ON THE ROLE OF DIFFERENT SIGNAL TRANSDUCTION PATHWAYS IN INDUCTION OF APOPTOSIS BY ANTICANCER DRUGS

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ABSTRACT

Anticancer drug treatment gives rise to complex cellular responses in tumor cells. An improved understanding of the mechanisms underlying these responses is important in order to improve treatment. It is also of importance to investigate novel mechanisms of drug action that could potentially represent new concepts for treatment. In this thesis, both clinically used anticancer agents as well as other agents inducing tumor cell death were studied.

The DNA damaging agents cisplatin and ellipticine were shown to also exert non-nuclear effects important for apoptotic signaling such as induction of endoplasmic reticulum stress and for cisplatin also generation of reactive oxygen species. Cisplatin was shown to trigger distinct cellular outcomes depending on the concentration used. Our data suggest that DNA damage signaling primarily induces senescence. Acute apoptosis is mostly independent of DNA damage responses and is rather triggered by cytoplasmic events such as generation of reactive oxygen species.

A chemical library was screened for compounds inducing apoptosis independently of p53. It was found that lysosomes often are involved in p53-independent apoptotic signaling. Lysosomal apoptosis signaling was further examined by screening for compounds that induce cathepsin-dependent apoptosis. Among the most potent agents identified were two compounds with similar chemical structures that were found to induce the accumulation of polyubiquitins in cells. Our data raise the possibility that inhibition of the ubiquitin-proteasomal system may lead to the triggering of lysosomal membrane permeabilization.

Apoptosis induction by the anticancer agent paclitaxel was examined with a focus on the potential involvement of the proapoptotic protein Bad. We found Bad to be phosphorylated at Ser 128, a site reported to enhance its proapoptotic activity, after paclitaxel treatment. Our data does not however suggest an important role for this phosphorylation event in paclitaxel-induced apoptosis.

The studies in this thesis illustrate the complexity of cellular responses triggered by anticancer drugs. The signaling pathways often involve different cellular organelles and the outcome of treatment can include various forms of antiproliferative responses.
LIST OF PUBLICATIONS

I  Berndtsson M, Konishi Y, Bonni A, Hägg M, Shoshan M, Linder S, Mandic Havelka A.
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FEBS Letters 2005;579:3090-3094

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but is not associated with damage to nuclear DNA
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Induction of lysosomal membrane permeabilization by compounds that
activate p53-independent apoptosis
PNAS 2005;102:192-197

V  Berndtsson M, Beaujouin M, Mandic Havelka A, Larsson R, Westman J,
Liaudet-Coopman E, and Linder S.
Induction of cathepsin-dependent apoptosis by inhibitors of the ubiquitin-
proteasome system
Manuscript
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LIST OF ABBREVIATIONS

AIF  Apoptosis-inducing factor
AP-1  Activator protein 1
Apaf-1  Apoptotic protease activating factor-1
ASK1  Apoptosis-signaling-regulated kinase
ATF  Activating transcription factor
ATM  Ataxia telangiectasia mutated protein
ATP  Adenosine three phosphate
ATR  ATM- and Rad3-related protein
Bcl-2  B-cell lymphoma-2
BH  Bel-2 homology
Cdc2  Cell division cycle 2
CHK  Cell cycle checkpoint kinase
CHOP  C/EBP homologous protein
CML  Chronic myelogenous leukaemia
EGFR  Epidermal growth factor receptor
eIF2  Eukaryotic initiation factor
ER  Endoplasmic reticulum
ERSE  Endoplasmic reticulum stress response element
GRP  Glucose-regulated protein
HDAC  Histone deacetylase
Hsp  Heat shock protein
IAPs  Inhibitors of apoptosis proteins
IP3R-1  Inositol triphosphate receptor type 1
IRE1  Inositol-requiring enzyme 1
JNK  c-Jun N-terminal kinase
MAPK  Mitogen-activated protein kinase
NFκB  Nuclear factor kappa beta
PARP  Poly(ADP-ribose)polymerase
PERK  Pancreatic ER kinase (PKR)-like ER kinase
PDGF  Platelet-derived growth factor receptor
PI3′K  Phosphatidylinositide-3′-OH kinase
PIDD  p53-induced protein with a death domain
PKA  Protein kinase A
ROS  Reactive oxygen species
Rsk  Ribosomal S6 kinase
Ser  Serine
SERCA  Sarcoendoplasmic-reticulum Ca^{2+}-ATPase
SMases  Sphingomyelinases
TNF  Tumor Necrosis Factor
TRAF2  TNF receptor-associated factor 2
UPR  Unfolded protein response
XBP1  X box-binding protein 1
6-PA-ELL  6-propanamine ellipticine
1 INTRODUCTION

Cancers arise as a result of normal cells acquiring genetic and epigenetic alterations that enables evasion of the physiologic control mechanisms that govern the life of a cell. The process of tumor development can be depicted as a form of Darwinian evolution where clones of cells with acquired alterations conferring growth advantages will be expanded, leading to the progressive conversion of normal cells into cancer cells (Foulds 1954; Nowell 1976). A number of different acquired capabilities overcoming normal restrictions have been suggested to be shared by all advanced human tumors. These are: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. In order for a cell to acquire these alterations within a reasonable time, most cancers are believed to be genetically unstable (Hanahan and Weinberg 2000). The numerous variations of genetic and epigenetic changes that can arise in a tumor explain the heterogeneous nature of cancer diseases and complicate cancer treatment.

1.1 CANCER TREATMENT

The main strategies for cancer treatment are surgery, radiation, chemotherapy, hormonal therapy and immune therapy. Often different therapies are used in combination. Surgery and radiation are used in the treatment of localized cancer, whereas systemic therapy is generally the only way to potentially cure cancers that have metastasized to distant sites. Chemotherapy is given with different objectives. It can be curative or used to slow down the progression of the disease and alleviate symptoms when cure is not possible. Chemotherapy is often given after surgical removal of the primary tumor but can also be given before localized treatment to reduce the tumor size and to target micrometastases.

1.1.1 Anticancer drugs

Chemotherapy has played an important role in cancer treatment during a long time and many of the drugs discovered several decades ago are still among the most clinically effective anticancer agents available. These drugs were usually identified empirically and the molecular actions responsible for their anti-tumor effects have been poorly understood. A major obstacle for the successful use of chemotherapy in cancer treatment is tumor cell resistance. Resistance can be developed after tumor cell exposure to chemotherapy or the cells can be inheritantly resistant to certain drugs probably as the result of alterations that have provided the tumor cells with survival advantages. Another challenge of chemotherapy is the need for tumor cell specificity to avoid toxicity to normal tissues. A better understanding of the cellular signaling induced by various anticancer drugs and the development of new drugs that induce antiproliferative responses in tumor cells by novel mechanisms is therefore important for improving therapy.

Chemotherapeutic drugs are generally classified according to the type of damage they confer on the tumor cell. However, many of these drugs give rise to complex responses involving several cellular targets.
1.1.1.1 Alkylating agents and platinum compounds

Cancer chemotherapy evolved out of the observed effects on bone marrow and lymphoid tissues after exposure to sulphur mustard gas during World War I. Because of these findings, sulphur mustard and the related nitrogen mustards were evaluated as antitumor agents and the search for other chemicals with antitumor activity was stimulated. The sulphur and nitrogen mustards have been described to act as alkylating agents forming covalent bonds to nucleic acid bases and proteins. The related compound cyclophosphamide is commonly used in the treatment of several different forms of cancer (Colvin 1999).

Cisplatin (cis-diamminedichloroplatinum(II)) was the first platinum antitumor compound discovered as the result of studies of effects of electrical current on bacterial growth (Rosenberg, Vancamp et al. 1965). Cisplatin is one of the most potent antitumor agents known and one of few curative drugs. It is especially effective in the treatment of testicular carcinoma for which the cure rate exceeds 90%, but is also used in the treatment of other forms of cancer, including ovarian, cervical, head and neck, and non-small-cell lung cancer (Boulikas and Vougiouka 2004; Wang and Lippard 2005).

Cisplatin is a neutral inorganic complex that is activated upon entry into the cell, where the low chloride concentration facilitates replacement of the chloro-ligands of cisplatin with water molecules. The aquated form is highly reactive, positively charged and forms covalent bonds to N7 positions of DNA purines to generate intra- and interstrand crosslinks. DNA is generally accepted as the primary target of cisplatin. However, only about 1% of the intracellular cisplatin react with nuclear DNA (Eastman 1991) and cisplatin is known to also interact with other cellular components including proteins, RNA and membrane phospholipids (Wang and Lippard 2005).

![Cisplatin structure](image)

Figure 1. Cisplatin structure.

1.1.1.2 Topoisomerase-inhibitors

Processes such as transcription and replication produce alterations in the DNA supercoiling that are regulated by topoisomerase enzymes. These enzymes produce transient DNA strand breaks to resolve sterical hindrance within the DNA structure. Topoisomerase inhibitors generally have the effect of stabilizing the normally transient intermediate complex in which the enzyme is covalently bound to DNA and the DNA strands have been cleaved. These complexes are subsequently converted by cellular processes into DNA damage harmful to the cell (Walker and Nitiss 2002). Examples of topoisomerase-targeting drugs are the anthracyclin doxorubicin, the epipodophyllotoxin etoposide and the plant alkaloids camptothecin and ellipticine (5,11-dimethyl-6H-pyrido(4,3-b)carbazole). Several topoisomerase targeting drugs are used clinically in the treatment of various forms of cancer. Ellipticine derivatives have been investigated
as candidate agents for treatment. These compounds have shown preferential
cytotoxicity to brain tumor cell lines (Acton, Narayanan et al. 1994) but have also
shown activity in breast cancer treatment (Rouesse, Le Chevalier et al. 1985).
Topoisomerase inhibition is generally regarded as the main mechanism of cytotoxicity
for ellipticines (Tewey, Chen et al. 1984) although a variety of cellular activities have
been reported for these agents, including protein binding (Dodin, Andrieux et al. 1990)
and uncoupling of oxidative phosphorylation (Schwaller, Allard et al. 1995).

1.1.1.3 Antimetabolites

Antimetabolites act by mimicking the structure of nucleic acid bases or folic acid,
thereby interfering with their normal biochemical function. This can result in inhibition
of nucleic acid synthesis as well as the incorporation of analogues into DNA or RNA
that can inhibit replication and lead to miscoding. Drugs from this group in clinical use
include the folic acid analogue metotrexate, the purine analogues 6-mercaptopurine and
thioguanine as well as the pyrimidine analogue 5-fluorouracil.

1.1.1.4 Microtubuli-interfering drugs

The plant derived vinca alkaloids and taxanes target the microtubules of the cells
cytoskeleton. The vinca alkaloids have been described as microtubule-depolymerizing,
since these agents can prevent tubulin polymerization. Taxanes, including paclitaxel,
binding to tubulin and instead stabilize the microtubules. The suppression of microtubule
dynamics in either case lead to the arresting of cells in mitosis (Dumontet and Sikic
1999). Paclitaxel is being used in the treatment of tumors including those of breast,
arovian, lung, head and neck, bladder and esophageal origin (Rowinsky 1997). Though
the primary mechanism of these drugs is well known, the signaling events downstream
of paclitaxel binding to microtubules leading to cell death is not well understood.

1.1.2 New anticancer drugs

The major improvements in the understanding of mechanisms underlying cancer
development have resulted in a number of novel therapy concepts being evaluated or
approved for clinical use, a few examples of which are given below.

1.1.2.1 Targeting growth factor signaling

A successful group of recently developed targeted anticancer drugs are those that
interfere with various growth and survival promoting kinases, in particular the tyrosine
kinases. For tumors whose growth is driven by activated tyrosine kinases, the use of
kinase inhibitors has proved valuable for treatment. Imatinib (Gleevec®) inhibits BCR-
ABL, a fusion protein with constitutively active tyrosine kinase activity expressed in
chronic myelogenous leukaemia (CML) cells. Trastuzumab (Herceptin®) is a
monoclonal antibody that binds the receptor tyrosine kinase HER2 and inhibits growth
of HER2-overexpressing breast cancer cells. In addition, a number of monoclonal
antibodies and small-molecule inhibitors of the epidermal growth factor receptor
(EGFR) have been developed of which a few have been approved for treatment of
specific cancers (Baselga 2006)
The complexity of advanced tumors and the existence of interactions between signaling pathways suggests that combinations of molecularly targeted drugs as well as the use of drugs with activity on more than one single target is likely to be more potent than single agent target-specific therapy. Imatinib is in fact known to inhibit not only BCR-ABL, but also additional tyrosine kinases, including the c-Kit receptor and the platelet-derived growth factor receptor (PDGF) (Baselga 2006). Sorafenib (Nexavar®) was developed to target Raf kinase involved in mitogenic signaling and has shown clinical efficiency in renal cell carcinoma. However, further characterization of sorafenib has revealed that it also affects several other tyrosine kinases (Schreck and Rapp 2006). The cellular chaperone heat shock protein 90 (Hsp90) has been recognized as an interesting target in cancer therapy since it is overexpressed in cancer cells and is important for the conformational maturation of several oncogenic proteins with key roles in survival signaling. The clinical usefulness of the Hsp90 inhibitor 17-AAG (17-(allylamino)-17-demethoxygeldanamycin) is currently being evaluated (Workman 2004).

1.1.2.2 Proteasome inhibition

Inhibition of the proteasome represents a new anticancer approach that can potentially arrest tumor growth and induce cell death. Protein degradation within the ubiquitin-proteasome system is a mechanism by which the cell controls degradation of key regulators of cellular processes such as cell cycle progression and apoptosis. Many types of malignant cells are more sensitive to proteasome inhibition than normal cells. Rapidly proliferating tumor cells may accumulate defective proteins at a higher rate and thereby may be more dependent on the proteasome for elimination of such damaged proteins (Adams 2004). In accordance with this, enhancement of the proteasomal activity has been reported in certain leukemic cells (Masdehors, Merle-Beral et al. 2000). Nuclear factor kappa beta (NFκB) activation can protect tumor cells against apoptosis by upregulation of prosurvival proteins and its constitutive activation has been reported in some tumor cells (Ni, Ergin et al. 2001). The activity of NFκB is regulated by association with its inhibitor IκB that is a degraded by the proteasome upon stress stimulation. Proteasome inhibitors may overcome NFκB-mediated drug resistance by inhibition of IκB degradation and thereby prevention of NFκB translocation to the nucleus (Dou and Li 1999). Bortezomib or Velcade® is a selective inhibitor of the proteasome in clinical use for treatment of patients with multiple myeloma. The mechanism of the anticancer effect is not clear although it seems to involve a combination of effects on proapoptotic and antiapoptotic pathways (Caravita, de Fabritiis et al. 2006). The recent identification of apoptosis-inducing compounds acting as inhibitors of ubiquitin isopeptidases suggest that in addition to direct inhibition of the proteasome, the degradation pathway also contains other molecular targets potentially interesting for anticancer therapy (Mullally and Fitzpatrick 2002; Aleo, Henderson et al. 2006).
Ubiquitin is attached to target proteins through a process involving three enzymes called E1, E2 and E3 (Adams 2004). Proteasomes consist of a central cylinder formed from multiple distinct proteases whose catalytically active sites are facing the lumen of the complex. The core proteasome particle associates with regulatory subunits (Adams 2004) that select proteins for destruction by binding polyubiquitin chains and controlling access of substrates to the active sites of the proteasome (DeMartino and Slaughter 1999). Ubiquitin isopeptidases are involved in cleavage of the isopeptide bonds connecting the individual ubiquitin monomers and the ubiquitin polymer to its target protein. This enables the recovery of ubiquitin for its reuse by the proteasome system by maintaining a cellular pool of monoubiquitin (Hershko and Ciechanover 1998; Mullally and Fitzpatrick 2002).

In addition to the fact that many of the newly developed drugs showing clinical efficiency affect several targets in the cell and elicit complex cellular responses, these drugs are in many cases being used in combination with standard chemotherapeutics. This supports the idea that multiple signaling pathways often need to be activated in tumor cells for obtaining efficient antiproliferative responses.

1.2 OUTCOMES OF TREATMENT

The increased knowledge about cell damage responses is providing new insight into chemotherapeutic drug-action. It is now known that anticancer agents can induce a series of responses affecting proliferation and survival in the tumor cell of which apoptosis is the most studied.

1.2.1 Apoptosis

Apoptosis was first described in 1972 by Kerr et al as a controlled cell death mode characterized by certain morphological features (Kerr, Wyllie et al. 1972). The dying cell undergoes nuclear and cytoplasmic condensation, plasma membrane blebbing and nuclear fragmentation. The process is energy-requiring and results in formation of membrane-enclosed particles called apoptotic bodies. These are rapidly recognized and phagocytosed by professional phagocytes or neighbouring cells (Fadeel and Orrenius 2005). Apoptotic cells are thereby eliminated in an orderly manner. Apoptosis has been
appreciated as a remarkably conserved cell death process essential not only during embryonic development but also for maintaining tissue homeostasis in the adult organism. Apoptosis has been regarded as the main mechanism by which chemotherapies induce tumor cell death and defects in apoptosis are believed to be important for drug resistance (Johnstone, Ruefli et al. 2002). It is however becoming increasingly clear that also other tumor cell responses may be important for the antiproliferative effects of chemotherapy (Brown and Attardi 2005). Apoptosis is discussed in further detail below.

1.2.2 Necrosis

Necrosis has been referred to as a form of cell death that is uncontrolled and often occurs in response to profound damage or a physical insult. It is characterized by swelling of cytoplasm and loss of membrane integrity, resulting in cell rupture and release of cellular contents into the surrounding tissue. A major consequence of this is activation of an inflammatory response (Fadeel and Orrenius 2005). Recent data indicate that necrosis may also be a regulated process triggered by stimuli such as intracellular Ca\textsuperscript{2+} overload, excessive production of reactive oxygen species (ROS) and cellular energy depletion (Ricci and Zong 2006). Whether a cell will die an apoptotic or necrotic cell death is influenced by the severity of the insult and the availability of ATP (Leist, Single et al. 1997). Induction of necrotic cell death is attracting interest in anticancer therapy. An approach under investigation is the use of alkylating agents to induce necrosis via poly(ADP-ribose)polymerase (PARP)-mediated energy depletion. This approach exploits differences in energy metabolism between tumor cells and normal cells for selectivity (Ha and Snyder 1999; Zong and Thompson 2006). Whether the inflammatory response associated with necrosis is beneficial for the treatment outcome or not is controversial. Inflammatory responses induced by necrosis may be associated with systemic toxicity but can also increase the efficiency of tumor cell death (Jin and El-Deiry 2005).

1.2.3 Autophagy

Autophagy is an evolutionarily conserved process in which cytoplasmic contents of a cell are sequestered within double membrane vacuoles called autophagosomes, which subsequently fuse with lysosomes where degradation of the sequestered material occurs. Autophagy functions as a survival mechanism in starving cells, in which the degradation of cytoplasmic material generates nutrients and energy. At the same time, extensive autophagy can be observed in dying cells, which has led to its classification as an alternative form of programmed cell death (Debnath, Baehrecke et al. 2005). Although autophagy is believed to represent a form of cell death distinct from apoptosis, several lines of evidence demonstrate the existence of cross-talk between autophagic and apoptotic cell death pathways (Kondo and Kondo 2006). Accumulating evidence demonstrate the induction of autophagy in tumor cells by anticancer therapies including radiation and the new alkylating agent temozolomide (Paglin, Hollister et al. 2001; Kanzawa, Germano et al. 2004). However, whether autophagy in response to therapy causes death or protects cells is still controversial.
1.2.4 Mitotic catastrophe

Mitotic catastrophe has been suggested to be a form of death occurring during mitosis as a result of cellular damage in combination with deficient cell-cycle checkpoints that would normally arrest progression into mitosis unless the damage has been repaired (Castedo, Perfettini et al. 2004). Micronucleated, nonviable cells arise after aberrant mitosis through formation of nuclear envelopes around clusters of missegregated chromosomes (Ricci and Zong 2006). Mitotic catastrophe can be induced by agents interfering with microtubule function as well as by DNA damage (Lock and Stribinskiene 1996; Morse, Gray et al. 2005). Tumor cells that often are deficient in cell cycle checkpoints may be particularly susceptible to induction of mitotic catastrophe (Ricci and Zong 2006). Notably, mitotic catastrophe is generally considered to be a trigger for cell death rather than a specific process by which cell death occurs. Cells going through mitotic catastrophe might eventually die by apoptosis or necrosis (Brown and Attardi 2005; Mansilla, Priebe et al. 2006).

1.2.5 Senescence

Senescence is a condition of permanent growth arrest first described in cultured human fibroblasts that failed to divide after a limited number of cell divisions. The limited replicative potential is caused by the progressive shortening of telomeres upon cell division, and growth arrest induced in this way is referred to as replicative senescence (Hayflick and Moorhead 1961). The senescent cells remain metabolically active and show characteristic changes in morphology such as enlarged and flattened cell shape and increased granularity. In addition to the telomere-dependent induction of replicative senescence, DNA-damaging treatment and agents that induce other types of stress responses also trigger a senescence-like phenotype (Wang, Wong et al. 1998; Chang, Broude et al. 1999). This type of stress-induced, telomere-independent growth arrest is believed to represent a premature form of senescence (von Zglinicki, Saretzki et al. 2005). Interestingly, senescence induced by both non-telomeric DNA damage and shortened, dysfunctional telomeres have been shown to be accompanied and maintained by activation of a DNA damage response (d'Adda di Fagagna, Reaper et al. 2003; von Zglinicki, Saretzki et al. 2005). This response involves signaling leading to stabilization of p53 that induce growth arrest (Shiloh 2003). With tumor suppressors such as p53 acting as important regulators of senescence, it is being accepted as a tumor suppressor mechanism that cells need to bypass for cancer development (Dimri 2005). Tumor cells usually avoid replicative senescence through upregulation of telomerase (Kim, Piatyszek et al. 1994) but can be forced into stress-induced premature senescence in response to chemotherapy and radiation (Wang, Wong et al. 1998; Chang, Broude et al. 1999). Induction of tumor cell senescence by anticancer drugs is not limited to cells in culture but has also been reported in vivo and suggested to be a relevant factor in determining treatment outcome (Schmitt, Fridman et al. 2002; te Poele, Okorokov et al. 2002). Senescent cells have been shown to secrete many factors with both tumor-suppressing and tumor-promoting activities that can affect the growth of neighboring cells. The relative expression of different secreted factors may influence the outcome of treatment (Roninson 2003).
1.3 APOPTOSIS SIGNALING

Two main apoptosis pathways have been described in mammals. The extrinsic, or death receptor-mediated pathway, is essential for maintaining tissue homeostasis especially in the immune system. This pathway is triggered by ligand binding to cell surface death receptors of the Tumor Necrosis Factor (TNF) receptor family (Danial and Korsmeyer 2004). The intrinsic pathway utilizes organelles such as mitochondria to amplify the death signals and is triggered by a variety of stimuli including DNA damage, reactive oxygen species (ROS) and activation of oncogenes (Ricci and Zong 2006).

1.3.1 Bcl-2 family proteins

The B-cell lymphoma-2 (Bcl-2) family proteins play important roles in regulating the initiation of caspase activation and apoptosis. The role of these proteins in controlling mitochondrial-dependent apoptosis by governing the release of caspase activating proteins from mitochondria is well established. In addition to the mitochondrial membrane, Bcl-2 family proteins also localize to other intracellular membranes such as the endoplasmic reticulum (ER) where they influence apoptosis signaling. The Bcl-2 family proteins consist of antiapoptotic members, such as Bcl-2 and Bcl-XL, and proapoptotic members such as Bax, Bak and Bad. The proteins can be divided into subgroups defined by the homology shared within four conserved regions termed Bcl-2 homology (BH) domains. The antiapoptotic members display conservation in all four BH domains whereas the proapoptotic proteins are either multidomain proteins, possessing the BH1-3 domains, or BH3-only proteins having homology only within the BH3 domain. The proapoptotic multidomain proteins are characterized by possessing intrinsic death-inducing activity and can be exemplified by Bax and Bak (Reed 2006).

1.3.2 Regulation of Bcl-2 family proteins

In response to death stimuli, Bax that is normally found in the cytosol, undergoes a conformational change and translocates to mitochondria. The conformational change allows the insertion of Bax into the mitochondrial outer membrane in its oligomerized active state. Bak is constitutively associated with membranes but also undergoes conformational activation and oligomerization in response to death signals (Danial and Korsmeyer 2004). Antiapoptotic Bcl-2 proteins interact with the proapoptotic proteins, thereby preventing their oligomerization. The relative amounts of pro- and antiapoptotic proteins will thus influence the susceptibility of cells to death stimuli. The BH3-only family consists of a large group of proteins, including Bid, Bad and Bim, which serve as upstream sensors selectively responding to specific death and survival signals. These proteins appear to require the multidomain proteins for the execution of cell death (Wei, Zong et al. 2001; Fadeel and Orrenius 2005). Bid and Bim have been suggested to directly activate Bax and Bak, whereas other BH3-only proteins such as Bad have an indirect activating effect by binding and neutralizing the effects of antiapoptotic proteins (Huang and Strasser 2000; Kuwana, Boucher-Hayes et al. 2005). Recent data favor a model in which the most important proapoptotic effect also of Bid and Bim are not direct activation, but an indirect effect of binding proapoptotic Bcl-2 proteins (Willis, Fletcher et al. 2007). The binding and occupying of antiapoptotic proteins by BH3-only proteins will cause release of Bax and Bak allowing their activation to proceed (Gogvadze and Orrenius 2006).
The BH3-only proteins are often found in inactive states in the cell and require appropriate stimuli for their activation. Bid is cleaved and activated by caspase-8 upon stimulation of cell surface death receptors, whereas the proapoptotic activity of Bim is controlled by sequestration to microtubule-associated dynein motor complexes. In response to a variety of proapoptotic stimuli, Bim translocates to mitochondria (Puthalakath, Huang et al. 1999; Gogvadze and Orrenius 2006).

The proapoptotic activity of Bad is regulated by phosphorylation. In response to survival factor signaling, Bad is phosphorylated at serines 112, 136 and 155, and sequestered in the cytoplasm bound to the adaptor protein 14-3-3. These phosphorylations promote survival by preventing Bad from binding antiapoptotic proteins, mainly Bcl-X\textsubscript{L} (Zha, Harada et al. 1996; Adachi and Imai 2002). Several kinases involved in survival signaling have been proposed to mediate Bad phosphorylation. Among these are Akt, that is activated in response to phosphatidylinositide-3’-OH kinase (PI3’K) signaling (Datta, Dudek et al. 1997), pp90 ribosomal S6 kinases (Rsks) acting within the mitogen-activated protein kinase (MAPK) signaling (Bonni, Brunet et al. 1999) as well as the cAMP-dependent protein kinase A (PKA) (Harada, Becknell et al. 1999; Lizcano, Morrice et al. 2000).

In addition to the survival-promoting phosphorylations of Bad, a proapoptotic phosphorylation of Bad at serine 128 (Ser 128) has been identified. This phosphorylation inhibits the cytoplasmic sequestration of Bad, thereby promoting apoptosis. The cell cycle regulated kinase Cdc2, which is involved in controlling the transition of proliferating cells through mitosis, has been identified to mediate Ser 128 phosphorylation of Bad. In postmitotic neuronal cells, Cdc2 activates apoptosis upon activity deprivation by a mechanism involving Bad Ser 128 phosphorylation (Konishi, Lehtinen et al. 2002). Cdc2-induced phosphorylation of Bad at Ser 128 results in apoptosis of lymphoid cells when there is a lack of growth factors at the G2/M phase of the cell cycle (Hashimoto, Hirose et al. 2005). The c-Jun N-terminal kinase (JNK), involved in stress-induced cellular responses within the MAPK pathways, has also been reported to phosphorylate Bad at Ser 128 (Donovan, Becker et al. 2002; Bhakar, Howell et al. 2003).

1.3.3 Mitochondrial control of apoptosis

Many proapoptotic signals converge on mitochondrial membranes with the resulting permeabilization of the outer mitochondrial membrane, release of proapoptotic proteins that normally reside in the space between the two mitochondrial membranes and activation of caspases. Cytochrome c is a component of the mitochondrial electron transport chain that initiates caspase activation upon release from mitochondria. Cytochrome c participates together with the cytosolic protein Apoptotic protease activating factor-1 (ApaF-1) and dATP/ATP in the formation of the apoptosome complex. The Apaf-1 proteins contain caspase recruitment domains that become exposed in the apoptosome complex and recruit pro-caspase-9 molecules to the complex where these are activated (Zou, Li et al. 1999). Activated caspase-9 can then cleave and activate downstream executioner caspases such as caspase-3. In addition to cytochrome c, several other apoptogenic proteins are released from mitochondria such as
Smac/Diablo that binds inhibitors of apoptosis proteins (IAPs) preventing their inhibition of caspases. Other proteins released are Apoptosis-inducing factor (AIF) and endonuclease G that can translocate to the nucleus and cause DNA fragmentation (Wang 2001).

1.3.4 Mitochondrial membrane permeabilization

The proapoptotic proteins Bax and Bak appear to be the main effectors of the mitochondrial cell death pathway. Multimeric oligomerization of these proteins in mitochondrial membranes correlates with mitochondrial membrane permeabilization and cells deficient in Bax and Bak are resistant to numerous stimuli known to cause mitochondria-dependent cell death (Wei, Zong et al. 2001). The precise mechanisms underlying mitochondrial outer membrane permeabilization are not clear. Bax and Bak have pore-forming properties and according to one model, oligomerized Bax and Bak may form channels that could potentially directly allow passage of proteins across the outer mitochondrial membrane. According to an alternative model, cross-talk between Bax and Bak in the outer mitochondrial membrane and components of the inner membrane are important for causing mitochondrial outer membrane permeabilization (Reed 2006). A complex called the permeability transition pore, formed in sites of interaction between the inner and outer mitochondrial membranes is believed to drive an increase in the permeability of the inner mitochondrial membrane (Forte and Bernardi 2006). Permeability transition results in disruption of the inner membrane function leading to ATP depletion as well as outer mitochondrial membrane rupture. Bax has been shown to cooperate with components of the permeability transition pore to increase mitochondrial membrane permeability (Marzo, Brenner et al. 1998).

However, the importance of the inner mitochondrial permeability transition for the release of cytochrome c during apoptosis has been questioned and it has been suggested that this process may instead lead to necrosis (Gogvadze and Orrenius 2006).

1.3.5 Caspases

Caspases are expressed as proenzymes that must be proteolytically processed into their activated forms. Upstream caspases, known as initiators, undergo autocatalytic activation, whereas downstream effector caspases are activated by the initiators. The caspase cascade can be triggered by stimulation of death receptors that activate initiator caspases such as caspase -8/-10 or alternatively by the intrinsic pathway in which caspase-9 is first activated. These caspases cleave and activate other caspases leading to activation of the effector caspases; caspase 3, 6 or 7. The effector caspases cleave a large number of substrates in the cell. This leads to disassembly of cell structures and the characteristic cellular changes occurring during apoptosis. Caspases also cleave proteins that normally would be protecting cells from apoptosis and inactivate them or produce fragments that promote cell death, thereby further amplifying the apoptotic signal (Thornberry and Lazebnik 1998; Fadeel and Orrenius 2005; Chowdhury, Tharakan et al. 2006).

1.4 ORGANELLE CONTROL OF APOPTOSIS

Several organelles possess sensors that can detect alterations and relay stress signals to the rest of the cell and cross-talk between different organelles appears to represent an
important concept in the control of cell death. When apoptosis is induced, organelle-
specific stress signals either locally activate caspases or indirectly activate caspases
after induction of mitochondrial membrane permeabilization and cytochrome c release
(Ferri and Kroemer 2001). The nucleus-initiated DNA damage response has long been
recognized as an apoptosis trigger. Accumulating evidence establish that organelles
such as the endoplasmic reticulum and lysosomes also sense stress and activate
proapoptotic signaling pathways.

### 1.4.1 Nucleus initiation of cell death

Cells respond to DNA damage by activating a complex system of signaling pathways
that will only briefly be touched upon here. DNA damage signaling is initiated by
kinases including ataxia telangiectasia mutated protein (ATM) and ATM- and Rad3-
related protein (ATR). These enzymes are recruited to sites of DNA damage and
phosphorylate diverse proteins, either directly or through the transducing kinases
CHK1 and CHK2 (Kastan and Bartek 2004). The phosphorylation of histone H2A.X at
Ser 139 occurs early in response to double strand breaks and facilitates assembly of
checkpoint and DNA repair factors (Shiloh 2003). Phosphorylation of p53 leading to its
stabilization and increased activity as a transcription factor is a key event in response to
DNA damage. p53 plays a central role in a cells decision to either induce cell cycle
arrest or apoptosis. The p53-mediated apoptotic response involves induction of
proapoptotic Bcl-2 family proteins including Bax, Noxa and Puma and repression of
antiapoptotic proteins such as Bcl-2. These effects together with p53-induced
upregulation of ROS-generating enzymes all promote mitochondrial membrane
permeabilization (Ferri and Kroemer 2001). In addition, p53 can have transcription-
independent mechanisms of action activating proapoptotic proteins at mitochondria
(Chipuk, Kuwana et al. 2004; Leu, Dumont et al. 2004). A role for caspase-2 during
DNA damage-induced apoptosis upstream of mitochondrial events has been established
(Robertson, Enoksson et al. 2002). p53 can play a role also in the activation of caspase-
2, since it induces the expression of the p53-induced protein with a death domain
(PIDD) which is part of a caspase-2 activating complex, the PIDDosome (Tinel and
Tschopp 2004).

### 1.4.2 The endoplasmic reticulum

The ER comprises a membranous network in the cytoplasm involved in synthesis of
proteins, in the folding and sorting of newly synthesized proteins as well as in Ca
storage and signaling. Transmembrane and secretory proteins as well as some
organelle-targeted proteins are translocated into the lumen of the ER where post-
translational modification, folding and oligomerization occurs (Kaufman 1999). In the
ER, numerous protein chaperones reside that promote proper protein folding and
minimize misfoldings. Stresses that disturb energy levels, the redox state or the Ca
concentration will negatively affect the protein folding capacity of the ER, leading to
accumulation of unfolded proteins. This stress condition will trigger protective
responses, collectively called the unfolded protein response (UPR). If the stress
condition persists and is not resolved, pro-apoptotic signaling will be initiated.
1.4.2.1  ER as a sensor of cellular stress

The signaling pathways constituting the UPR are initiated by three ER transmembrane receptors that sense the conditions in the ER lumen. The receptors are associated with the ER chaperone glucose-regulated protein 78 (grp78) in unstressed conditions, but upon accumulation of unfolded proteins grp78 dissociates from the receptors and receptor activation follows.

The Pancreatic ER kinase (PKR)-like ER kinase (PERK) receptor undergoes dimerization and autophosphorylation after grp78 dissociation. Activated PERK phosphorylates eukaryotic initiation factor (eIF2). This leads to inhibition of protein translation, thereby decreasing the load of new proteins arriving at the ER. However, some genes carrying certain regulatory sequences in their 5´untranslated regions can bypass the eIF2-dependent translational inhibition. One such example is the gene encoding the transcription factor ATF4, which can induce the expression of pro-survival genes as well as the apoptosis-promoting transcription factor C/EBP homologous protein (CHOP) (Harding, Novoa et al. 2000).

Activating transcription factor 6 (ATF6) is processed to its active form in the Golgi apparatus after dissociation from grp78 (Chen, Shen et al. 2002). It then translocates to the nucleus where it induces genes that contain an ER stress response element (ERSE) within their promoters. Examples of these are genes encoding the chaperones grp78 and grp94 as well as the transcription factor X box-binding protein 1 (XBP1) (Szegedi, Logue et al. 2006).

The receptor inositol-requiring enzyme 1 (IRE1) possess endonuclease activity and cause splicing of XBP1 into an active form (Yoshida, Matsui et al. 2001). XBP1 induce the expression of chaperones, genes involved in degradation of misfolded proteins (Yoshida, Matsui et al. 2003) and the gene encoding the co-chaperone p58\textsuperscript{IPK} that also interacts with PERK and relieves the PERK-mediated translational inhibition (Lee, Iwakoshi et al. 2003). The induction of p58\textsuperscript{IPK} could represent the termination of the UPR after which the cell either returns to normal functioning or, if the stress condition has not been resolved, commits to apoptosis (Szegedi, Logue et al. 2006).

1.4.2.2  ER stress-induced apoptotic signaling

The mechanisms regulating the switch from the protective UPR response into proapoptotic signaling appear complex. Activated IRE1 has been shown to form a complex with the adaptor protein TRAF2, which is involved in activation of JNK (Urano, Wang et al. 2000). Apoptosis-signaling-regulated kinase 1 (ASK1) has been shown to be recruited to the IRE1-TRAF2 complex and to mediate JNK activation (Nishitoh, Matsuzawa et al. 2002). JNK has been implicated in the apoptotic response to various stress stimuli and exerts proapoptotic functions through phosphorylation of AP-1 transcription factors that enhances their activity (Pulverer, Kyrnikis et al. 1991). In addition to transactivating effects, JNK also regulate Bcl-2 family proteins through phosphorylation (Davis 2000).

UPR-induced changes in the levels and activation of Bcl-2 family proteins may sensitize mitochondria to proapoptotic stimuli. Observations that several ER stress
agents cause mitochondrial membrane permeabilization also suggest that apoptotic signaling initiated in the ER can relay to mitochondria (Boya, Cohen et al. 2002). In addition to apoptosis-regulating functions at the mitochondria, Bcl-2 family proteins also localize to the ER membrane. In a recent report, Bax and Bak were shown to directly interact with IRE1 and to be essential for its activation (Hetz, Bernasconi et al. 2006). Much evidence indicates that the Bcl-2 family proteins play important roles regulating movement of $\text{Ca}^{2+}$ across the ER membrane. It is possible that Bak and Bax form pores in the ER membrane mediating release of luminal proteins and controlling ER $\text{Ca}^{2+}$ stores (Breckenridge, Germain et al. 2003). Alternatively, Bel-2 family proteins can interact with $\text{Ca}^{2+}$ channels such as the inositol triphosphate receptor type 1 (IP3R-1). Bel-2 has been found to interact with and affect the phosphorylation status of IP3R-1 at ER membranes, thereby enhancing Ca$^{2+}$ leak through the receptor. This interaction is enhanced in the absence of Bak and Bax, suggesting that these proapoptotic proteins regulate ER $\text{Ca}^{2+}$ stores by binding and displacing Bel-2 from the IP3R-1 receptor (Oakes, Scorrano et al. 2005).

$\text{Ca}^{2+}$ released from the ER can function as a trigger and modulator of apoptotic death signaling. The cytosolic cysteine protease calpain is activated by $\text{Ca}^{2+}$ and has a role in caspase activation as well as cleavage and activation of Bel-2 family proteins such as Bid (Mandic, Viktorsson et al. 2002; Breckenridge, Germain et al. 2003). The Ca$^{2+}$ activated phosphatase calcineurin dephosphorylates Bad promoting its translocation to mitochondria (Wang, Pathan et al. 1999). The ER and mitochondria form contact sites in which $\text{Ca}^{2+}$ released from the ER can be taken up by mitochondria (Rizzuto, Pinton et al. 1998) and promote mitochondrial permeability transition and subsequent apoptosis (Hajnoczky, Csordas et al. 2000). Bel-2 family proteins influence the apoptotic crosstalk between the ER and mitochondria by regulating both the amount of $\text{Ca}^{2+}$ in the ER that can be released and the tolerance of mitochondria to $\text{Ca}^{2+}$ (Breckenridge, Germain et al. 2003; Scorrano, Oakes et al. 2003).

Mouse procaspase-12 is localized to the ER and is specifically activated during ER stress stimuli in murine cells (Nakagawa, Zhu et al. 2000). Caspase-12 has thus been proposed to function as an initiator caspase during ER stress, activated by calpain-dependent cleavage or via interaction with IRE1-TRAF2 (Nakagawa and Yuan 2000; Yoneda, Imaizumi et al. 2001). The gene in the human genome showing the largest homology to caspase-12 gene was however found to have mutations preventing the expression of a functional protein (Fischer, Koenig et al. 2002). Instead, caspase-4 has been proposed to fulfil the function of caspase-12 in humans (Hitomi, Katayama et al. 2004).

1.4.2.3 ER stress in cancer

Activation of UPR signaling has been reported in various tumors and could be induced by limited oxygen and nutrients (Ma and Hendershot 2004). It is possible that UPR responses could act protecting and favour the survival of tumors, but this requires suppression of the pro-apoptotic responses which are also part of the UPR signaling. Activation of UPR has been shown to alter the chemosensitivity of tumors, making them more sensitive to certain drugs while more resistant to others (Ma and Hendershot 2004). Several agents have been reported to directly induce ER stress. These include
inhibitors of glycosylation, such as tunicamycin, agents that impair disulfide bond formation, such as dithiothreitol, and thapsigargin that depletes ER Ca\(^{2+}\) by inhibition of the sarcoendoplasmic-reticulum Ca\(^{2+}\)-ATPase (SERCA) (Breckenridge, Germain et al. 2003). ER stress responses may also be part of the overall effects induced by other chemotherapeutic agents, including cisplatin (Mandic, Hansson et al. 2003).

Figure 3. ER stress responses.

1.4.3 Lysosomes and cathepsins

In lysosomes degradation of both exogenous proteins and particles occur, as well as digestion of endogenous proteins and cellular organelles (Ciechanover 2006). This makes the lysosomes important organelles for the recycling of intra- and extracellular material. Numerous proteases responsible for degradation exist within the lysosomes. The main class of lysosomal proteases is the cathepsins, which can be divided into three subgroups according to their active-site amino acid. Most of the cathepsins are cysteine proteases (cathepsin B, C, H, F, K, L, O, S, V, W and X/Z). Two of them are aspartyl proteases (cathepsin D and E) and one is a serine protease (cathepsin G). Cathepsins are synthesized as inactive precursors that are activated during transport through endosomes to lysosomes by proteolytic removal of an N-terminal propeptide (Ishidoh and Kominami 2002). In addition to the function of cathepsins in general protein turnover, certain cathepsins also have more specific functions in various
processes such as bone remodeling, antigen presentation and hair follicle morphogenesis (Turk, Turk et al. 2001).

1.4.3.1 Cathepsins in cancer development

Several cathepsins have been implicated in cancer progression, in particular cathepsins B, L and D (Fehrenbacher and Jaattela 2005), which are the most abundantly expressed lysosomal proteases (Turk, Stoka et al. 2002). High levels of cathepsin D in tumors have been shown to be a prognostic factor for metastasis of breast cancer (Syratos, Maudelonde et al. 1989). Cathepsin B overexpression has been demonstrated in different human cancers, and has in some tumors been found to correlate with tumor progression (Yan, Sameni et al. 1998). Secreted cathepsins have been suggested to participate in the degradation of extracellular matrix, thereby promoting invasion and angiogenesis (Fehrenbacher and Jaattela 2005). In contrast to these tumor promoting activities, cathepsins also have death inducing functions.

1.4.3.2 Lysosomes as death signal integrators

Lysosomes have long been regarded as cellular “suicide bags” that release unspecific digestive enzymes after cell damage. This concept has been reassessed and a role for lysosomes and lysosomal enzymes in the regulation and execution of different modes of cell death has now been established. The extent of the lysosomal membrane permeabilization and thereby the amount of active cathepsins released into the cytosol is believed to influence what type of cell death is being triggered (Li, Yuan et al. 2000; Fehrenbacher and Jaattela 2005). Complete rupture of the lysosome with massive release of lysosomal enzymes into the cytosol is believed to cause necrosis, whereas partial lysosomal membrane permeabilization leads to limited release of enzymes and to apoptosis (Bursch 2001). For the cysteine proteases, endogenous inhibitors, cystatins, exist that control enzyme activity in the cytoplasm, whereas no endogenous inhibitors have been described for aspartyl cathepsins. Cathepsins are optimally active in the acidic pH of lysosomes (pH 4-5), but can also function in neutral pH outside lysosomes, generally with less efficacy (Fehrenbacher and Jaattela 2005). Notably, lysosomal leakage has been shown to cause a decrease in cytosolic pH, likely favorable for the activity of released cathepsin (Nilsson, Johansson et al. 2006).

Diverse stimuli such as TNFα (Guicciardi, Deussing et al. 2000; Foghsgaard, Wissing et al. 2001), oxidative stress (Brunk and Svensson 1999), Fas activation (Brunk and Svensson 1999), lipid mediators (Kagedal, Zhao et al. 2001), sphingosine, (Kagedal, Johansson et al. 2001), p53 activation (Yuan, Li et al. 2002) and chemotherapeutic drugs (Emert-Sedlak, Shangary et al. 2005) have been demonstrated to trigger release of cathepsins into the cytosol. The roles of the individual cathepsins are likely to vary in between different cell types and may be determined by the inducing death trigger. Most studies on lysosomal enzymes in apoptosis signaling implicate the cathepsins B, D and L.

The exact mechanisms causing destabilization and permeabilization of lysosomal membranes remain elusive. ROS can be responsible for destabilizing lysosomal membranes (Persson, Yu et al. 2003) and several stimuli that cause lysosomal leakage, such as TNFα, also promotes induction of ROS (Manna, Zhang et al. 1998). Formation
of the sphingolipid products ceramide and sphingosine by sphingomyelinase enzymes can be stimulated by death triggers such as TNFα (Adam-Klages, Adam et al. 1996) and chemotherapeutics (Modrak, Gold et al. 2006). Five sphingomyelinases (SMases) have been described in mammalian cells of which one has an acidic pH optimum and is found primarily in lysosomes (Modrak, Gold et al. 2006). Sphingosine accumulates in lysosomes and has been suggested to permeabilize lysosomal membranes via detergent-like mechanisms (Kagedal, Zhao et al. 2001). Several studies suggest that the Bcl-2 family proteins also play a role in the control of lysosomal membrane stability. Bax has been reported to insert into lysosomal membranes and promote release of lysosomal enzymes after staurosporine treatment (Kagedal, Johansson et al. 2005). Bcl-2 can suppress oxidative stress-induced lysosomal rupture via inhibition of phospholipase A2, which can have destabilizing effects on membranes (Zhao, Brunk et al. 2001; Zhao, Eaton et al. 2001).

Several studies have shown that cathepsins, once released into the cytosol, are involved in the triggering of mitochondrial membrane permeabilization, caspase activation and subsequent apoptotic cell death (Guicciardi, Deussing et al. 2000; Boya, Gonzalez-Polo et al. 2003; Emert-Sedlak, Shangary et al. 2005). There are, however, also indications of cathepsin involvement in caspase-independent cell death (Foghsgaard, Wissing et al. 2001). Little is known about the specific substrates that cathepsins could cleave in the cytosol and that would activate cell death pathways. The cysteine cathepsins have been demonstrated to be able to cleave and activate Bid, which has accordingly been suggested to be a link connecting lysosomal leakage to mitochondrial membrane permeabilization (Cirman, Oresic et al. 2004). However, lysosomal leakage can also trigger mitochondrial membrane permeabilization independently of Bid (Boya, Gonzalez-Polo et al. 2003) suggesting the existence of additional connecting pathways. Cathepsin D has been shown to trigger Bax activation in response to staurosporine treatment (Bidere, Lorenzo et al. 2003). Another possibility could have been direct processing of procaspases by cathepsins. This has proved not to be a likely mechanism for activation of apoptosis since cathepsins mainly have been demonstrated to process caspases involved in inflammatory responses rather than apoptosis (Stoka, Turk et al. 2001).

1.4.3.3 Lysosomal cell death in tumor cells

Several characteristics of tumor cells suggest that they may be preferentially sensitive to triggering of lysosomal death pathways. Many human tumors have increased levels of cathepsins (Spyratos, Maudelonde et al. 1989; Yan, Sameni et al. 1998) and lysosomal rupture will therefore lead to release of larger amounts of cathepsins. It has been demonstrated that both immortalized and transformed mouse embryo fibroblasts show greater sensitivity to TNFα treatment compared with primary cells. This increased sensitivity did not apply to cathepsin B -/- fibroblasts, which remained the resistant phenotype of primary cells (Fehrenbacher, Gyrd-Hansen et al. 2004). Metastatic tumor cells within an acidic tumor microenvironment have been described to contain larger lysosomes (Glunde, Guggino et al. 2003), and larger lysosomes are known to rupture more easily (Ono, Kim et al. 2003). In addition, the high protein turnover in rapidly growing cells could potentially enhance the probability of lysosomal leakage. Turnover of iron-containing proteins leads to lysosomal accumulation of iron.
that via Fenton chemistry can cause increased oxidative stress, destabilizing lysosomal membranes (Zdolsek, Zhang et al. 1993; Brunk, Neuzil et al. 2001). Cancer cells normally produce high levels of ROS and are thereby more sensitive to drug-induced increases in oxidative stress (Pelicano, Carney et al. 2004).

Consequently, tumorigenesis sensitizes cells to lysosomal death pathways in a number of ways and activation of cathepsin-dependent cell death could therefore prove useful in therapy. There are however also examples of changes occurring during tumorigenesis that could confer resistance to cathepsin-mediated cell death, such as increased expression of endogenous cysteine cathepsin inhibitors (Kuopio, Kankaanranta et al. 1998) and translocation of heat shock protein 70 (hsp70) to lysosomal membranes where it stabilizes them (Nylandsted, Gyrd-Hansen et al. 2004).

Figure 4. Lysosomes in apoptotic signaling
2 AIMS OF THE THESIS

The aim of this work was to achieve a better understanding of the mechanisms of action of clinically used chemotherapeutic agents as well as to identify agents with novel mechanisms of action. Clinically used chemotherapeutic drugs generally induce complex responses in tumor cells. Studying the mechanisms underlying these responses is important in order to understand how drugs should be optimally used and why tumor cells become resistant. It is also important to examine novel mechanisms of drug action that could potentially represent new concepts for treatment.
3 RESULTS

3.1 PAPER I

Paclitaxel is an effective anticancer agent known to act by interfering with microtubule function. However, the mechanisms leading to apoptosis induction by paclitaxel are not clear. In this study we addressed the question of how paclitaxel induces apoptosis and focused on the potential involvement of the proapoptotic protein Bad. Cdc2- and JNK-mediated phosphorylation of Bad at Ser 128 was recently described to enhance its proapoptotic activity (Donovan, Becker et al. 2002; Konishi, Lehtinen et al. 2002; Bhakar, Howell et al. 2003; Hashimoto, Hirose et al. 2005). Since both Cdc2 and JNK have been implicated in paclitaxel-induced apoptosis (Donaldson, Goolsby et al. 1994; Wang, Popp et al. 1999), we speculated that Bad Ser128 phosphorylation may occur in response to paclitaxel and be important for the apoptosis induction.

We found that phosphorylation of Bad at Ser 128 occurs in mitotic fibroblasts, probably mediated by Cdc2 which has a high activity during mitosis (Morgan 1997). In contrast to the neuronal cells, the phosphorylation of Bad in proliferating fibroblasts was not correlated with apoptosis induction. We also demonstrated that Bad Ser 128 phosphorylation occurs after treatment with apoptosis-inducing concentrations of paclitaxel in fibroblasts and MDA-MB-231 cancer cells. JNK may mediate the phosphorylation of Bad in response to paclitaxel, since we observed a slight inhibition in the phosphorylation-specific signal after cotreatment with a JNK inhibitor. It is also possible that Cdc2 contributes to the paclitaxel-induced Bad Ser 128 phosphorylation. We observed a decreased phosphorylation signal also after cotreatment with a Cdc2 inhibitor. However, we cannot discriminate between direct inhibitory effects on the phosphorylation and indirect effects due to cell cycle inhibition. To test whether the Bad phosphorylation occurring in response to paclitaxel is of importance for the apoptotic response, we transfected cells with a wild-type Bad vector or a dominant negative Bad vector mutated at the Ser 128 site. To specifically measure apoptosis in transfected cells, we cotransfected the Bad vectors with a caspase substrate, cytokeratin 18, not endogenously expressed by fibroblasts. We found no inhibition of the apoptotic response in cells expressing the mutant Bad construct, suggesting that the phosphorylation of Bad at Ser 128 is probably not of importance for paclitaxel-induced apoptosis. Together our results suggest that, in contrast to neuronal cells, Bad phosphorylation at Ser 128 do not appear to promote apoptosis in the cells we have studied and the effects on apoptosis are therefore cell type-specific.

3.2 PAPER II

Ellipticines are plant alkaloids known to act as inhibitors of topoisomerase II-β (Tewey, Chen et al. 1984). This inhibition resulting in DNA damage is considered to be the main mechanism of cytotoxicity for these drugs, although a variety of cellular activities have been reported for these agents. The potent ellipticine derivative 6-propanamine ellipticine (6-PA-ELL) was demonstrated to induce rapid apoptosis in MDA-MB-231 accompanied by cytochrome c release indicating involvement of mitochondria in the apoptotic response. In addition, Bak was found to be activated in response to treatment
and MEF Bak-/- cells were relatively resistant to 6-PA-ELL treatment. 6-PA-ELL treatment was shown to induce a number of cellular responses characteristic of conditions of ER stress.

The expression of protein chaperones was induced after 6-PA-ELL treatment and more specifically, the expression of the chaperone grp78 was demonstrated to be induced via stimulation of the ERSE in the grp78 promoter. Importantly, the upregulation of grp78 was an early event occurring before apoptotic activity measured as caspase-cleaved cytokeratin 18 was detected, suggesting that the ER stress responses can have a role in initiating the apoptotic response. 6-PA-ELL was further shown to induce ER stress-characteristic splicing of mRNA encoding the transcription factor XBP1. Drug treatment also caused cleavage of the ER associated caspase-12 in CT51 mouse colon cancer cells as well as cleavage of a protein detected by caspase-12 antibodies in human MDA-MB-231 breast cancer cells. Studies have shown that the human caspase-4 plays a similar role as was earlier reported for caspase-12 in rodent cells (Hitomi, Katayama et al. 2004). It is possible that the protein detected in the MDA-MB-231 cells represent caspase-4, although we cannot be certain about this. We demonstrate a role for the Ca\(^{2+}\) dependent protease calpain in 6-PA-ELL-induced apoptosis. Calpain inhibition slightly reduces the processing of caspase-12/4 and since calpain inhibitors also significantly lower the apoptotic response to 6-PA-ELL, the actions of calpain in the apoptotic response are likely to be important. We finally show that 6-PA-ELL treatment of enucleated cytoplasts gives rise to Bak and caspase activation, thereby demonstrating that proapoptotic effects of 6-PA-ELL can be independent of nuclear DNA damage.

In summary, we demonstrate that ellipticines possess ER stress-inducing activities and our data indicate that these activities can be important for the cytotoxicity of this group of compounds since apoptosis-inducing potency of different derivatives seems to correlate with induction of ER stress.

### 3.3 PAPER III

Cisplatin is classified as a DNA damaging agent but it also reacts with several other cellular components, including proteins, RNA and membrane phospholipids (Wang and Lippard 2005). Since DNA damage is regarded as the main mechanism responsible for the cytotoxicity of cisplatin, effects of drug interactions with other cellular components has not been the focus of much attention. However, a role for non-nuclear targets important for apoptosis induction has been proposed by data demonstrating caspase activation in cisplatin-treated enucleated cytoplasts (Mandic, Hansson et al. 2003). Cisplatin is widely used in studies of DNA damage-dependent apoptosis, often at concentrations that are at least 10 times higher than the growth inhibitory (G\(_{100}\)) concentrations of cisplatin. In this study, we investigated the mechanisms underlying the responses to different concentrations of cisplatin and the importance of nuclear and non-nuclear targets for these responses.

We found that cisplatin concentrations of \(\geq10\mu\text{M}\) were required to elicit acute apoptosis occurring up to 48 hours after initiation of treatment of HCT116 colon carcinoma and 224 melanoma cells, whereas lower concentrations caused a delayed apoptotic response.
evident at 72 hours. We found that cisplatin within the first few hours of treatment induced production of ROS species at concentrations $\geq 10\mu\text{M}$, thus correlating to induction of acute apoptosis. We also measured caspase activation in enucleated cytoplasts and found that caspase activation is initiated at similar concentrations in intact cells and cytoplasts, suggesting that important apoptosis determinants are present in the cytoplasts. Furthermore, ROS scavengers were found to reduce the apoptotic response to cisplatin in both intact cells and cytoplasts. This suggests that ROS species are important mediators of the nucleus-independent response to cisplatin. We then examined what concentrations of cisplatin are required for induction of DNA damage signaling. DNA damage signaling, measured as phosphorylation of histone H2A.X and ATM and stabilization of p53, was induced by cisplatin already at lower concentrations that do not induce significant acute apoptosis. Instead we found premature senescence to be induced by lower cisplatin concentrations and this possibly accounts for much of the antiproliferative responses at these concentrations. The DNA damage signaling observed already at these lower doses is likely an underlying mechanism for the senescence response. Acute apoptosis induced by higher cisplatin doses may mostly be independent of DNA damage responses, but rather be triggered by cytoplasmic events such as ROS generation.

### 3.4 PAPER IV

Since many cancers have dysfunctional p53 contributing to therapy resistance, we aimed to investigate apoptotic signaling by compounds inducing p53 independent apoptosis. We found that 175 compounds from the NCI mechanistic set drug library induced apoptosis, measured as caspase cleavage of cytokeratin 18, at concentrations $\leq 5\mu\text{M}$ in HCT116 cells. Most of these drugs elicited stronger apoptosis in HCT116 p53 wild-type cells, but none of the drugs were totally dependent on p53 for apoptosis induction since apoptotic responses were seen also in p53 null cells. From the initial screen, 20 compounds showing varying degrees of p53 dependence for apoptosis induction were selected for further studies.

Twelve of the selected compounds induced the expression of p53 to various degrees and among these 8 drugs also caused signs of DNA damage, measured as phosphorylation of histone H2A.X. We did not find a simple correlation between p53 induction and the dependency on p53 for apoptosis, since some of the p53-inducing drugs caused similar apoptotic responses in p53 wild-type and p53null cells. Of 15 compounds inducing similar levels of apoptosis in p53 wild-type and p53null cells, 11 induced caspase activation also in enucleated cells, indicating the existence of non nuclear targets. Among these compounds, lysosomal membrane permeabilization appeared to be an important cytoplasmic target, since 7 compounds induced release of lysosomal cathepsins, and apoptosis induced by these compounds was partially inhibited by a cathepsin inhibitor. From these data, we suggest that involvement of lysosomes may be a common mechanism for drugs inducing p53-independent apoptosis.

### 3.5 PAPER V

In this study we continue to investigate apoptosis signaling involving lysosomes. We screened a drug library for compounds inducing cathepsin-dependent apoptosis. Using
cathepsin inhibitors and cathepsin B and D siRNA, we identified four compounds for which apoptosis was significantly inhibited by cathepsin D siRNA. These compounds were selected for further studies in which we demonstrate induction of lysosomal membrane permeabilization, measured as cathepsin D release, as well as mitochondrial involvement including Bak activation and mitochondrial depolarization. The two most potent apoptosis-inducing agents of the four compounds studied in detail have very similar structures, both containing a core structure that have shown inhibitory activities on ubiquitin isopeptidase enzymes (Mullally and Fitzpatrick 2002). We found that treatment with these two compounds caused accumulation of polyubiquitins likely due to disturbances in the normal function of the ubiquitin-proteasomal system upon isopeptidase inhibition. We also tested seven structurally related compounds and found a correlation between their ability to induce apoptosis and to cause accumulation of polyubiquitins. In addition, we found three agents known to act as inhibitors of the proteasomal system to induce lysosomal membrane permeabilization. This suggests that induction of apoptosis involving lysosomes may be common to various inhibitors of the ubiquitin-proteasomal system.

Preliminary studies have shown that one of the agents (NSC 687852) inducing cathepsin D-dependent apoptosis and accumulation of polyubiquitins also induce apoptosis of tumor cells in vivo. Caspase-cleaved cytokeratin 18 fragments can be used as biomarkers for apoptosis of epithelially derived cells (Linder, Havelka et al. 2004). Increased levels of caspase-cleaved cytokeratin 18 were detected after drug treatment in the plasma of tumor bearing mice but not in mice without tumor (Fig. 5). This suggests that this type of agent may be interesting for anticancer therapy.

**Figure 5.** SCID mice were injected subcutaneously with human squamous cell carcinoma FaDu cells and tumors were formed before treatment with the indicated drugs for 48 hours. Caspase-cleaved cytokeratin 18 (CK18-Asp396) levels were analyzed in plasma samples collected before (gray bars) and after treatment (black bars) by the M30 Apoptosense ELISA (Peviva).
4 DISCUSSION

4.1 INVOLVEMENT OF DIFFERENT CELLULAR ORGANELLES IN APOPTOSIS

In several of our studies we have found involvement of different cellular organelles in the proapoptotic signaling triggered by various drugs. The contribution of different cellular organelles to the apoptotic response will be discussed in the following section with an emphasis on the endoplasmic reticulum and lysosomes.

4.1.1 ER stress mediated by ellipticine and cisplatin

The ellipticine derivative, 6-PA-ELL, was found to induce a condition of ER stress as judged by the presence of several characteristic cellular activities. In addition, 6-PA-ELL treated cells showed signs of mitochondrial involvement in the apoptotic response. It is well known that proapoptotic signals generated at the ER can be transmitted to mitochondria. These signals can consist of Ca\(^{2+}\) released from the ER taken up by mitochondria, promoting mitochondrial membrane transition and cytochrome c release. Although we did not perform any Ca\(^{2+}\) measurements in 6-PA-ELL-treated cells, we demonstrate a role for the Ca\(^{2+}\) activated protease calpain in 6-PA-ELL-induced apoptosis. Calpain can activate Bcl-2 family proteins such as Bid, which in its turn promotes activation of multidomain proapoptotic proteins including Bak. Bak activation occurs early in response to 6-PA-ELL, before signs of caspase activity, and cells lacking Bak were found to be partially resistant to the drug. These observations suggest Bak to play a critical role in 6-PA-ELL-induced apoptosis. Bak can, in addition to its established proapoptotic role at mitochondrial membranes also influence apoptotic activities at the ER membrane (Breckenridge, Germain et al. 2003; Oakes, Scorrano et al. 2005). The Bak proteins that we show are activated after 6-PA-ELL treatment could include both mitochondria-localized and ER membrane localized proteins.

ER stress has also been suggested to be involved in the nucleus-independent responses to cisplatin (Mandic, Hansson et al. 2003). Cisplatin treatment induces a rise in intracellular Ca\(^{2+}\) followed by Bid activation. Ca\(^{2+}\) release is important for apoptosis induction by cisplatin since a Ca\(^{2+}\) chelator significantly decreases the apoptotic response (Mandic, Viktorsson et al. 2002; Mandic, Hansson et al. 2003). Cisplatin has further been shown to cleave ER-associated caspases and to cause an increase in the expression of the chaperone grp78 (Mandic, Hansson et al. 2003; Nawrocki, Carew et al. 2005). In the present study we further examined the responses induced by cisplatin that may indicate an ER stress response. Ire1-mediated splicing of XBP1 mRNA is known to represent one of the later events during the UPR (Szegezdi, Logue et al. 2006). In contrast to 6-PA-ELL, we could not detect splicing of XBP1 mRNA after cisplatin treatment, indicating that cisplatin effects do not show signs of a complete UPR.

The mechanisms underlying the ER stress effects of 6-PA-ELL and cisplatin are not clear. Both drugs are reported to have protein binding properties (Dodin, Andrieux et
al. 1990; Wang and Lippard 2005) and it can be speculated that such activities could be involved in the triggering of ER stress conditions. However, for cisplatin we suggest that the ER stress responses may be secondary to the observed cisplatin-induced ROS generation. ROS are constantly generated during respiration and mitochondria are considered to be a major source of ROS in cells although it can be produced by a number of other cellular oxidase enzymes (Pelicano, Carney et al. 2004). How ROS is generated in response to cisplatin is not clear, but cisplatin is thiol reactive and might affect redox buffering systems in the cell by binding to proteins such as glutathione (Arner, Nakamura et al. 2001; Sedletska, Giraud-Panis et al. 2005). The cisplatin-induced increase in intracellular Ca\textsuperscript{2+} concentration was slightly reduced upon cotreatment with a ROS scavenger, suggesting that ROS generation could occur upstream of Ca\textsuperscript{2+} release. This could be explained by Ca\textsuperscript{2+} channels in the ER membrane being sensitive to oxidation, leading to cytosolic Ca\textsuperscript{2+} release (Waring 2005).

In summary, compared to 6-PA-ELL that was positive for all tested activities characteristic of ER stress, cisplatin did not show signs of a complete UPR. Instead, ROS seem to be important mediators in the cisplatin-induced apoptotic signaling acting upstream of the ER-related effects such as Ca\textsuperscript{2+} release.

The ER is believed to be a cellular compartment highly sensitive to stresses that disturb its normal functions. ER stress-induced apoptosis may have evolved as a defence mechanisms against viral infection. Upon synthesis of large amounts of viral proteins in infected cells, misfolded proteins can trigger ER stress responses and viruses have in many cases developed ways to modulate these responses (He 2006). The sensitivity of the ER to various perturbations may be the reason for its sensitivity to various drugs and its involvement in apoptotic responses.

4.1.2 Identification and characterization of compounds inducing lysosomal apoptotic signaling

We found that the majority of the compounds studied in detail in paper IV that induced p53-independent apoptosis caused caspase activation also in enucleated cytoplasts. For several of these drugs involvement of lysosomes appeared to be important for apoptotic signaling. Much evidence suggest that cathepsins, once released into the cytosol, are involved in the triggering of mitochondrial membrane permeabilization (Boya, Gonzalez-Polo et al. 2003) and subsequent apoptosis. Cathepsin D-mediated activation of Bax is one example of how signals can be transmitted from lysosomes to mitochondria (Bidere, Lorenzo et al. 2003). In our study, all compounds failed to induce significant apoptosis in HCT116 cells lacking Bax, suggesting a critical role for mitochondrial involvement and for this proapoptotic protein. In addition, we cannot exclude direct effects of Bax at lysosomes since Bax has been reported to insert into lysosomal membranes and promote release of lysosomal enzymes in response to certain stimuli (Kagedal, Johansson et al. 2005). Our finding that apoptosis induction via lysosomal membrane permeabilization can occur independently of p53 together with observations suggesting that tumor cells can be preferentially sensitive to lysosomal cell death pathways suggest this mechanism of drug action as interesting to explore in drug development.
In paper V we identified four compounds for which cathepsin D plays a role in the apoptosis induction. Apoptosis induced by these compounds was however only slightly, although significantly, decreased in cathepsin D siRNA transfected cells. This indicates that additional targets are likely important for the apoptotic signaling induced by these drugs. Similar to the lysosomally acting drugs identified in the previous study, apoptosis induction by these agents also involved mitochondrial events, here measured as mitochondrial membrane permeabilization and Bak activation. An intriguing finding was that two of the compounds identified for which apoptosis was sensitive to cathepsin D downregulation had very similar structures, including a core structure characteristic for ubiquitin isopeptidase inhibitors (Mullally and Fitzpatrick 2002). In agreement with this, we found that these two compounds affected the ubiquitin-proteasomal system since drug treatment caused accumulation of polyubiquitins. We believe that this effect is due to their inhibition of ubiquitin isopeptidases, although a direct inhibition of enzyme activities by these compounds remains to be demonstrated. Effects on the ubiquitin-proteasomal system are likely to be important for the apoptotic activity of these agents as induction of polyubiquitins correlated to apoptotic activity in a set of structurally related compounds. The finding that two out of four compounds identified as inducers of cathepsin-dependent apoptosis also exerted effects on the ubiquitin-proteasomal system prompted us to examine whether induction of lysosomal enzyme release is a general feature of inhibitors of the ubiquitin-proteasomal system. We indeed found that a few other drugs with proteasome inhibitory actions also triggered release of cathepsin D, although we have not demonstrated that this release is important for the apoptotic activities of these drugs. In agreement with our findings, the clinically used proteasome inhibitor bortezomib was recently demonstrated to cause lysosomal membrane permeabilization. This activity was found to contribute to the apoptosis induction by bortezomib (Yeung, Huang et al. 2006).

Together these findings suggest that inhibition of the ubiquitin-proteasomal system and the triggering of lysosomal membrane permeabilization may be related effects. Since both the proteasome and lysosome are important for protein degradation it is possible that inhibiting the proteasome would lead to increased requirement for lysosomal degradation of polyubiquitinated proteins. It has been shown that when a cell’s capacity to degrade misfolded proteins is exceeded, misfolded ubiquitinated proteins aggregate into cytoplasmic structures termed aggresomes. These types of structures can be observed in cells treated with inhibitors of the proteasome and of deubiquitinating enzymes (Johnston, Ward et al. 1998; Wang and Figueiredo-Pereira 2005). Aggresomes recruit refolding and degradative components to aid in the clearance of the aggregated proteins. This process may however be inefficient and prolonged presence of aggresomes instead triggers an autophagic pathway in which the protein aggregates are incorporated into autophagosomes and delivered to lysosomes for degradation (Garcia-Mata, Gao et al. 2002). We are currently investigating whether aggresome formation and autophagic transport of ubiquitinated proteins to lysosomes occurs after treatment with the identified compounds and whether such processes can promote permeabilization of lysosomal membranes.

Proteasomal activity may not only influence lysosomes. Since proteasomes are important for the clearance of misfolded proteins translocated from the ER (Plemp...
and Wolf 1999), inhibition of proteasomal function may cause ER stress (Nawrocki, Carew et al. 2005). It is therefore possible that the compounds in our study interfering with the function of the ubiquitin-proteasomal system also cause ER stress responses that may contribute to the apoptotic effect of these drugs.

4.1.3 Nucleus-initiated apoptotic signaling
We have observed nucleus-independent apoptotic signaling in response to treatment with DNA damage-inducing drugs. These observations raise questions about the role of the DNA damage response in apoptosis induction. In the study on cisplatin, this question was addressed. Our data on cisplatin do not favour a model in which nuclear DNA damage has a major influence on the acute apoptosis response. This is based on observations that the dose requirements for triggering of apoptotic signaling were similar in intact cells and enucleated cytoplasts. In addition, activation of DNA damage signaling was detected at low doses where no significant acute apoptosis was observed. Our observations do not however imply that DNA damage caused by cisplatin is not an important determinant of the antiproliferative response of tumor cells to this drug. DNA damage may be the main mechanism underlying delayed apoptotic responses and other antiproliferative responses as will be discussed below.

4.2 COMPLEX CELLULAR OUTCOMES OF TREATMENT
Apoptosis has been recognized as the main mechanism by which chemotherapies induce tumor cell death. It is however becoming increasingly clear that the complex effects that many drugs have on tumor cells are likely to cause various forms of antiproliferative responses. As discussed above, apoptosis can occur directly in response to organelle initiated stress signaling. In addition, after some drug-induced effects such as DNA damage and interference with microtubule function apoptosis can occur both directly or be a delayed effect occurring after cells have gone through abnormal cell division (Woods, Zhu et al. 1995; Endlich, Radford et al. 2000; Castedo, Perfettini et al. 2004). In this section, cellular responses to microtubule-interfering drugs and DNA-damaging treatments will be discussed.

4.2.1 Cellular responses to inhibition of microtubule function
Drugs including paclitaxel that interferes with the normal function of microtubules have been reported to cause mitotic catastrophe and apoptosis (Banerjee, Fallis et al. 1997; Torres and Horwitz 1998). Mitotic catastrophe generally refers to cell death caused by aberrant mitosis, although the definition is not clear. As pointed out earlier, cell death by mitotic catastrophe can be executed by apoptosis or necrosis (Brown and Attardi 2005; Mansilla, Priebe et al. 2006) and should therefore rather be considered a trigger for death than a cell death process. Paclitaxel-induced apoptosis can occur directly from mitotic arrest or can occur following aberrant mitotic exit into a multinucleated state (Woods, Zhu et al. 1995; Castedo, Perfettini et al. 2004). Whether the conditions leading to apoptosis in response to paclitaxel should generally be defined as mitotic catastrophe depends on how this term is interpreted. In our study on paclitaxel we observed heterogenous cellular responses involving both micronucleated and apoptotic cells. We focused on examining the mechanisms underlying the apoptotic responses to paclitaxel. The apoptotic response measured can include both cells
committing to apoptosis directly from mitotic arrest as well as cells undergoing apoptosis after progression through abnormal mitosis.

Activation of both JNK and Cdc2 occurs at mitosis and both enzymes have been associated with paclitaxel-induced apoptosis (Donaldson, Goolsby et al. 1994; Wang, Popp et al. 1999; MacCorkle and Tan 2005). JNK is involved in apoptosis-regulating responses including transcriptional activation of c-Jun-induced genes and post-translational effects of several proteins within the Bcl-2 family (Pulverer, Kyriakis et al. 1991; Davis 2000). Phosphorylation of Bad at Ser 128 is thus only one example of many potential apoptosis-regulating activities of JNK. Although we did not observe an apoptotic effect of Bad phosphorylation, involvement of JNK in paclitaxel-induced apoptosis could be explained by other mechanisms. Cdc2 has in several studies been reported to be activated by paclitaxel treatment and inhibitors of Cdc2 can reduce the apoptotic response to paclitaxel (Donaldson, Goolsby et al. 1994; Shen, Huang et al. 1998; Yu, Jing et al. 1998). By phosphorylation of Bad at Ser 128, Cdc2 may have a role in sensitizing cells to cell death at mitosis, thereby preventing inappropriate cell proliferation. In our study there were no signs of apoptosis induction coupled to Bad Ser 128 phosphorylation in mitotic fibroblasts. It is possible that additional signals are required for apoptosis induction to occur in response to Bad Ser 128 phosphorylation. Supporting this are data from a recent study reporting that Bad dissociates from 14-3-3 at each G2/M phase of proliferating lymphoid cells in response to Cdc2-mediated Bad Ser 128 phosphorylation. However, only if there is a lack of growth factors at the G2/M phase is the dissociation followed by apoptosis induction (Hashimoto, Hirose et al. 2005). In addition to its involvement in apoptosis, a role for Cdc2 in mitotic catastrophe has also been suggested. Abnormal activation of Cdc2 has been shown to induce mitotic catastrophe and the prolonged activation of Cdc2 activation in paclitaxel treated cells may have the same effect (Castedo, Perfettini et al. 2002). The responses to treatment with paclitaxel for patients with p53 mutant cancers have been shown to be as good as for patients with wild-type p53 cancers (Safran, King et al. 1996). This is in agreement with a role for mitotic catastrophe in the triggering of cell death by paclitaxel, since cell death involving mitotic catastrophe occur independently of p53 (Merritt, Allen et al. 1997).

4.2.2 Cellular responses to DNA damage

The generally accepted view that tumor cell death in response to DNA damaging anticancer treatment mainly constitutes of apoptosis has in recent years been challenged by data on different cellular responses to radiation-induced DNA damage in various tissues. Radiation usually causes rapid apoptosis in tumors such as lymphomas and some haematological tumors. However, many solid tumors do not primarily undergo apoptosis in response to radiation, but rather responses such as senescence-like growth arrest and mitotic catastrophe. Mitotic catastrophe can then be followed by death modes such as delayed apoptosis or necrosis (Gudkov and Komarova 2003; Brown and Attardi 2005). It has been shown in several studies that decreasing the sensitivity to apoptosis e.g. by upregulation of Bcl-2 or deletion of p53 causes little or no change in overall survival of epithelial tumor cells exposed to radiation as well as to some DNA damaging anticancer agents (Lock and Stribinskiene 1996; Merritt, Allen et al. 1997).
These studies emphasize the importance of other tumor cell responses than apoptosis to DNA damaging treatment.

In agreement with these reports, our data on cisplatin indicate that DNA damage is not a major trigger for induction of the acute apoptosis response. We found senescence to be induced at low cisplatin concentrations that do not induce acute apoptosis. Since DNA damage signaling was activated at these doses we believe that DNA damage may underlie the senescence response. An association between DNA damage and senescence was not unexpected since DNA-damaging agents can trigger premature senescence and both replicative and premature senescence have been shown to be accompanied and maintained by activation of a DNA damage response (d'Adda di Fagagna, Reaper et al. 2003; von Zglinicki, Saretzki et al. 2005). Our data show decreased senescence-like growth arrest and increased clonogenic survival in p53null HCT116 cells compared to p53 wild-type cells. Since mitotic catastrophe likely is p53-independent (Merritt, Allen et al. 1997), we believe that senescence might be the predominant cellular effect underlying growth inhibition at low cisplatin doses in our system. However, we cannot exclude a role also for mitotic catastrophe. It is possible that the delayed apoptotic response observed at 72 hours in cells treated with doses below 10μM could be occurring secondary to conditions of mitotic catastrophe.

Although ROS and Ca\(^{2+}\) appear to be important mediators of the acute apoptotic response caused by cisplatin, neither a ROS scavenger nor a Ca\(^{2+}\) chelator had any major influence on senescence induction by cisplatin. These observations strengthen our hypothesis that the acute apoptosis and senescence triggered by cisplatin have distinct underlying mechanisms. We believe that our data may explain why cisplatin has to be used at much higher doses than the GI\(_{50}\) to induce acute apoptosis. Senescence can be triggered by DNA damage occurring already at lower doses while higher doses lead to cytoplasmic effects such as ROS generation and Ca\(^{2+}\) release important for the acute apoptosis response.

It is possible that different cellular responses to cisplatin exposure can be important for the treatment outcome in vivo. Apoptosis may occur close to blood vessels in solid tumors, whereas senescence may occur more distant, where the drug concentrations are likely to be lower. Since cisplatin is usually administered in repeated treatment cycles, it is relevant to know whether cells that have become senescent during one treatment cycle can undergo apoptosis upon subsequent cycles when these cells may be exposed to higher drug concentrations. Little is known about this possibility. One report demonstrates that senescent human fibroblasts were resistant to serum withdrawal-induced apoptosis (Wang 1995). Our preliminary data suggest that senescent cells can die in response to exposure to acute apoptosis-inducing doses of cisplatin although we have not examined the mode of cell death induced in the senescent cells.

4.3 SUMMARY

From our studies it is clear that apoptosis-inducing compounds often exert various complex effects on cells and that different organelles can be involved in the initiation and propagation of the apoptotic signal. Ellipticine and cisplatin are both DNA damaging but both agents also affect non-nuclear targets leading to the triggering of
apoptosis. The apoptosis-inducing compounds examined in paper IV also illustrate the complexity of cellular effects being triggered in tumor cells upon drug treatment. Although nuclear signaling involving DNA damage and p53 induction are commonly seen, apoptotic signaling can generally be induced without p53 and in many cases even in the absence of the cell nucleus. Several apoptosis-promoting effects appear to occur in parallel and the effect having most impact on the cell could be determined by the context including the genotype of the affected cell. Although apoptosis can be initiated at different organelles, a general theme seems to be the relaying of apoptotic signals to mitochondria, since signs of mitochondrial participation were detected in all our studies. In addition to induction of apoptosis, the drug-induced effects on tumor cells can cause various forms of antiproliferative responses. As exemplified by cisplatin, the context including the concentration of drug to which cells are exposed strongly influence the cellular responses being triggered.
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