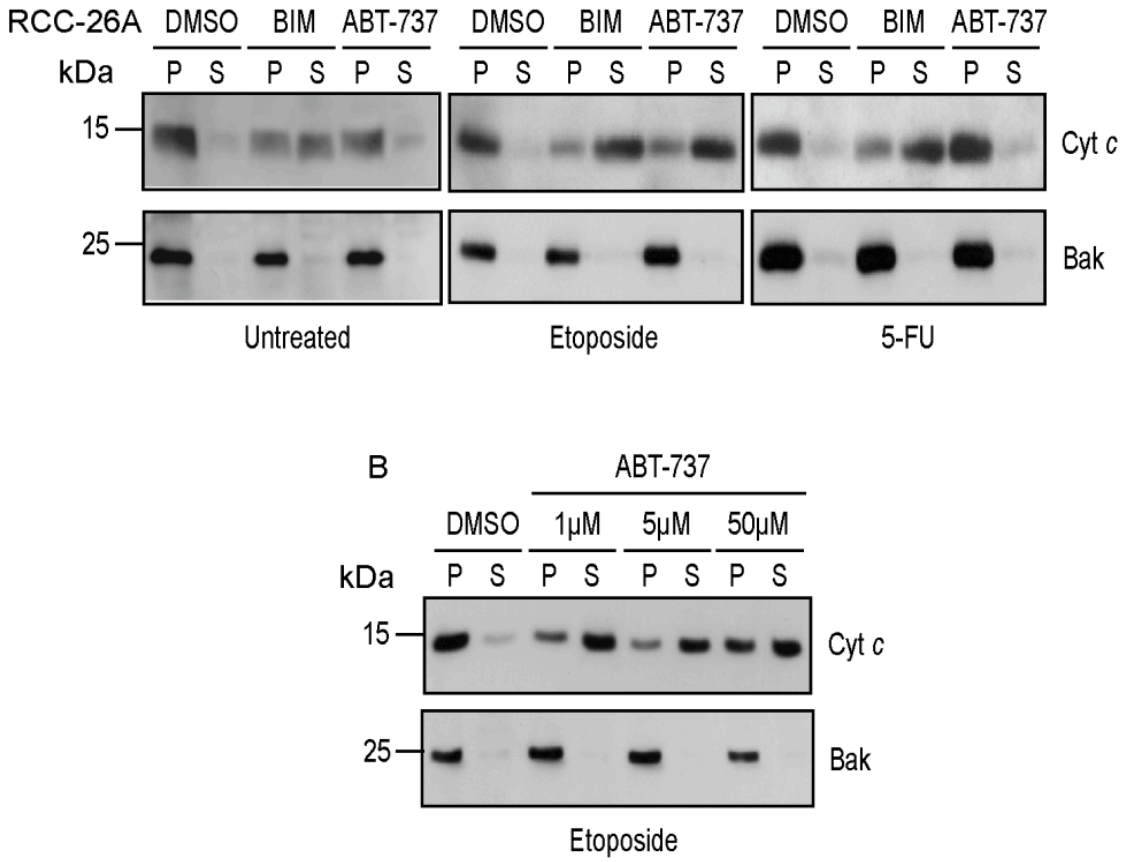

Figure 16 - Etoposide sensitizes mitochondria to cyt *c* release by ABT-737

Cells from the RCC-26A cell line were left untreated or treated with 200 μM etoposide or 1 mM 5-FU for 24 h. Cells were then permeabilized using 200 μg/ml digitonin in the presence of solvent (DMSO, dimethyl sulfoxide), a peptide derived from the BH3 domain of the BH3-only protein Bim (100 μM) or ABT-737 (in A 50 μM, in B concentrations as shown). After 1 h incubation at 30 °C the samples were separated into cytosol and insoluble pellets and subjected to Western blotting and detection of cyt *c*. The membranes were re-probed with an antibody specific for Bak as a mitochondrial marker. P, pellet containing mitochondria; S, supernatant containing cytosol with released cyt *c*. The blots shown here are representatives of three independent experiments.

3.2.4 Levels of Bcl-2 proteins during treatment with drugs

Etoposide treatment on its own was less effective to induce apoptosis in RCC cells, but appeared to have the activity of neutralizing the anti-apoptotic function of Mcl-1 and A1, thereby synergizing with ABT-737. This would suggest that some BH3-only proteins become activated by etoposide, but do not efficiently target other Bcl-2-like proteins. This observation indicates a role of the endogenous Mcl-1 antagonist, the BH3-only protein Noxa to account for neutralization since its binding pattern is specific for Mcl-1 and A1 (Chen et al., 2005). Moreover, it has been proposed by other reports that functional elimination of Mcl-1 requires Noxa-induced proteasomal degradation of Mcl-1 upon ultraviolet (UV) radiation (Nijhawan *et al.*, 2003; Willis *et al.*, 2005). Reduction of Mcl-1 levels by treatment with drugs through induction of Noxa would be one mechanism that may explain the here observed sensitization to ABT-737.

To identify changes in the expression levels of Bcl-2 proteins, the four RCC cell lines (section 3.2.2) were treated with the indicated chemotherapeutic drugs for 24 h and levels of Mcl-1 and Noxa were followed by Western blotting (Figure 17). Noxa was nearly undetectable in untreated cells from any of the RCC cell lines analyzed. Of the four drugs tested, etoposide treatment most strongly induced Noxa in RCC cell lines. However, only in the RCC-26A cell line Mcl-1 was degraded concomitantly upon etoposide treatment, suggesting that functional inactivation of Mcl-1 does not occur via degradation primarily, since the other cell lines also underwent synergistic apoptosis without having Mcl-1 necessarily degraded. Nonetheless, degradation of Mcl-1 was a selective effect in RCC-26A cells, since the levels of other Bcl-2-like proteins like Bcl-2 itself or Bcl-x_L were not substantially affected by any of the drugs used (RCC-26A, Figure 18). In the two RCC cell lines 26A and 30, vinblastine, paclitaxel and 5-FU also caused some detectable induction of Noxa, but there was no clear difference between the drugs that synergize with ABT-737 and 5-FU, which did not synergize. Thus, induction of Noxa seems to participate during treatment with chemotherapeutic drugs, but does not explain the lack of sensitization between 5-FU and the other drugs. At least in RCC, the appearance of Noxa does not stringently correlate with reduced levels of Mcl-1. The results suggest that functional elimination of Mcl-1 occurs via neutralization primarily.

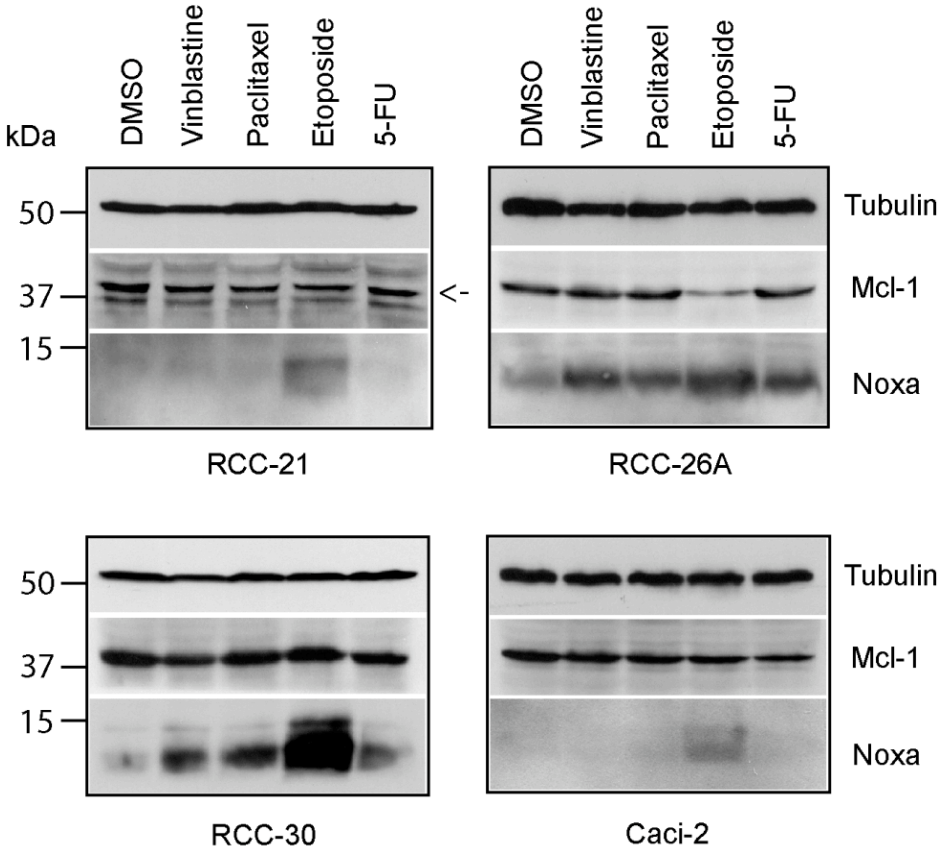

Figure 17 - Levels of Mcl-1 and Noxa during treatment with drugs

Cells from RCC cell lines were treated with solvent (DMSO, dimethyl sulfoxide) or with the indicated drugs for 24 h (100 nM vinblastine, 200 nM paclitaxel, 200 µM etoposide, 1 mM 5-FU) and expression of Mcl-1 and Noxa was detected by Western blot analysis. Detection of Tubulin served as a control for equal loading of samples. The immunoblots shown here are representatives of three separate experiments.

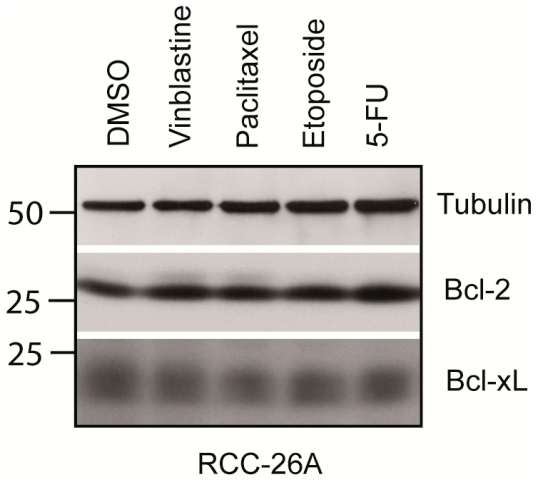

Figure 18 - Levels of Bcl-2-like proteins in RCC-26A cells during treatment with drugs

RCC-26A cells were treated with drugs as indicated in Figure 17 and expression of Bcl-2-like proteins was analyzed by Western blotting with specific antibodies for Bcl-2, Bcl-x_L. Detection of Tubulin served as a control for equal loading of samples. The immunoblots shown here are representatives of three separate experiments.

3.2.5 Synergism between ABT-737 and etoposide or vinblastine requires Noxa

The results above suggested that Noxa is induced upon treatment with chemotherapeutic drugs and might interact with Mcl-1 and A1 to neutralize their function. It has been demonstrated that also the BH3-only proteins Bim and Puma can bind to Mcl-1 and A1 and might account for their neutralization (Chen et al., 2005). Down-regulation of BH3-only proteins may be expected to reduce the level of synergistic apoptosis induced, if a member of this class has the ability of neutralizing Mcl-1 and A1. To identify the BH3-only proteins responsible for Mcl-1 and A1 neutralization Bim, Puma and Noxa were down-regulated individually by transfection with specific siRNAs. Upon transfection with siRNAs the RCC cell lines 26A and 30 were treated with the combination of ABT-737 plus etoposide or vinblastine and cell death was determined by propidium iodide staining. As shown in Figure 19 the targeted proteins Bim and Puma were substantially reduced at 48h post transfection in both RCC cell lines analyzed; since basic levels of Noxa protein were not detectable, knockdown efficiency was shown in the presence of etoposide to induce Noxa (Figure 19C). A clear reduction of Noxa protein levels could be achieved by transfection of specific siRNA.

No reduction of cell death was observed for the knockdown of Bim or Puma in RCC-26A cells respectively, treated with the combination of ABT-737 and etoposide (Figure 20). However, Noxa-specific siRNA was able significantly to reduce cell death induced by this combination of drugs. Further, Noxa-specific siRNA also inhibited cell death induced by the combination of ABT-737 plus vinblastine in the same line. A similar protective effect was observed in the RCC-30 cell line for the combination of ABT-737 plus etoposide and ABT-737 plus vinblastine. These results strongly suggest that neutralization of either Mcl-1 or A1 by Noxa is a significant mechanism through which chemotherapeutic agents sensitize RCC cell lines to ABT-737 mediated apoptosis.

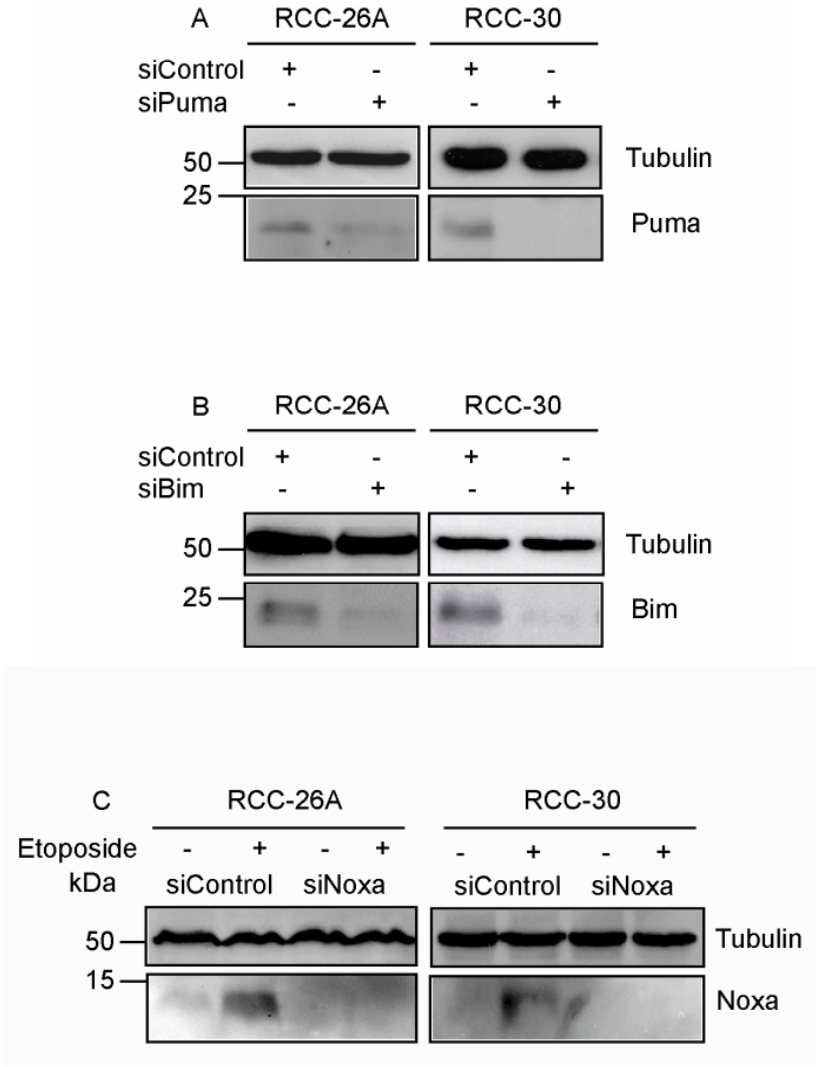

Figure 19 - Efficiency of the targeting of Bim, Puma or Noxa by RNAi

Expression levels of Puma (A), Bim (B) and Noxa (C) in the RCC cell lines 26A and 30 upon transfection with control or specific siRNAs. Cells were analyzed by Western blotting 48 h post transfection (A and B) or 24 h later upon treatment with 200 µM etoposide to induce Noxa (C).

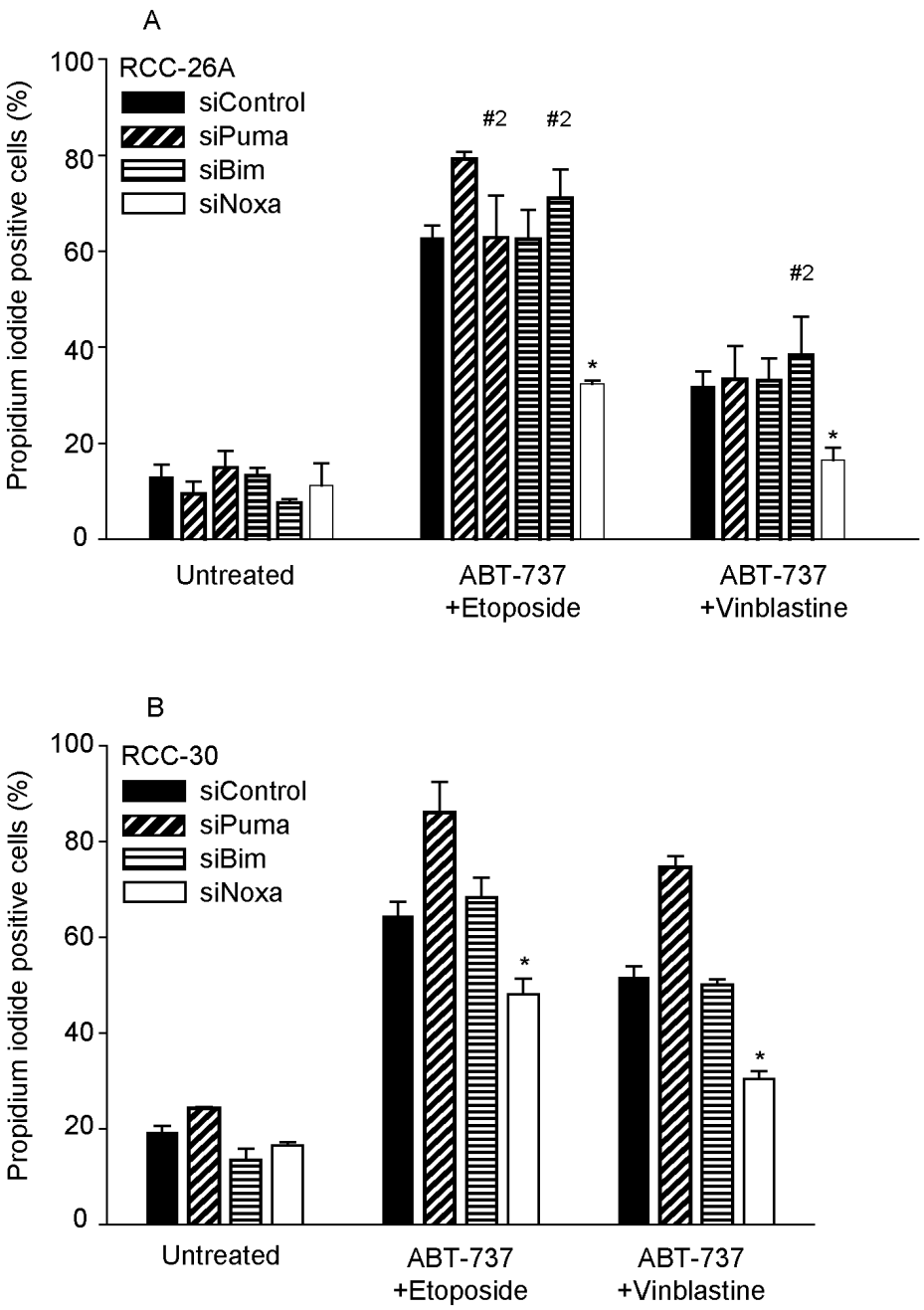

Figure 20 - Knockdown of Noxa but not Bim and Puma protects from combination treatment

Cells from RCC cell lines 26A (A) and 30 (B) were transfected with 20 nM of control siRNA or siRNA specific for Bim, Puma or Noxa. In some experiments a second siRNA sequence targeting a different site in Bim or Puma has been included (#2). 48 h post transfection, cells were treated with the combination of ABT-737 (1 µM) plus etoposide (200 µM) or vinblastine (100 nM) as indicated. Cell death was determined by propidium iodide staining 24 h later. Values are the means/SEM of at least three independent experiments. * $P < 0.03$; transfection of control siRNA versus Noxa siRNA.

3.2.6 Knockdown of Mcl-1 or A1 sensitizes RCC cell lines to ABT-737

It has been demonstrated in several human tumor cells that high expression of anti-apoptotic Mcl-1 correlates with protection against ABT-737 (for a recent review see (Cragg *et al.*, 2009)); while one publication has shown that *de novo* synthesis of A1 induced a strong resistance against ABT-737 in chronic lymphocytic leukemia cells (CLL), cultured under conditions that mimic the lymph node microenvironment (Vogler et al., 2009). Other reports have suggested that A1 is not expressed by most solid tumors entities and is rather involved in hematological malignancies (Su *et al.*, 2002; van Delft *et al.*, 2006). By contrast, in this work expression of A1 mRNA could be easily detected in RCC cell lines (see below) and was also detected by microarray analysis in multiple primary tumor samples from patients with clear cell RCC (Lenburg *et al.*, 2003). Clearly, A1 is the less well-characterized member within the group of anti-apoptotic Bcl-2-like proteins and functional data concerning A1-activity is rare.

To directly test for the contribution of Mcl-1 and A1 to resistance against ABT-737, both proteins were knocked down in RCC cell lines individually by RNAi and apoptosis was measured using active caspase-3 and propidium iodide stainings. The transfection reagent RNAi MAX produced by Invitrogen induced cell death of Caci-2 cells in the absence of any siRNA and this cell line was therefore excluded from the experiments. Transfection of siRNA specific for Mcl-1 achieved a good although incomplete reduction of Mcl-1 protein in the three RCC lines tested (Figure 21). A1 protein was not detectable by Western blotting, which may be the result of low expression in RCC cell lines or low sensitivity of the antibodies used. However, A1 mRNA was easily detectable in RCC cell lines by quantitative RT-PCR and a good reduction could be achieved by transfection with specific siRNA (Figure 21B). To confirm that reduction of A1 does not influence the expression of Mcl-1, mRNA levels of Mcl-1 were determined in the RCC cell line 26A and were not affected by transfection with A1-specific siRNA (Figure 21B, right panel).

Knockdown of Mcl-1 increased the apoptosis sensitivity of all three RCC cell lines to single treatment with ABT-737, suggesting that the expression level of Mcl-1 determines the susceptibility of RCC cells to ABT-737 (Figure 22A). A sensitizing effect was even observed in RCC-30 cells where ABT-737 at 5 µM on its own induced substantial cell death. Thus, the effect of chemotherapeutic drugs used in the previous experiments was indeed similar to loss of function of Mcl-1 and was therefore likely the neutralization of Mcl-1 function. Unexpectedly, reduction of A1 protein had a similar sensitizing effect as the down-regulation of Mcl-1 (Figure 22B). The data suggest for the first time, that expression of anti-apoptotic A1 in the RCC tumor entity protects against ABT-737 on a scale similar to Mcl-1. Cell death induced by siRNA transfection and treatment with ABT-737 depended on caspases, as it could be blocked with the pan-caspase inhibitor zVAD-fmk. Further, there was even significant apoptosis induced in the RCC-26A line by sole transfection of A1 siRNA in the absence of ABT-737, suggesting that RCC-26A cells depend on A1 for their survival. Also a second

siRNA, targeting another site in the A1 mRNA, induced apoptosis in the absence of an additional stimulus and had a similar sensitizing effect to ABT-737 (Figure 23).

As transfection of siRNAs to either gene produced similar results, i.e. increased apoptosis sensitivity of RCC cell lines to ABT-737, it may be possible that both genes contribute to resistance in an additive fashion. To address this question of cooperativity, Mcl-1 was first knocked down stably by shRNA followed by transient knockdown of A1. Stable RCC-26A cell line derivatives were generated that either expressed a control shRNA targeting luciferase, or Mcl-1 specific shRNA (Figure 21A, right panel). This stable cell line showed a good reduction of Mcl-1 and was sensitive to increasing amounts of ABT-737, too (Figure 22C). Additional targeting of A1 by transient transfection of specific siRNA further increased the sensitization towards ABT-737, even by using a reduced concentration of ABT-737 (0.2 µM, Figure 22D). Therefore, the two anti-apoptotic proteins appeared to have complementary function. The data suggested that at least in RCC cell lines resistance to ABT-737 is not only regulated by Mcl-1 but also A1, and targeting A1 in concert with ABT-737 may be an approach more promising than targeting Mcl-1.

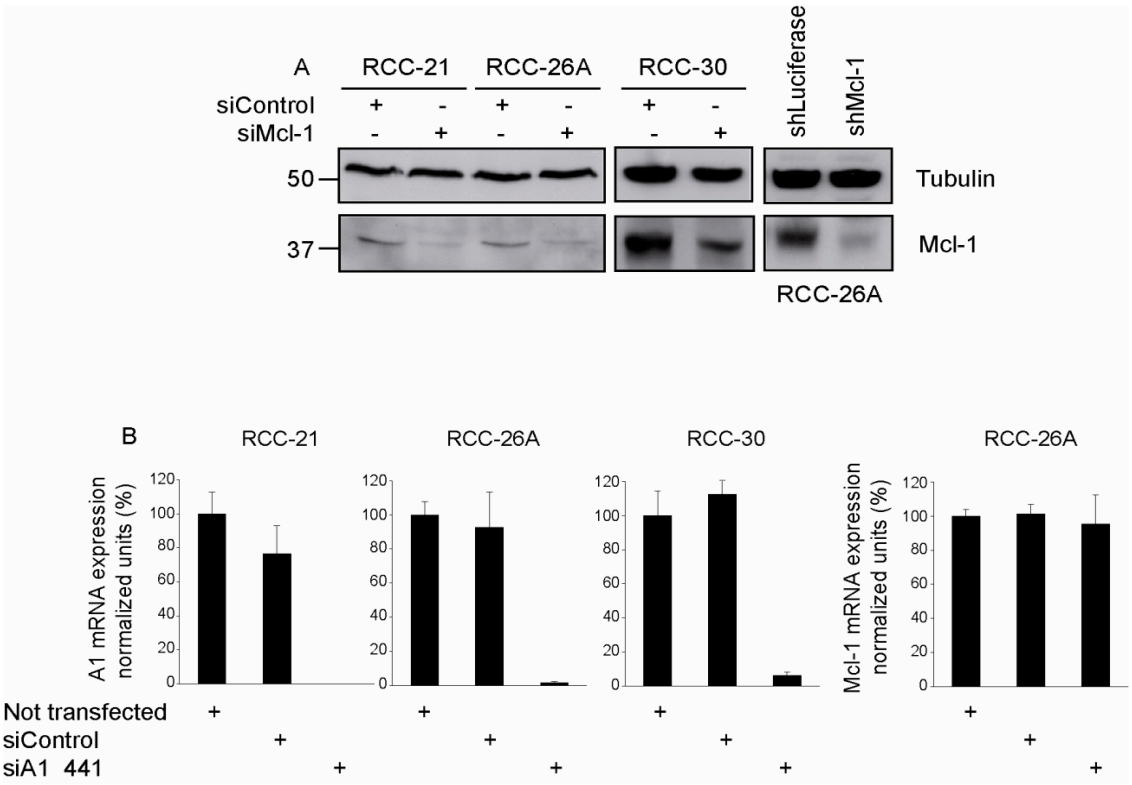

Figure 21 - Efficiency of the targeting of Mcl-1 or A1 by RNAi

Expression levels of Mcl-1 protein (A) or A1 mRNA (B) in cells from the RCC cell lines 21, 26A, 30, 26A shLuciferase and 26A shMcl-1. The cells were analyzed by Western blotting (A) or quantitative RT-PCR (B) 48 h post transfection with siRNAs. Tubulin served as a loading control for Western blotting. The immunoblots shown here are representatives of four separate experiments. B, mRNA levels of A1 (three left panels) and Mcl-1 (right panel) were determined by quantitative RT-PCR in not transfected cells and in RCC cells transfected with control siRNA and A1-specific siRNA (nucleotides 441 to 460 in the A1 mRNA). Relative A1 mRNA expression was normalized to expression levels of hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA in the same sample. Mean values of not transfected controls were normalized to 100%. Results are the mean/SEM of three independent transfections.

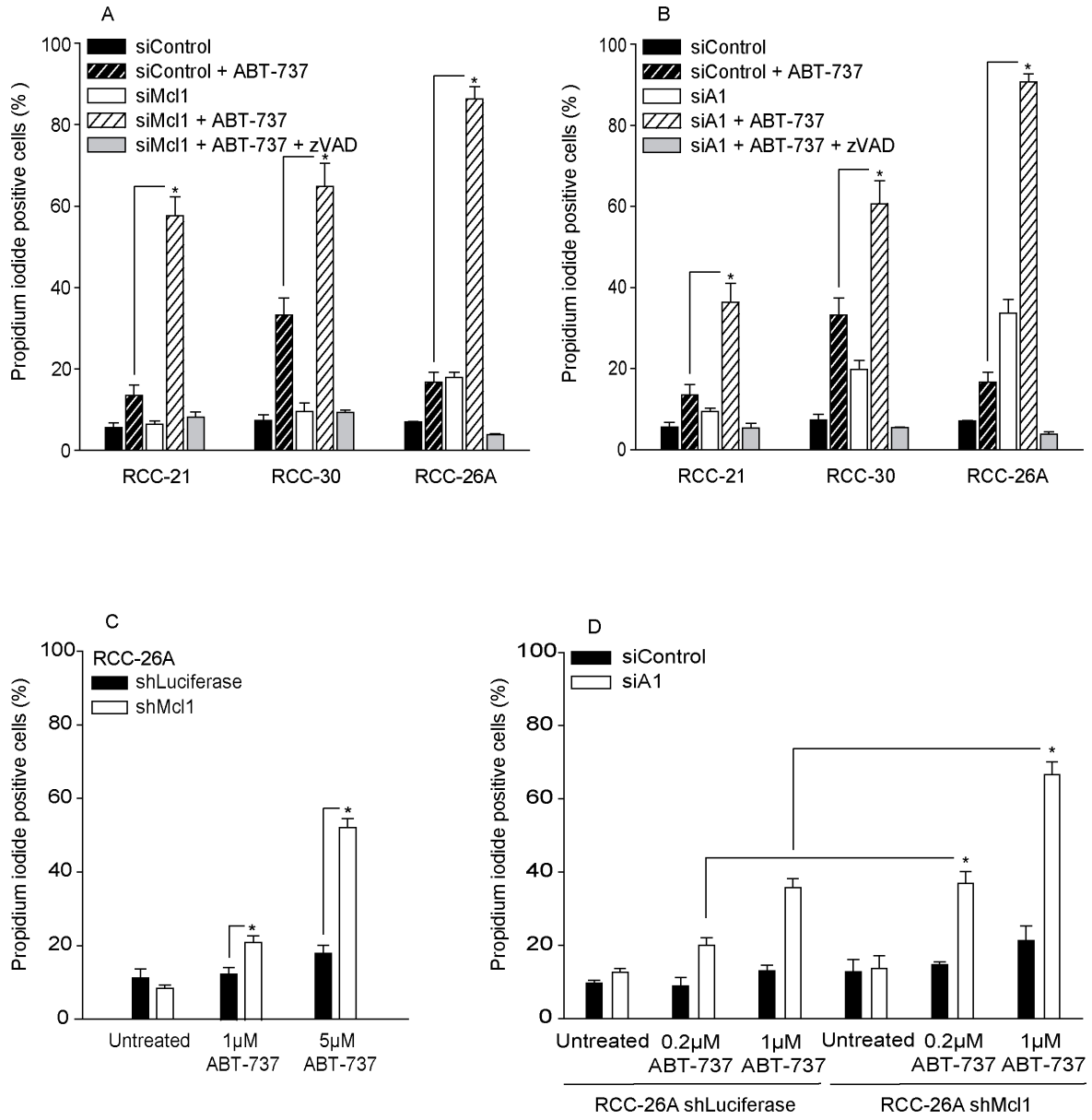

Figure 22 - Loss of Mcl-1 and A1 correlates with increased sensitivity to ABT-737

Cells from RCC cell lines 21, 26A and 30 were transfected with 20 nM of a control siRNA or siRNAs specific for Mcl-1 (A) or A1 (B). 48 h post transfection, RCC cells were treated with 5 μM ABT-737 for further 24 h and cell death was measured by propidium iodide staining. 100 μM zVAD-fmk was added to the cells 1 h prior treatment with ABT-737 to inhibit caspase activation. C, stable polyclonal RCC-26A cell line derivatives were generated by transduction of cells with a control shRNA (specific for firefly luciferase) or Mcl-1 specific shRNA (targeting a site in the Mcl-1 mRNA different from the siRNA used in A). Cells from the RCC-26A shLuciferase and shMcl-1 cell line were treated with 1 μM and 5 μM ABT-737 for 24 h and cell death was measured by propidium iodide staining. D, Cells from the RCC-26A shLuciferase and shMcl-1 cell line were transfected with control siRNA and A1-specific siRNA. 24 h post transfection cells were treated with 0.2 μM and 1 μM ABT-737 and cell death was determined by propidium iodide staining 24 h later. Data represent the mean/SEM of three independent experiments. * $P < 0.04$, transfection of control siRNA versus Mcl-1 siRNA and A1 siRNA respectively, or shLuciferase versus shMcl-1.

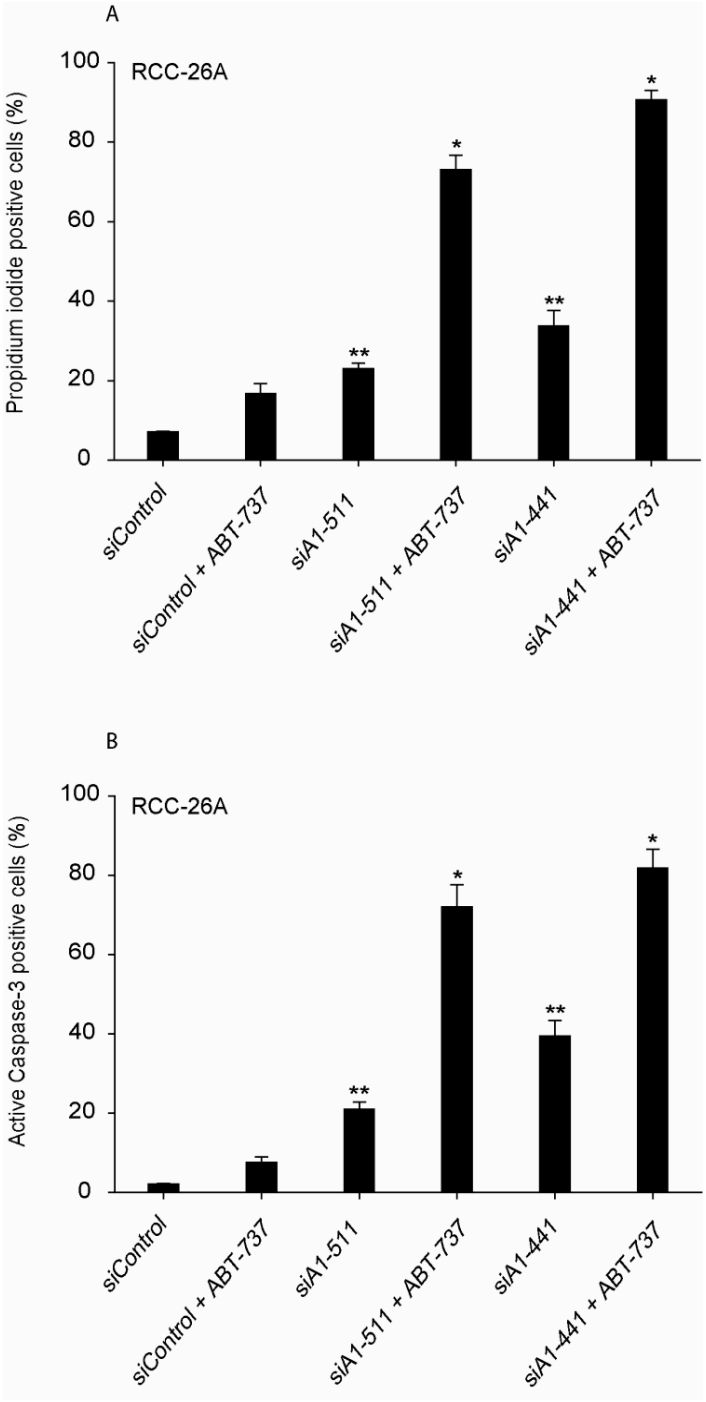

Figure 23 - Targeting A1 in RCC cells by two different siRNAs induces apoptosis

RCC-26A cells were transfected with two different siRNA sequences targeting A1 mRNA (nucleotides 511 to 530 or nucleotides 441 to 460). 48 h post transfection cells were treated with 5 µM ABT-737 and cell death was quantified by staining for propidium iodide 24 h later (A) or activated caspase-3 (B). Data represent the means/SEM of three experiments. (* $P < 0.01$, ** $P < 0.02$, control siRNA versus A1 siRNA).

3.2.7 Inhibition of the proteasome sensitizes for ABT-737 induced apoptosis

Previous data suggested that pro-apoptotic Noxa was at least in part responsible for neutralizing the anti-apoptotic activity of Mcl-1 and A1 in RCC cell lines (Figure 20), while degradation of Mcl-1 occurred only in the cell line RCC-26A upon treatment with etoposide (Figure 17). Several studies point to the possibility that Mcl-1 and its endogenous interaction partner Noxa are primarily regulated through protein turn-over. Accordingly, proteasome inhibition has been shown to increase the levels of these proteins and to protect from degradation of Mcl-1 (Willis *et al.*, 2005). Since both interaction partners are expected to be induced under proteasome inhibition, one prediction would be that Noxa induces the neutralization of Mcl-1 in absence of Mcl-1 degradation.

Therefore, it was tested whether proteasome inhibition had the effect of sensitizing RCC-26A cells to ABT-737. As demonstrated in Figure 24A, the proteasome inhibitor MG-132 on its own increased the protein levels of Mcl-1 and Noxa and was able to protect from the etoposide-induced degradation of Mcl-1 in the RCC-26A cell line, suggesting that both processes are regulated through the proteasome. Mcl-1 can also be a target of activated effector caspases, but the degradation of Mcl-1 during treatment with etoposide was not a direct consequence of cell death, since it was not blocked in the presence of the caspase family inhibitor zVAD-fmk (Figure 24B). As predicted, MG-132 on its own was almost inactive to induce apoptosis as determined by staining for active caspase-3, but the combination with ABT-737 further sensitized RCC-26A cells to apoptosis on a scale similar to the combination of ABT-737 and etoposide at 12 h (Figure 24C). MG-132 on its own was capable of a very strong induction of Noxa protein, much more than etoposide in a time-course experiment over 6 to 24 h (Figure 24B). Despite the significant induction of Noxa, sensitization to MG-132 was clearly slower than for etoposide at 6 h (Figure 24C). This may be a result of Mcl-1 protein not being degraded during proteasome inhibition. Since in the combination of MG-132 with ABT-737, Mcl-1 was not degraded but the cells remained sensitive to ABT-737, it could be that Noxa still binds to Mcl-1 and probably A1 and neutralizes their pro-survival function. Thus, proteasome inhibition in RCC by MG-132 was able to sensitize for ABT-737 induced apoptosis.

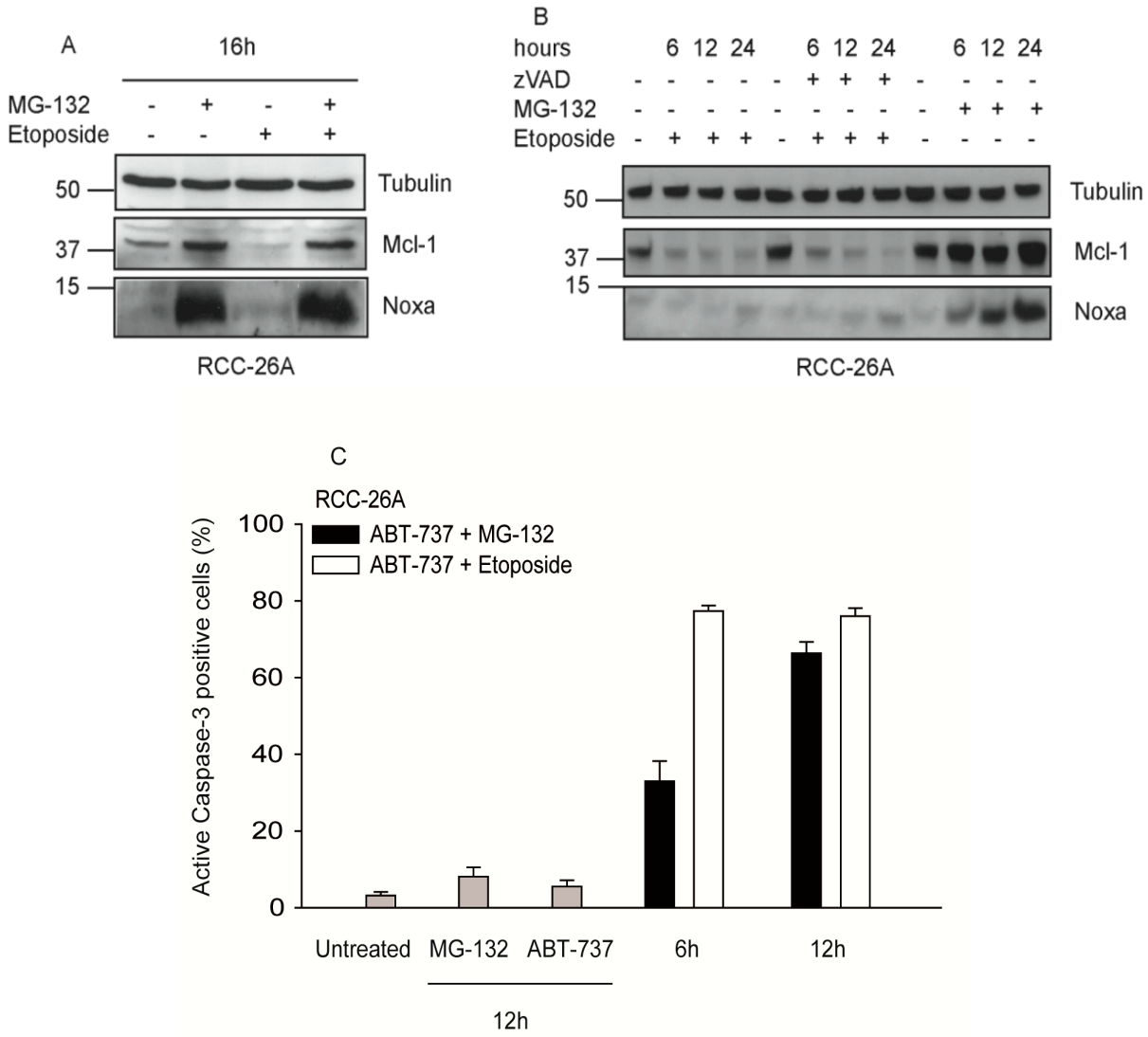

Figure 24 - Proteasome inhibition sensitizes for ABT-737 induced apoptosis

A, RCC-26A cells were left untreated or treated with 10 µM MG-132, 200 µM etoposide or with the combination of etoposide plus MG-132 for 16 h and expression levels of Mcl-1 and Noxa were determined by Western blot analysis. B, RCC-26A cells were treated in a time course experiment over 6 h, 12 h and 24 h with 10 µM MG-132, 200 µM etoposide or with the combination of 200 µM etoposide plus 100 µM zVAD-fmk and expression levels of Mcl-1 and Noxa are shown. Detection of tubulin served as a loading control. The immunoblots shown here are representatives of three separate experiments. C, RCC-26A cells were left untreated or treated with 10 µM MG-132 and 1 µM ABT-737 for 12 h (grey bars) or with the combination of 1 µM ABT-737 plus 10 µM MG-132 (black bars) or 200 µM etoposide (open bars) for 6 h and 12 h. Apoptosis was measured by active caspase-3 staining. Data represent the means/SEM of three independent experiments.

3.2.8 p53-independent induction of Noxa

The tumor suppressor protein p53 has been reported to be one of the major transcription factors for the *noxa* gene during treatment with genotoxic agents (Oda *et al.*, 2000; Shibue *et al.*, 2003; Villunger *et al.*, 2003a). This would imply that p53 is responsible for the induction of Noxa in RCC cell lines upon treatment with chemotherapeutic drugs, but also p53-independent mechanisms of Noxa induction have been described in other tumors (Ploner *et al.*, 2008). To directly test whether p53 was required for Noxa induction by treatment with genotoxic drug etoposide, p53 was down-regulated in the RCC cell line 26A using RNAi (Figure 25). Etoposide treatment had the effect of a strong induction of p53 and Noxa, but the induction of Noxa was clearly independent of p53 activity. Although p53 was completely reduced by transfection with siRNA, the strength of Noxa induction by etoposide was similar in cells transfected with p53-specific siRNA compared to transfection with control siRNA. Thus, for the first time in RCC a p53-independent mechanism of Noxa induction was detected, making it likely that Noxa could be still induced in the RCC tumors with inactivated p53 to account for neutralization of Mcl-1 and/or A1 in combination with ABT-737.

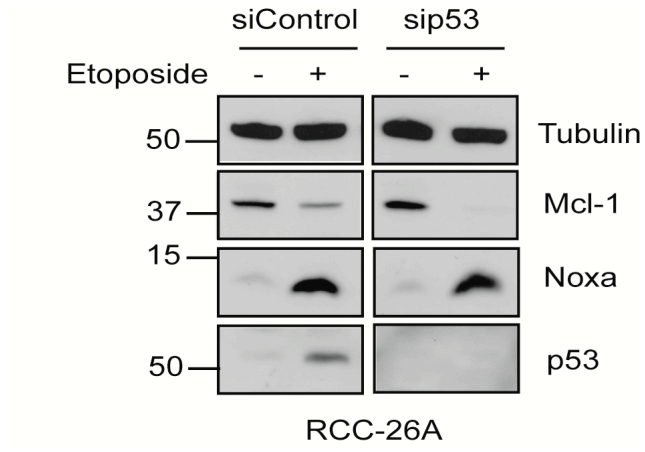

Figure 25 - Induction of Noxa by etoposide does not depend on p53

RCC-26A cells were transfected with control or p53-specific siRNA and were treated 24 h post transfection with 200 µM etoposide for further 24 h. Expression levels of Mcl-1, Noxa and p53 were determined by Western blot analysis. The white line between the bands indicates that samples were on the same blot, but were not consecutive. The immunoblots shown here are representatives of three separate experiments.

4 Discussion

4.1 Loss of Bim in renal cell carcinoma

The results in this work demonstrated a low expression of Bim both in primary tumor cells from clear cell RCC and in tumor-derived RCC cell lines (Figure 7). As in approx. 80% of all tumor samples a reduced expression of Bim was detected as compared to normal kidney epithelium, it may be that Bim is specifically down-regulated in RCC disease (Zantl *et al.*, 2007). Thus, a likely speculation would be that Bim-loss is involved in development of RCC. Low levels of Bim may further contribute to resistance against chemotherapeutic drugs. Evidence to confirm this idea came from the observation that low expression of Bim correlated with reduced apoptosis sensitivity to etoposide; the expression levels of other pro- and anti-apoptotic Bcl-2 proteins were not predictive for the varying apoptosis sensitivity in RCC cell lines (Figure 10). This led to the model that expression of Bim was a determinant of apoptosis sensitivity in RCC cell lines. Moreover, the relevance of Bim-expression was analyzed by functional studies. First, over-expression of Bim_S induced apoptosis in a resistant cell line, indicating that the presence of Bim was required to undergo apoptosis. Since Bim_S-induced apoptosis in a resistant cell line was similar to a cell line with high expression of Bim (Figure 11), the expression of other Bcl-2 proteins was likely not involved in determining the apoptosis sensitivity of RCC cell lines. Second, Zantl *et al* confirmed this hypothesis by reporting that upon reduction of Bim in the high expressing RCC cell line 1M, the cells underwent significantly reduced apoptosis in response to several external stimuli, supporting that Bim was relevant in the apoptotic response to these experimental stimuli and that loss of Bim expression may contribute to conferring resistance to apoptotic stimuli (Zantl *et al.*, 2007). Thus, the expression of Bim appears to be involved in determining the apoptosis sensitivity of RCC cell lines, not the expression of other Bcl-2 proteins.

4.1.1 BH3-only proteins in RCC

Apoptosis resistance in RCC is has been linked to a failure to release cyt *c* from mitochondria upon treatment with apoptosis-inducing chemotherapeutic drugs (Gerhard *et al.*, 2003), suggesting that RCC cells fail to activate the BH3-only proteins or possess elevated levels of Bcl-2-like proteins, which might compensate the apoptotic signals generated. Our data demonstrated that differential expression of Bcl-2-like proteins is not a responsible factor for the varying apoptosis sensitivity of RCC cell lines. Alternatively, a loss of BH3-only expression may account for the strong resistance of RCC cell lines against induction of apoptosis. It has been demonstrated by others that low expression of the BH3-only protein Nbk was a common aspect of RCC. In the study of Sturm *et al* it was noted that deletion of the chromosome arm 22q13.2, which harbors the *nbk* gene occurred in many cases of clear cell RCC. Chromatin methylation was also a mechanism that contributed to inactivation of the *nbk* gene. Interestingly, it was speculated that loss of Nbk may coincide with low expression of Bim (Sturm *et*

al., 2006). In addition to loss of Nbk expression, the here observed reduction of Bim would further enhance the resistance to apoptosis. In this context, it may be important to note that mice deficient for *nbk* are phenotypically normal in terms of induction of apoptosis (Coultas *et al.*, 2004) but the combined loss of Nbk and Bim leads to infertility in males, due to impaired spermatogenesis (Coultas *et al.*, 2005). Thus, it may be that Nbk-loss co-operates with the loss of Bim in RCC cells to achieve a strong inhibition of apoptosis. Loss of Puma and Noxa due to dominant-negative inhibition of p53 may further leave RCC cell lines refractory to apoptosis (Gurova *et al.*, 2004). Thus, the impressive apoptosis resistance of RCC cells may arise from a combined defect in the expression of BH3-only proteins.

4.1.1.1 Physiological role of Bim and molecular regulation of its activation

Bim is a BH3-only protein with a broad pro-apoptotic capacity that may interact with pro-survival Bcl-2-like proteins to mediate mitochondrial damage or may directly activate Bax and/or Bak to induce apoptosis (Certo *et al.*, 2006; Chen *et al.*, 2005). The physiological role of Bim became first apparent by gene targeting in mice. Together with other BH3-only proteins, expression of Bim has been reported to be involved in hematopoietic development and homeostasis of B cells, T cells, granulocytes and other cells of the hematopoietic lineage. Mice deficient for Bim have elevated numbers of these cell types. Survival of hematopoietic progenitor cells correlates with the down-regulation of Bim through cytokines, including IL-3 and GM-SCF (Inaba, 2004). The impaired Bim expression causes abnormal immune responses including autoimmunity or reactions to infections agents (Bouillet *et al.*, 1999; Bouillet *et al.*, 2002; Hildeman *et al.*, 2002; Strasser, 2005). Moreover, Bim deficiency was shown to cooperate in the induction of follicular lymphoma and to accelerate *myc*-induced lymphomagenesis in a mouse model (Egle *et al.*, 2004). The tumor suppressive function of Bim has further been demonstrated in epithelial tumors of mice and Bim was here found to be frequently lost in RCC (Tan *et al.*, 2005; Zantl *et al.*, 2007).

Impaired mechanisms of apoptosis induction have now been recognized to be involved in tumor development. Bcl-2, which is over-expressed, for instance, by chromosomal translocation in human follicular lymphoma cells, promotes tumor development by inhibiting apoptosis but not by promoting proliferation (Strasser *et al.*, 1990; Vaux *et al.*, 1988). Loss of Bim in these cells is strongly oncogenic. First it was noted that B lymphoid cells expressing the *c-myc* transgene have elevated levels of Bim, suggesting that induction of Bim was required for apoptosis of *c-myc* expressing cells. However, cells that were additionally deficient for Bim were protected against apoptosis induced *in vitro* by cytokine deprivation. Thus, Bim is induced by the *c-myc* transgene and is required to induce apoptosis. In this context even the inactivation of a single allele of Bim accelerated *c-myc*-induced development of tumors (Egle *et al.*, 2004). These findings suggest that Bim can act as a tumor suppressor, at least in B lymphocytes. The results shown in RCC further support the idea that Bim suppresses tumor formation and that loss of Bim in solid tumors may lead to abrogation of a major

apoptotic program. In addition, many tumors have defects in the response to genotoxic damage, due to an inactivation of p53, which in turn leads to failure to induce Puma and Noxa. Hence, Bim reduction may further support the loss of p53 function, which could lead to an increased apoptosis-resistant phenotype of RCC cells. These findings support a possible therapeutic approach involving the restoration of Bim function in resistant RCC cells, as shown by using the BH3 mimetic ABT-737 (section 3.2).

There are at least three major isoforms of Bim produced by alternative splicing of the primary transcript (Bim_{EL}, Bim_L and Bim_S) (Bouillet *et al.*, 2001; O'Connor *et al.*, 1998), which are differentially expressed depending on the tissue analyzed. The most abundantly expressed isoform is Bim_{EL}, which is also the most abundant form found in RCC. The strength of apoptosis induction varies between the three isoform. Bim_S, the shortest isoform is considered the most strongly pro-apoptotic (based on a study in factor dependent mouse myeloid cells, (O'Connor *et al.*, 1998)), perhaps due to its pro-apoptotic activity not being regulated by post-translational mechanisms. The pro-apoptotic activity of the two other Bim isoforms is regulated transcriptionally, post-transcriptionally and post-translationally. FoxO3a is one of the major transcription factors for the Bim gene, at least in hematopoietic cells and is phosphorylated by Akt, which also inhibits its nuclear translocation from cytoplasm in healthy cells (Gilley *et al.*, 2003; Stahl *et al.*, 2002). For post-translational regulation, Bim_{EL} and Bim_L proteins have been reported to be sequestered to the microtubule-associated dynein motor complex by binding to DLC8 (Puthalakath *et al.*, 1999; Puthalakath *et al.*, 2001) although it has to be said that this finding has not been reproduced by others. Erk-1/2 kinases have also been reported to phosphorylate Bim_{EL} and Bim_L and to target them for ubiquitination and proteasomal degradation, while inhibition of Erk leads to accumulation of dephosphorylated Bim_{EL} which is then no longer a target for ubiquitination (Ley *et al.*, 2003; Ley *et al.*, 2004). However, deletion of these phosphorylation sites in Bim_{EL} by a knock-in approach had only minimal effect on apoptosis induction (Hubner *et al.*, 2008). Another study has shown that endoplasmatic reticulum (ER) stress induces the phosphatase PP2A, which can dephosphorylate Bim to increase its stability, but it is not clear whether the induction of Bim is mainly achieved by activation of the transcription factors CEBPα and CHOP (Puthalakath *et al.*, 2007). Therefore, inhibitors of the Ras/Erk pathway, inducers of ER stress like thapsigargin and the proteasome inhibitors are capable of causing the accumulation of Bim_{EL} and Bim_L, and should be tested for their capacity of inducing Bim expression as a treatment option in clear cell RCC. Although the molecular details of Bim activation are not clear at the moment, it has been reported that Bim is required for apoptosis induced by various chemotherapeutic agents. Therefore, further effort is needed to determine the mode of Bim activation in RCC.

4.1.1.2 Noxa and Puma are regulated by p53 and by p53-independent mechanisms

Noxa was initially found as a p53 up-regulated target gene, which participates in apoptosis induction upon DNA damage (Oda *et al.*, 2000; Shibue *et al.*, 2003; Villunger *et al.*, 2003b). p53-independent

activation of Noxa has also been demonstrated to be involved in apoptosis induced by HDACIs, proteasome inhibitors, or Notch inhibitors in myelomas or lymphomas (Gomez-Bougie *et al.*, 2005; Nefedova *et al.*, 2008; Perez-Galan *et al.*, 2006; Qin *et al.*, 2004; Qin *et al.*, 2005). Mice deficient for Noxa do not exhibit a broad apoptotic phenotype. Puma is also responsible for p53-mediated apoptosis resulting from DNA damage (Nakano and Vousden, 2001; Villunger *et al.*, 2003b; Yu *et al.*, 2001) and is involved in apoptosis caused by p53-independent cytotoxic insults in various cell types, including cytokine deprivation, glucocorticoids, or staurosporine (Jeffers *et al.*, 2003). Puma knockdown has been demonstrated to dramatically accelerate Myc-induced lymphomagenesis, indicating that Puma has a tumor suppressive function (Hemann *et al.*, 2004). Puma knockout mice are more strongly protected from apoptosis induced by DNA damage than mice deficient for Noxa. Thus Puma is the main p53 induced mediator of apoptosis (Villunger *et al.*, 2003a).

In accordance with published results the genotoxic drug adriamycin was able to up-regulate p53, but failed to up-regulate the target genes *noxa* and *puma* in RCC (Zantl *et al.*, 2007). This failure of p53 to up-regulate these genes has been connected to a dominant negative inhibitor expressed in RCC cell lines (Gurova *et al.*, 2004). Furthermore, it has been suggested that this inhibitor is activated by an NFκB-dependent pathway (Gurova *et al.*, 2005). These findings make it unlikely that Puma and Noxa may step in when cells have lost Bim. This would further enhance the apoptosis-resistant phenotype of RCC cells.

4.1.2 Up-regulation of Bim by inhibition of HDAC

Data in this work suggested that inactivation of Bim expression may be achieved through epigenetic silencing. Inhibition of histone deacetylation or chromatin methylation increased the expression levels of Bim, suggesting that these processes contribute to silencing of the Bim gene. Since induction of Bim by TSA or AZA respectively was not only detected in RCC cell lines but also in primary tumor samples, inactivation of the Bim gene may occur during development of clear cell RCC.

Chromosomal deletion of genes is a prominent mechanism for inactivation of tumor suppressor genes. The *bim* gene is localized on the short arm of chromosome 2q, and its deletion has been demonstrated in RCC (Phillips *et al.*, 2001). Thus, besides epigenetic regulation of *bim*, deletion of the gene locus may also contribute to inactivation or loss of Bim.

During treatment of HDAC inhibitors, it may be that transcription factors become expressed that function as a *bim* gene-specific trans-activator. To exclude an involvement of other protein factors in the induction of Bim, the translation inhibitor CHX was co-administered. Treatment with TSA plus CHX demonstrated the same induction of all three major splice forms of Bim mRNA, as TSA alone. Thus, adding the protein synthesis inhibitor failed to block the induction of Bim mRNA. This demonstrated that induction of Bim does not depend on the synthesis of other protein factors.

Alternatively, a recent study reported that transcription of Bim can be induced by treatment with HDAC inhibitors, which has been linked to activation of the Rb-E2F1 signaling pathway (Zhao *et*

al., 2005). The transcription factor E2F1 binds to the promoter region of Bim and activates its transcription. Similar to Bim, other BH3-only proteins have been demonstrated to become activated by HDAC inhibition, including Puma and Noxa. However, further investigation showed that knockdown of Bim protected against HDAC-induced apoptosis (Zhao *et al.*, 2005). Thus, Bim is the major contributor of apoptosis induced by HDAC inhibitors. Therefore, it is unclear presently whether the induction of Bim by HDAC inhibition primarily depends on reduced histone deacetylation or on activation of other transcription factors. Further research is needed to determine how Bim is transcriptionally activated and to establish inhibition of HDAC as a therapeutical option in clear cell RCC.

4.1.3 Regulation of Bim through the VHL protein

As outlined in the introduction (section 1.2.1), clear cell RCC is the most common form of kidney cancer arising from the renal tubule epithelium. The majority of cases are associated with mutations in the *von Hippel-Lindau* (*VHL*) tumor suppressor gene (Cohen, 1999). The VHL protein is an E3 ubiquitin ligase that targets HIF-1α and other substrates for proteasomal degradation (Maxwell *et al.*, 1999). HIF-1α is a transcription factor that regulates the transcription of genes that are involved in angiogenesis, glycolysis and other mechanisms (section 1.2.1). Under normoxic conditions, pVHL interacts with HIF-1α resulting in its poly-ubiquitination and degradation by the proteasome. Under hypoxia HIF-1α escapes ubiquitination by pVHL and becomes activated (Rini, 2009b). A low expression of Bim_{EL} was here found in the analyzed tumor samples and in patient-derived RCC cell lines, which has been connected to low apoptosis sensitivity of RCC cell lines (section 3.1.1 and 3.1.3). Very little is known of how RCC cells achieve a reduction in Bim_{EL}. It is also not known whether there is a connection between the low expression of Bim_{EL} in RCC cells and the activity of pVHL in these cells (Guo *et al.*, 2009). Interestingly, it was noted in a recent study that the high rate of pVHL inactivation in inherited and sporadic forms of clear-cell RCC may coincide with the low expression of Bim in a high percentage of clear-cell RCC sample (Guo *et al.*, 2009). They found that cells expressing pVHL had higher levels of Bim_{EL} protein than the same cells without pVHL. This suggested that Bim levels may be regulated by the activity of pVHL. Furthermore, knockdown of pVHL with RNAi induced a decrease in Bim_{EL}. Functional restoration of pVHL in RCC cell lines increased the apoptosis sensitivity to etoposide and UV radiation, which was again reduced by down-regulating either pVHL or Bim_{EL} with RNAi (Guo *et al.*, 2009). Thus, pVHL may participate in regulating Bim_{EL} stability, which can have an impact on apoptosis sensitivity. A loss of pVHL would then lead to a destabilization of Bim_{EL}. Thus, regulation of Bim by pVHL could be a likely mechanism. However, how Bim is regulated by pVHL has to be clarified in more detail in further studies.

4.2 Inhibition of Bcl-2 proteins in renal cell carcinoma

4.2.1 ABT-737 synergized with chemotherapeutic drugs in RCC

The results in this study demonstrated that ABT-737 was able to sensitize RCC cell lines to apoptosis with etoposide, vinblastine and paclitaxel, but not with 5-FU (Figure 12; Figure 13). All chemotherapeutic drugs have been used to treat clear cell RCC, although they have a low potential to induce clinical responses. The clinical resistance is probably reflected by the low apoptosis sensitivity of RCC cell lines to treatment withl chemotherapeutic drugs. Knockdown of either anti-apoptotic Mcl-1 or A1 proteins sensitized for ABT-737 induced apoptosis, suggesting that both proteins contributed to protection against ABT-737 and both need to be neutralized by the drugs to allow for synergistic killing of RCC cells (Figure 22). Except of 5-FU, the drugs were able to induce neutralization of Mcl-1 and probably A1, which depended at least in part on the induction of the pro-apoptotic BH3-only protein Noxa (Figure 20). This suggests that 5-FU failed to neutralize Mcl-1 and A1 (Figure 14), although there was some induction of Noxa detectable (Figure 17). Therefore, it may be possible to predict that the chemotherapeutic agent that most strongly induces neutralization of the Mcl-1/A1 axis is the best combination partner for ABT-737, not that which induces apoptosis most strongly on its own.

4.2.2 Neutralization of Mcl-1 and A1

Tumor development, progression and resistance to chemotherapeutic drugs have been linked to deregulation of Bcl-2 family proteins, which commonly leads to varying apoptosis sensitivity (Chonghaile and Letai, 2008; Hanahan and Weinberg, 2000). The pro-apoptotic BH3-only proteins are the most upstream triggers of mitochondrial apoptosis and can interact with other Bcl-2 proteins to mediate apoptosis and both groups of proteins are pivotal regulators of mitochondrial apoptosis. Their interaction determines the ability of tumor cells to undergo apoptosis. ABT-737 was the first small-molecule mimetic of the pro-apoptotic BH3 domain that exhibits high affinity to three proteins of the pro-survival group (Oltersdorf et al., 2005). Studies from numerous laboratories have established the activity of ABT-737 in a variety of single agent and combination settings (Certo *et al.*, 2006; Chauhan *et al.*, 2007; Del Gaizo Moore *et al.*, 2007; Kang *et al.*, 2007; Kline *et al.*, 2007; Kohl *et al.*, 2007; Oltersdorf *et al.*, 2005; Tse *et al.*, 2008; van Delft *et al.*, 2006). However, it has to be said that ABT-737 has a reduced bioavailability in preclinical animal models, which would affect therapy responses. Therefore, ABT-263 was developed, a second-generation inhibitor, which binds to serum albumin to increase its bioavailability (Tse et al., 2008). This small-molecule Bcl-2 inhibitor is currently in phase I and phase II clinical trials and its function is analogous to ABT-737, as ABT-263 binds in the nanomolar range to Bcl-x_L, Bcl-2, and Bcl-w, but not to Mcl-1 or A1 (Tse et al., 2008).

4.2.2.1 Mechanisms of ABT-737 sensitivity

ABT-737 was able to induce apoptosis in two RCC cell lines analyzed (Figure 12, Figure 22). How do ABT-737 and its structural analogue kill tumor cells in general? Two scenarios may explain the observations with ABT-737 in RCC cell lines. The results shown here and by others predict that increased levels of pro-apoptotic proteins sequestered by Bcl-2-like proteins and low endogenous expression of Mcl-1 are two factors that correlate with sensitivity to ABT-737 (Cragg *et al.*, 2009).

As a single agent ABT-737 induces apoptosis in some types of cells by inhibiting the interaction between pro-apoptotic and anti-apoptotic Bcl-2 family proteins (Tse *et al.*, 2008). This has been shown, for instance, in FL5.12 cells (Certo *et al.*, 2006; Tse *et al.*, 2008). These cells depend on continuous support of the cytokine IL-3 for survival. IL-3 withdrawal induces apoptosis, which can be blocked by over-expression of Bcl-2 or Bcl-x_L (Certo *et al.*, 2006). Furthermore, IL-3 withdrawal has been demonstrated to increase Bim levels and to reduce Mcl-1, supporting a likely mechanism for the induction of apoptosis (Harada *et al.*, 2004; Maurer *et al.*, 2006; Tse *et al.*, 2008). In this context Certo *et al* demonstrated that over-expression of Bcl-2 protected the cells from IL-3 withdrawal by sequestering Bim in a Bim:Bcl-2 complex (Certo *et al.*, 2006; Tse *et al.*, 2008). This state has been referred to "oncogene addiction", where the cells may become dependent on the over-expressed Bcl-2-like molecules. These proteins serve as a "sink" for activated BH3-only proteins in a state that has been termed "primed for death" (Certo *et al.*, 2006; Letai *et al.*, 2002). According to Tse *et al,* disruption of this complex by the BH3 mimetic ABT-737 and liberation of Bim either to directly activate Bax and/or Bak (as postulated by the direct model) or to further neutralize the remaining pro-survival proteins (that are not targeted by ABT-737) such as Mcl-1 and A1 to release activated Bak (as postulated by the indirect model) appears to be sufficient to induce apoptosis (Tse *et al.*, 2008), once the total sum of binding sites provided by Mcl-1 and/or A1 has been exceeded by the liberated pro-apoptotic molecules (Figure 26). Similar to these results, Tse *et al* have recently demonstrated that ABT-263 is ineffective in cells that have not previously been primed with pro-apoptotic stimuli, for instance in FL5.12 cells over-expressing Bcl-2 that have not been deprived of cytokine support, thereby inhibiting the induction of Bim (Mason *et al.*, 2008; Tse *et al.*, 2008). During cytokine deprivation, where Bim becomes activated, a reduction of Bim by RNAi may indeed protect from single-agent treatment with ABT-737 (Del Gaizo Moore *et al.*, 2007). It was noted in this work that Caci-2 cells were sensitive to treatment with 1 µM ABT-737, while apoptosis was induced in RCC-30 cells at 5 µM ABT-737 (Figure 12, Figure 22). A likely explanation would be that the sensitive cell lines have a greater amount of pro-apoptotic BH3-only proteins like Bim, tBid or Puma sequestered by Bcl-2, compared to a resistant cell line, i.e. the RCC-26A cell line. Indeed, the results demonstrated in section 3.1 showed some correlation of Bim expression with increasing sensitivity to single treatment with ABT-737. Alternatively, the amount of liberated pro-apoptotic Bak displaced from Bcl-2 or Bcl-x_L by ABT-737 may exceed the amount of Mcl-1 to allow for Bak-mediated apoptosis (Willis *et al.*, 2007). This mechanism of ABT-737 sensitivity is also supported by analysis of cells from patients

with CLL and follicular lymphoma (Del Gaizo Moore *et al.*, 2007; Vogler *et al.*, 2009). These cells typically carry the t(14,18) translocation leading to enforced expression of Bcl-2 and also express relatively little amounts of Mcl-1. As outlined in section 1.2.2, oncogene activation and cell-cycle regulation usually causes the activation of pro-apoptotic factors in form of activated BH3-only proteins (Certo *et al.*, 2006; Fesik, 2005; Hanahan and Weinberg, 2000). Therefore, normal non-malignant cells have to face continually generated apoptotic signals that determine the ability of a cell to undergo apoptosis (Chonghaile and Letai, 2008). If such cells have pre-activated apoptotic molecules like Bim, complexed to Bcl-2, they may become sensitive to single treatment with ABT-737 (Del Gaizo Moore *et al.*, 2007). Further support of this idea came from the observation that human SCLC cell lines with low expression of Mcl-1 and high dependency on Bcl-2 are very sensitive to treatment with ABT-737 (Oltersdorf *et al.*, 2005; Tse *et al.*, 2008).

4.2.2.2 Levels of Mcl-1 are a factor of resistance to ABT-737

As mentioned above, another important resistance factor of ABT-737 sensitivity is the endogenous expression of anti-apoptotic Mcl-1. The results obtained in RCC and by others suggest that ABT-737 is most effective in cells with low expression of Mcl-1. Indeed, ABT-737 and its relative ABT-236 demonstrated a good single-agent activity in some xenograft models of small lung carcinomas and acute lymphoblastic leukemia (Oltersdorf *et al.*, 2005; Tse *et al.*, 2008). ABT-263 induced complete tumor responses in all animals bearing tumors of the SCLC or ALL type. Intriguingly, Mcl-1 and A1 mRNA expression was significantly lower in tumors of these type compared to other tumors, which correlated with increased sensitivity to ABT-737 (Tse *et al.*, 2008). Further support of Mcl-1 as a resistance factor for ABT-737 came from the observation that ABT-737 was only effective in MEF cells, where Mcl-1 has been deleted by gene targeting but not in normal MEF cells (van Delft *et al.*, 2006). Furthermore, siRNA knockdown of Mcl-1 in a resistant SCLC cell line restored sensitivity to ABT-737 (Tahir *et al.*, 2007). Consistent with the binding profile of ABT-737 to Bcl-2-like proteins, tumors with high expression of Mcl-1 and high dependency on Mcl-1 for survival such as multiple myeloma (Mason *et al.*, 2008; Zhang *et al.*, 2002) are very resistant to single treatment with ABT-737 and need a combination partner to reduce Mcl-1 (Kline *et al.*, 2007). Other groups demonstrated in a variety of human tumor cells that down-regulation of Mcl-1 by RNAi increased sensitivity to single treatment with ABT-737 (Chen *et al.*, 2007; Konopleva *et al.*, 2006; Lin *et al.*, 2007; Tahir *et al.*, 2007; Tse *et al.*, 2008; van Delft *et al.*, 2006). Moreover, Mason *et al* found that mice transplanted with *myc* driven B cell lymphomas and with additional *bcl-2* transgene expression were sensitive to single treatment with ABT-737, but the *myc* lymphomas did not respond to ABT-737, very likely due to increased expression of Mcl-1 levels in these cells (Mason *et al.*, 2008). In their study, similar to findings obtained in RCC cell lines, the combination of ABT-737 with cyclophosphamide, which is a genotoxic drug, improved survival of animals transplanted with the *myc/bcl-2* lymphomas but also synergized in the *myc* lymphomas, demonstrating that combination therapy was able to overcome the

protection provided by Mcl-1 (Mason *et al.*, 2008). This could be achieved by inducing BH3-only proteins that have the potential ability to neutralize Mcl-1 and A1, for instance the p53-regulated Noxa and Puma. Analogues to these data Tse *et al* were able to reproduce this observation by using ABT-263. They also found similar that OPM-2 human myeloma cells with high endogenous expression of Mcl-1 were protected in a xenograft model from single treatment with ABT-263. Instead, ABT-263 significantly enhanced the response to the proteasome inhibitor bortezomib, which has been previously demonstrated to increase the levels of Noxa and to induce neutralization of Mcl-1 *in vitro* (Mason *et al.*, 2008; Tse *et al.*, 2008). This finding is similar to the data obtained in *myc*-driven lymphoma (Mason *et al.*, 2008) and to our data in RCC cell lines (Figure 12), which were mostly protected from single agent treatment with ABT-737, but underwent enhanced apoptosis by the combination of ABT-737 with etoposide and other drugs, if able to induce Noxa or possibly Bim and Puma or to activate other BH3-only proteins in response to chemotherapeutic drugs (Mason *et al.*, 2008). Interestingly, ABT-737 was able to sensitize the RCC cell lines 26A to proteasome inhibiton by MG-132, involving the induction of Noxa that could account for neutralization of Mcl-1. Noxa was even required for complete apoptosis induced by the combination of ABT-737 plus etoposide or vinblastine, but not Bim (Figure 20). Although it is often ignored, there is accumulating evidence that in particular Bim, which can also be induced by proteasome inhibition and other stimuli, cannot participate in neutralizing Mcl-1 and/or A1, despite the high affinity of its BH3 domain to these Bcl-2-like proteins (section 4.2.3) (Cragg *et al.*, 2008; Weber *et al.*, 2009).

4.2.2.3 Clinical relevance of Bcl-2 inhibition in RCC

From data in this work and in accordance to data obtained in *myc*-driven lymphoma (Mason *et al.*, 2008), in animal xenograft models (Tse *et al.*, 2008) or in other human tumor cells (Chen *et al.*, 2007; van Delft *et al.*, 2006) similar conclusion can be drawn in terms of the possible mechanism of ABT-737 for treating clear cell RCC and other tumors either as a single agent or in combination with chemotherapeutic drugs. In individual tumors that may acquire a high expression of Bcl-2 to compensate the continuous generated apoptotic signals, ABT-737 on its own could exhibit a good single-agent activity, probably without requiring the activation of the DNA damage response (Noxa and Puma) or other BH3-only proteins (Mason *et al.*, 2008; Certo *et al*., 2006), although it has to be said that the contribution of Bim is uncertain in this aspect. As mentioned above ABT-737 probably will be ineffective as a single agent for tumors in which Mcl-1 and/or A1 is expressed at relevant levels to confer resistance (Mason *et al.*, 2008; van Delft *et al.*, 2006). This postulates that the combination of ABT-737 with chemotherapeutic drugs should be highly synergistic if Noxa, Puma or other BH3-only proteins become activated to induce the neutralization of Mcl-1 and/or A1 (Mason *et al.*, 2008; Tse *et al*., 2008).

Thus, Bcl-2 inhibition by ABT-737 could be important for increasing the efficiency of chemotherapeutics drugs against resistant tumors cells, probably involving relatively high expression

of Mcl-1 and the induction of Noxa. Therefore, the expression of Mcl-1 or A1 and possibly Noxa in individual tumors should be a good prognostic marker for predicting the sensitivity to single treatment with ABT-737 (van Delft *et al.*, 2006). How well the combination with other chemotherapeutic drugs will be tolerated *in vivo* needs to be further analyzed (van Delft *et al.*, 2006). However, it has been reported that tumor cells that were initially sensitive to ABT-737 become resistant to long-time treatment by over-expression of Mcl-1 or other mechanisms (Mason *et al.*, 2008; Tahir *et al.*, 2007; van Delft *et al.*, 2006). The results obtained in RCC and in other tumors suggest that chemotherapeutic drugs inactivate the Mcl-1/A1 axis, while ABT-737 inhibits the other Bcl-2-like proteins. This could be important for clinical settings. One consequence could be that combination therapy with ABT-737 would be more effective at lower concentrations of the chemotherapeutics drugs, or would lead to more stable tumor regression with normal doses (Mason *et al.*, 2008; van Delft *et al.*, 2006).

4.2.2.4 Expression of Mcl-1 is maintained by cytokine signaling

Mcl-1 is a protein with a high turn-over rate, which is maintained in some cell types by cytokines (Kozopas *et al.*, 1993; Nijhawan *et al.*, 2003; van Delft *et al.*, 2006). It may be expected that blocking the activity of these growth factors may also sensitize to ABT-737. Accordingly, van Delft *et al* tested whether cytokine deprivation was able to sensitize tumor cells for ABT-737 and they achieved a strong sensitization, even with over-expression of Bcl-2 (van Delft *et al.*, 2006). RCC disease typically involves the constitutive activation of VEGF signaling. Therefore, inhibitors of cytokine or VEGF signaling may also sensitize RCC and other tumors to ABT-737 (Jourdan *et al.*, 2003; Le Gouill *et al.*, 2004). Since Mcl-1 mRNA and protein has a high turn-over it may be possible to combine ABT-737 with mechanisms that regulate its transcription or translation (van Delft *et al.*, 2006). For example, the cyclin-dependent kinase inhibitor Seliciclib inhibits RNA polymerase II-dependent transcription and down-regulates Mcl-1 due to its rapid turn-over (MacCallum *et al.*, 2005; Raje *et al.*, 2005). As expected, Seliciclib demonstrated a strong synergism with ABT-737 in HeLa cells (van Delft *et al.*, 2006). Blocking of *de novo* protein synthesis by adding cycloheximid (CHX) also enhanced ABT-737-induced apoptosis, very likely by reducing the amount of Mcl-1 (van Delft *et al.*, 2006). Thus, levels of Mcl-1 can be affected by different mechanisms that under some conditions regulate its stability, highlighting the importance of this factor. The E3 ubiquitin ligase Mule has been reported to target Mcl-1 for proteasomal degradation via its BH3 domain (Zhong *et al.*, 2005), whereas a recent study identified the deubiquitinase Usp9x that can bind to Mcl-1 and remove its polyubiquitin chain, thereby protecting Mcl-1 from proteasomal degradation and promoting cell survival (Schwickart *et al.*). Eventually it may be possible to target these functions therapeutically. Noxa preferentially interacts with Mcl-1 and A1 and was here found to be induced during treatment with chemotherapeutic drugs; a promising strategy would be to develop a BH3 mimetic that recapitulates the binding profile of Noxa in terms of neutralizing the function of Mcl-1 and/or A1 (Tse *et al.*, 2008).

4.2.2.5 A model of ABT-737 function in RCC

ABT-737 appeared to synergize with strategies that induce the neutralization of Mcl-1 and/or A1. How does inactivation of Mcl-1 and A1 allow for synergistic induction of apoptosis? Under certain conditions degradation of Mcl-1 may help to ensure irreversible commitment to apoptosis (Flinterman *et al.*, 2005; van Delft *et al.*, 2006), but drug-induced degradation of Mcl-1 is not an essential mechanism in RCC to allow for synergistic killing. The displacement model of apoptosis activation holds that Bak is kept in check by inhibition through Bcl-x_L and Mcl-1 and can be displaced from its sequestration to Bcl-2-like proteins by BH3-only proteins. If Bak is liberated from its sequestration to Bcl-x_L by the BH3 mimetic, the role of conventional drugs would be to further neutralize Mcl-1 and/or A1 to allow for Bak-mediated induction of apoptosis (Figure 26, indirect model). This could be achieved for instance by induction of Noxa and other BH3-only proteins. The direct activation model postulates that the activation of Bax and Bak requires the direct interaction with "activator" BH3-only proteins (Bim, tBid and probably Puma). In accordance with this model apoptosis is induced once the "activators" have been liberated from Bcl-2 and Bcl-x_L by ABT-737 to a level exceeding the binding capacity provided by Mcl-1 and A1, leaving liberated "activators" that could then bind to Bax and/or Bak (Figure 26, direct model). However, the latter model underscores the possibility that induction of Noxa could participate in neutralizing Mcl-1 and A1 to displace Bak and/or "activators". At least in RCC cell lines the induction of Noxa was required to allow for complete induction of synergistic apoptosis. This observation is in line with results obtained in melanoma cells (Weber *et al.*, 2009) and data demonstrating that ABT-737 induced cyt *c* release in MEF cells expressing Noxa, but not in cells expressing Bad (van Delft *et al.*, 2006).

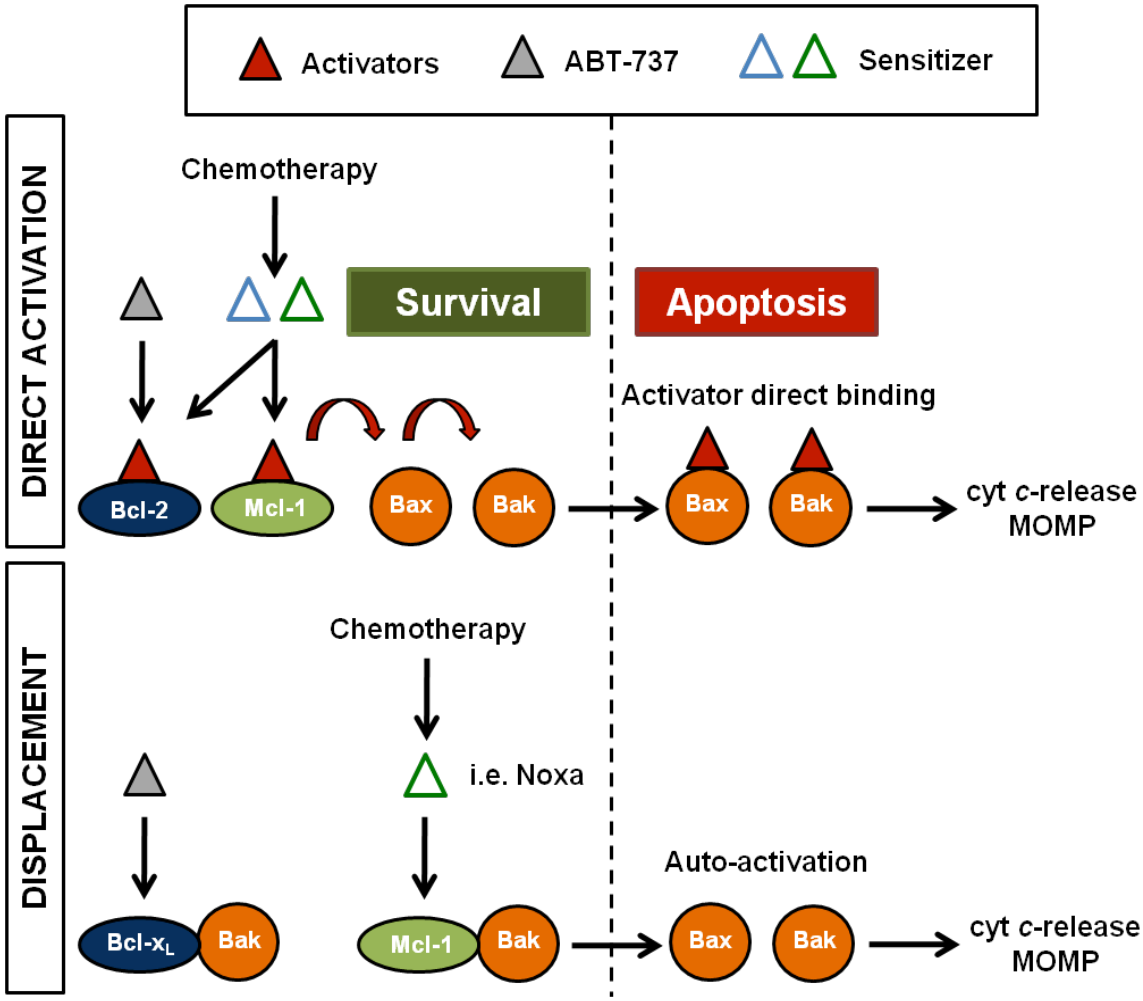

Figure 26 - Models for Bax/Bak activation by ABT-737 and chemotherapeutic drugs

According to the direct model "activators" (Bim, tBid and Puma) are constitutively bound to Bcl-2-like proteins to prevent apoptosis, until the combination of ABT-737 plus chemotherapeutic drugs induces "sensitizers" that bind to Bcl-2-like proteins and liberate the "activators" that could then directly activate Bax and/or Bak. In the indirect (displacement) model Bak is kept in check by inhibition through Bcl-2-like proteins, until ABT-737 and "sensitizers" induced by chemotherapeutic drugs bind to Bcl-2-like proteins, leaving liberated Bak for auto-activation of Bax and/or Bak.

4.2.3 Role of Bim during combination therapy with ABT-737

Studies with gene-deficient cells showed that loss of Bim has a strong phenotype. Bim appears to be a critical regulator of homeostasis in the hematopoietic system and Bim-deficient mice have abnormally elevated numbers of B cells, T cells, macrophages and granulocytes in their hematopoietic organs (Bouillet *et al.*, 1999). *Bim*$^{-/-}$ mice develop a fatal systemic lupus erythematosus (SLE)-like autoimmune disease, indicating that Bim might have a role in apoptosis of auto-reactive B and T cells (Bouillet *et al.*, 2002; Strasser, 2005). Bim has recently been implicated to have a tumor-suppressive function in epithelial tumors and was here shown to be low expressed in primary RCC and cell lines (Tan *et al.*, 2005; Zantl *et al.*, 2007).

Previous results found a low expression of pro-apoptotic Bim in RCC, which correlated with apoptosis sensitivity in RCC cell lines. The results suggested that chemotherapeutic agents required the activation of pro-apoptotic Bim to efficiently induce apoptosis in RCC cells (Zantl *et al.*, 2007). The function of ABT-737 indeed synergized with that of chemotherapeutic drugs limiting the anti-apoptotic function of Mcl-1 and A1, indicating that the remaining amount of Bim in RCC cells might be activated but is unable to neutralize Mcl-1 and A1. Chemotherapy or RNAi mediated knockdown appeared to provide the additional neutralization event that enables ABT-737 to kill intrinsically resistant RCC cells. Accordingly, the extent of synergistic apoptosis induced was similar or even higher in the Bim low expressing cell line 26A compared to the RCC-30 cell line with substantial expression of Bim. Thus, the strong requirement of RCC cells for Bim function can be overcome by using ABT-737 instead. This idea was further supported by showing that knock-down of Bim in the RCC-30 cell line did not protect from apoptosis induced by the combination of ABT-737 plus etoposide or vinblastine (Figure 20). This indicates that Bim does not participate in neutralizing Mcl-1 and A1 during treatment with these drugs. Recent results obtained in melanoma demonstrated the same effect, inhibition of MEK kinases required the activation of Bim which can be overcome by ABT-737 (Cragg *et al.*, 2008). Although knockdown of Bim in melanoma cell lines was able to protect from apoptosis induced by single treatment with the genotoxic agent dacarbazine, it failed to protect from combination treatment with ABT-737 (Weber *et al.*, 2009), suggesting that Bim was activated during treatment with chemotherapeutic drugs but was not involved in neutralization of Mcl-1 and A1. Thus, at least in RCC it seems that the remaining relatively low expression of Bim does not participate in antagonizing the anti-apoptotic function of Mcl-1.

4.2.4 5-FU did not synergize with ABT-737

5-FU did not synergize with ABT-737 in any of the RCC cell lines tested (Figure 12). Apoptosis was induced by 5-FU upon treatment for 48 h (Figure 14), which would imply that also BH3-only proteins become activated by 5-FU, but do not efficiently target the Mcl-1/A1 axis to induce synergistic apoptotic killing together with ABT-737. The strength of Noxa induction by 5-FU appeared similar to

that of vinblastine and paclitaxel, which would predict that also apoptosis sensitivity in the combination therapy would be similar (Figure 17). This was not the case. One explanation could be that other BH3-only proteins become preferentially activated during treatment with 5-FU, which are not involved in neutralizing Mcl-1 and A1 function. In contrast, the drugs that synergized with ABT-737 appeared to induce the Mcl-1 and A1 neutralization. However, at least reduction of Bim or Puma did not protect from combination therapy, which might be due to the high redundancy of these putative activator proteins (Figure 20). Therefore, knock-down of one single gene would not exhibit a resistant apoptotic phenotype because the function of the down-regulated BH3-only protein could be substituted by another. Although difficult to achieve experimentally, it should be tested whether the combined knockdown of several BH3-only genes will protect from synergistic killing. Second, it may be possible that additional mechanisms exist that regulate the Mcl-1/A1 neutralization subsequent to Noxa induction, but no such a mechanism has been described yet and its existence remains speculative. Third, it remains questionable whether Bim and Puma really have the physiological activity of interacting and neutralizing Mcl-1 and A1, despite the high affinity of their BH3 domains to all of the pro-survival Bcl-2-like proteins (Chen *et al.*, 2005). Intriguingly, a recent report showed that Bim induced by HDAC inhibition was primarily bound to Bcl-2 and Bcl-x_L in human leukemia and myeloma cells and that administration of ABT-737 reduced the association of these complexes. However, this did not correlate with increased binding of Bim to Mcl-1 (Chen *et al.*, 2009), suggesting that Noxa was essentially required for interaction and neutralization of Mcl-1 and A1.

4.2.5 A1 confers protection against ABT-737

Only few data are available in the literature regarding the role of A1. A1 may have an important role in neutrophilic development and in determining the survival of neutrophils (Chuang *et al.*, 1998). Certain inflammatory cytokines (TNF-α and IL-1β) or bacterial components like LPS can cause its up-regulation by an NF-κB-dependent mechanism that contributes to apoptosis protection (Karsan *et al.*, 1996). Inhibition of neutrophil apoptosis was reduced in mice deficient in one (out of four in mice) A1 gene (Hamasaki *et al.*, 1998). Its function appeared to be restricted to the hematological system, but A1 expression was also detected in endothelial cells (Lin *et al.*, 1993). A1 is reportedly not expressed in most solid tumor entities (van Delft *et al.*, 2006), but this may be a problem of low sensitivity of the antibodies used or may be due to very low expression of A1 in solid tumors, developing from epithelium. Our available A1 antibodies also failed to detect endogenous levels of A1 in RCC (a polyclonal antibody from Cell signaling and a yet not commercialized antibody kindly provided by Dr. J. Borst were used). However A1 mRNA was easily detectable and we achieved strong knockdown efficiencies, which correlated with increased sensitivity to single treatment with ABT-737 (Figure 21, Figure 22). Although restricted to leukemia models, two other studies demonstrated that A1 was able to provide resistance against ABT-737 (Vogler *et al.*, 2009; Whitecross *et al.*, 2009). Our data established for the first time that A1 had a similar effect in a solid tumor entity. How is A1 involved in

conferring resistance against ABT-737? Although the exact molecular mechanisms of A1 regulation are uncertain, a likely explanation would be that, similar to Mcl-1, A1 binds to BH3-only proteins or Bax/Bak displaced from the Bcl-2 axis by the BH3 mimetic (Figure 27). However, no interaction partner of physiological expressed levels of A1 has been found yet (Vogler *et al.*, 2009), but Simmons *et al* suggested that over-expressed A1 selectively interacts with Bak in healthy cells and can block TNF-mediated activation of Bax indirectly via its interaction with tBid. Another report has established that A1 can interact with Bax and Bak (Simmons *et al.*, 2008; Vogler *et al.*, 2009; Werner *et al.*, 2002; Zhai *et al.*, 2008; Zhai *et al.*, 2006). Reduction of A1 by RNAi and single agent treatment with ABT-737 would therefore liberate these pro-apoptotic molecules to induce apoptosis. Furthermore, two non-overlapping siRNA sequences targeting A1 mRNA induced apoptosis in RCC cells by mere transfection of siRNAs without an additional stimulus (Figure 23). This result is in accordance with results obtained in malignant B cells, where it was suggested that A1 may thus function as an essential Bcl-2-like protein on which the cells depend for survival (Brien *et al.*, 2007). However, this would imply that tumors of these types have pro-apoptotic proteins bound to A1 in untreated cells. Down-regulation of A1 by RNAi would then liberate these proteins to induce apoptosis. A similar mechanism could explain the results in RCC cells. The data presented in this work suggest that both pro-survival proteins need to be neutralized to allow for synergistic killing of RCC cells (Figure 22C). Noxa, which is activated by treatment with chemotherapeutic drugs, could account for neutralization, since it would predominantly bind to Mcl-1 and A1. Clearly more research has to be done to understand how A1 modulates apoptosis decisions.

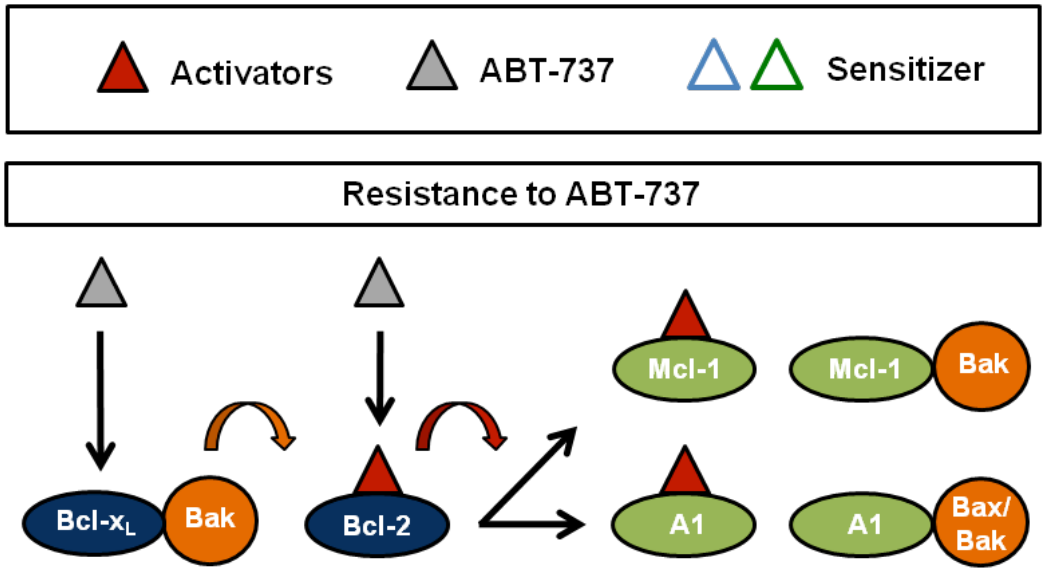

Figure 27 - Model of resistance to ABT-737 modulated by Mcl-1 and A1

In cells resistant to ABT-737 Mcl-1 and A1 may bind to liberated "activators" (Bim, tBid or Puma) or to Bax and/or Bak displaced from Bcl-2-like proteins by the BH3 mimetic to prevent apoptosis. The combination of ABT-737 and chemotherapeutic drugs may induce the "sensitizer" Noxa that would then bind to Mcl-1 and/or A1, leaving liberated pro-apoptotic proteins that could then induce apoptosis.

4.2.6 Noxa was induced during treatment with drugs

It may be important for clinical settings that Noxa was here found to be required for complete apoptosis induced by the combination of ABT-737 with etoposide or vinblastine. Noxa was first described in T cells following treatment with phorbol 12-myristate 13-acetate (PMA) (Hijikata *et al.*, 1990) and is a direct transcriptional target of p53 in response to DNA damage (Oda *et al.*, 2000). Its turn-over is rapidly regulated through proteasome-dependent and independent pathways (Wang and Sun, 2008). Cells deficient for Noxa do not exhibit a broad apoptotic phenotype and Noxa seems to have a more restricted role, being particularly involved in the apoptosis of fibroblasts and intestinal epithelial cells (Shibue *et al.*, 2003; Villunger *et al.*, 2003a). Inhibition of the proteasome leads to a rapid induction of Noxa, but in melanoma this has been suggested to be an indirect effect resulting from the activation of c-myc (Nikiforov *et al.*, 2007; Weber *et al.*, 2009). The proteasome inhibitor MG-132 induced Noxa in the RCC cell line 26A and was able to sensitize for ABT-737 in the same cell line, where etoposide induced the degradation of Mcl-1 (Figure 24). Thus, both mechanisms - either neutralization or degradation of Mcl-1 - may synergize with ABT-737 to account for synergistic induction of apoptosis in RCC cells.

Although Noxa has a weak pro-apoptotic capacity on its own, it appeared to modulate apoptosis decisions by inducing degradation of pro-survival Mcl-1 (Nijhawan *et al.*, 2003; Ploner *et al.*, 2008; Willis *et al.*, 2005). This event may be essential for apoptosis induced by growth factor deprivation or DNA damage (Willis *et al.*, 2005) but the only RCC cell line where such a reduction was found, was RCC-26A treated with etoposide (Figure 17). The other cell lines still underwent more than additive apoptosis although Mcl-1 was not degraded, making it likely that binding of Noxa to Mcl-1 suffices to neutralize its pro-survival function. Alternatively, Noxa may in these situations and cell lines preferentially target A1. Knock-down experiments in RCC cell line 30 confirmed that Noxa was required for sensitization towards ABT-737 without having Mcl-1 necessarily degraded (Figure 17, Figure 20). The molecular details of these differences are uncertain, but one explanation could be that the primary function of drug-induced Noxa was the neutralization of A1.

4.2.7 p53-independent induction of Noxa

Etoposide is a chemotherapeutic drug that is widely used and induces DNA alkylation. It is a semi-synthetic podophyllotoxin derived from the root of *Podophyllum peltatum* (May apple) and induces single-stranded DNA breaks, as well as DNA damage, through inhibition of DNA topoisomerase II (Strasser, 2005). Etoposide treatment had the effect of a strong induction of p53 and Noxa in the RCC cell line 26A, suggesting that Noxa was transcriptionally induced by p53, but this was not the case (Figure 25). Consistent with the described dominant negative effect of p53 function in RCC (Gurova *et al.*, 2004), probably due to an NF-κB-dependent mechanism of p53 inhibition (Gurova *et al.*, 2005), knockdown of p53 did not influence the induction of Noxa. Thus, some other p53-independent

mechanisms are responsible for up-regulation of Noxa during treatment with etoposide. For instance, the transcription factor E1A can activate p73 and Noxa in a p53-independent fashion (Flinterman *et al.*, 2005). It has been mentioned that RCC rely on constitutive activation of the transcription factor HIF-1α through inactivation of VHL protein. It has also been reported that Noxa is transcriptionally up-regulated under conditions of hypoxia and promotes p53-independent induction of cell death (Kim *et al.*, 2004). This process depends on HIF-1α via binding to hypoxia responsive elements (HRE) in the promoter region of Noxa and might explain the results obtained in RCC. A more detailed analysis is expected to resolve how chemotherapeutic drugs used here activate Noxa transcriptionally.

4.2.8 Conclusion remarks

In conclusion, RCC cell lines that are resistant to many chemotherapeutic drugs underwent enhanced apoptosis when Bcl-2 inhibition was combined with the function of conventional drugs. Apoptosis induced by combination therapy was much stronger than apoptosis induced by either of the single agents. The role of chemotherapeutic drugs is to achieve neutralization of Mcl-1 and/or A1 for instance by activation or induction of BH3-only proteins, in particular Noxa. Knockdown of Mcl-1 and A1 confirmed that these pro-survival proteins are responsible for resistance to single-agent treatment with ABT-737. If their function becomes neutralized during combination therapy, synergistic apoptosis will be induced. Expression of the endogenous Mcl-1/A1 antagonist Noxa was induced in RCC cell lines by treatment with etoposide by a p53-independent mechanism and could account for neutralization of Mcl-1 and A1. RNAi experiments confirmed that drug-induced Noxa was required for full synergistic apoptosis. Thus, a vulnerable axis in RCC was detected, that may predict the *in vivo* sensitivity of RCC to ABT-737 in a combination with chemotherapeutic drugs.

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6 Acknowledgements

I am very grateful to Prof. Dr. Georg Häcker for giving me the opportunity to pursue my doctoral thesis in his laboratory, for his generous support, profound discussions and overall excellent supervision even after he moved to Freiburg.

I want to thank Prof. Dr. Michael Groll for his excellent supervision of my doctoral thesis.

I would like to thank Dr. Robert Besch for the cooperation in the A1 project and helpful advice on designing siRNA.

Special thanks go to my former and current colleagues Christine, Eva, Juliane, Stephie, Susanne, Zofia, Arnim, Florian, Jan, Pedro, Stefan, Songmin and Tobias who made the years in the labarotary such an enjoyable time. Thank you for the friendly and inspiring environment.

Finally, I would like to thank my parents and my brother for their constant, infinite encouragement and support throughout my studies and this doctoral thesis.

7 Publications

Parts of the work presented in this doctoral thesis have been published in *Molecular Cancer* or *Oncogene*.

Zall H, Weber A, Besch R, Zantl N, Häcker G. "Chemotherapeutic drugs sensitize human renal cell carcinoma cells to ABT-737 by a mechanism involving the Noxa-dependent inactivation of Mcl-1 or A1." **Mol Cancer. 2010 Jun 24;9(1):164. [Epub ahead of print]**

Zantl N, Weirich G, **Zall H**, Seiffert BM, Fischer SF, Kirschnek S, Hartmann C, Fritsch RM, Gillissen B, Daniel PT, Häcker G. "Frequent loss of expression of the pro-apoptotic protein Bim in renal cell carcinoma: evidence for contribution to apoptosis resistance."
Oncogene; 2007 Oct 25; 26(49):7038-48. Epub 2007 May 7.