J Bioenerg Biomembr (2007) 39:13–21
DOI 10.1007/s10863-006-9055-9

MINI REVIEW

The role of the mitochondria in mediating cytotoxicity of anti-cancer therapies

Dao M. Nguyen · Mustafa Hussain

Published online: 9 February 2007

Abstract Optimal cytotoxic anticancer therapy, at the cellular level, requires effective and selective induction of cell death to achieve a net reduction of biomass of malignant tissues. Standard cytotoxic chemotherapeutics have been developed based on the observations that mitotically active cancer cells are more susceptible than quiescent normal cells to chromosomal, microtubular or metabolic poisons. More recent development of molecularly targeted drugs for cancer focuses on exploiting biological differentials between normal and transformed cells for selective eradication of cancers. The common thread of "standard" and "novel" cytotoxic drugs is their ability to activate the apoptosis-inducing machinery mediated by mitochondria, also known as the intrinsic death signaling cascade. The aim of this article is to provide an overview of the role of the mitochondria, an energy-generating organelle essential for life, in mediating death when properly activated by cytotoxic stresses.

Keywords Mitochondria · Cancer · Chemotherapy · Apo2L/TRAIL · Bcl2 superfamily · Apoptosis · Caspase

Abbreviations

Bax	Bcl2-associated X protein
Bak	Bcl2-antagonist/killer
Bcl2	B cell lymphoma protein 2
Bid	Bcl2 inhibitory domain
Bad	Bcl2 antagonist of cell death
Bim	Bcl2-interacting mediator of cell death
Omni/HtrA2	high temperature requirement protein A2
Puma/Bbc3	p53-upregulated modulator of apoptosis/ Bcl2-binding component 3
Smac/Diablo	second mitochondria derived activator of caspases/direct inhibitor of apoptosis-binding protein with low isoelectric point
AIF	apoptosis inducing factor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
MOMP	mitochondria outer membrane permeability
MEK	MAPK kinase
MAPK	mitogen activated protein kinase
ERK	extracellular signal regulated kinase
Apaf-1	apoptosis-protease activating factor 1
JNK	Jun N-terminus kinase
ANT	adenine nucleotide transporter
VDAC	voltage-dependent anion channel

D. M. Nguyen (✉) · M. Hussain
Section of Thoracic Oncology, Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Room 4W-4-3940, 10 Center Drive, Bethesda, MD 29892
e-mail: dao_nguyen@nih.gov

Introduction

Cancer chemotherapy – the use of naturally occurring or synthetic chemicals to treat malignancies- is the major component of the modern oncologists' armamentarium. Technically speaking, cancer chemotherapeutics are metabolic, microtubular or chromosomal poisons that kill cancer cells by interfering with their ability to divide and thus blocking tissue growth. The therapeutic index of cancer chemotherapy depends on the selectivity of individual agents in targeting malignant cells and sparing normal cells. This "tissue selectivity" is influenced by many factors including the fact that malignant cells are more mitotically active than normal cells and thus easier poisoned. Hematopoietic tissues and gastrointestinal epithelial cells have high mitotic indices and thus are frequently "collaterally damaged" by cancer

chemotherapeutic drugs. The process of chemotherapeutic drug developments has undergone tremendous transformation owing to better understanding of the cell and molecular biology of cancer and the identification of therapeutic targets. Molecularly targeted anticancer drugs are developed with the expectation that they more effectively kill cancer cells with minimal adverse side effects to normal tissues. Successful treatment of Philadelphia chromosome-positive chronic myelogeneous leukemia using Gleevec (Johnson et al., 2003) or meaningful clinical response of stage IV non-small cell lung cancer bearing activating mutations of the EGFR gene to Tarceva (Shepherd et al., 2005; Tsao et al., 2005) are prime examples of the potentials of targeted molecular therapy for cancers refractory to standard cytotoxic therapies. Despite the diversity of the physico-chemical properties or the intended mechanisms of action, the majority of chemotherapeutic drugs kill cancer cells by activating built-in machinery regulated by the mitochondria to induce cell death via apoptotic and/or non-apoptotic pathways. This article focuses on describing the molecular mechanism by which mitochondria regulate death responses to cytotoxic stresses in cancer cells. The molecular components that regulate the intrinsic (mitochondria-dependent) death signaling pathways will be briefly described followed by a description of the role of the mitochondria in mediating cytotoxicity of standard (currently in use for standard-of-care therapy) as well as novel (under pre-clinical or clinical development) anticancer drugs.

Anatomy of the mitochondria-dependent cell death signaling pathways

Eukaryotic cells execute cell death programs in response to cytotoxic stress or extracellular cues via well-defined extrinsic (receptor-mediated) and intrinsic (mitochondria-dependent) pathways. As further described below, these pathways closely interact with each other at the mitochondrial level leading to efficient induction of apoptosis. Apoptosis is an evolutionarily conserved intrinsic program of cell death that occurs in various physiological and pathological conditions (Fulda and Debatin, 2006). Apoptosis is an energy-dependent orderly cellular process characterized by typical biochemical and morphological features including cell shrinkage and nuclear DNA fragmentation culminating in the elimination of apoptotic bodies by macrophages (Hengartner, 2000).

Intrinsic death signaling pathway

Cytotoxic stresses, via intermediary signaling molecules particularly the p53 tumor suppressive protein, converge on the mitochondria to engage its apoptogenic activity by inducing mitochondrial outer membrane permeabilization (MOMP). The molecular basis of cytotoxic treatment-induced MOMP remains controversial and generally falls under two classes of mechanism. Each may function under different circumstances (Green and Kroemer, 2004). In the first model of MOMP, permeability transition (PT) occurs in response to apoptosis-induced stress by the formation of a transmembrane pore bridging both the outer membrane and the inner membrane of the mitochondria. The permeability transition pore complex is composed of the adenine nucleotide transporter (ANT) and other proteins in the inner membrane along with the voltage-dependent anion channel (VDAC) and perhaps other proteins in the outer membrane. Opening of the pore allows water to enter the mitochondrial matrix and ions to equilibrate causing the loss of mitochondria inner transmembrane potential ($\Delta\Psi$). The opening/closing of the PT pore is regulated by many factors including caspase activation, nitric oxide, amphipathic peptides, and members of the Bcl2 superfamily (Kroemer et al., 1998; Galluzzi et al., 2006). The mitochondria matrix swells following opening of the PT pore causing rupture of the outer membrane leading to the release of intermembrane apoptosis-inducing proteins. In the second model of MOMP, only the outer mitochondria membrane is involved. In this model, members of the Bcl2 pro- and anti-apoptotic proteins play crucial roles. The BH123 pro-apopototic proteins Bax (exists in the cytosol and translocates to MOM upon activation) and Bak (normally resides on the mitochondrial outer membrane in association with VDAC2 (Cheng et al., 2003) and fully inserts into the MOM following activation) are the effectors of the outer membrane permeabilization by forming a pore via protein hetero- or homo-oligomerization allowing the release of pro-apoptotic proteins in the intermembrane space such as cytochrome c, Smac/Diablo, AIF into the cytoplasm. The BH3-only pro-apoptotic proteins such as Bid, Bim, Bad, Bik, Noxa, Hrk, Bmf, Puma play important regulatory roles in Bak/Bax-induced MOMP. Activation of Bax and Bak is mainly mediated by the apoptosis activators Bid or Bim while others (Bad, Bik, Hrk, Noxa, Bmf, Puma - frequently referred to as apoptosis sensitizers) assist in the process of Bax, Bak activation and MOMP by binding to and displacing Bid/Bim or Bax/Bak from antiapoptotic proteins (Certo et al., 2006; Letai, 2005; Kuwana et al., 2005). Antiapoptotic members of the Bcl2 superfamily suppress death by sequestering BH3 apoptosis activators and also perhaps BH123 proteins Bax and Bak (Cheng et al., 2001). Multiple proteins one of which is the well-described tumor suppressor protein p53, act as second messengers upstream of MOMP connecting cytotoxic stress to mitochondrial activation (Galluzzi et al., 2006; Fulda and Debatin, 2006). Apoptotic signals from DNA-damaging agents can be transmitted via p53 to the mitochondria as p53 can indirectly engage the intrinsic death signaling pathway by transcriptionally upregulating the expression of Bid, Puma, Noxa and Bax (Chipuk et al.,

2005; Sax et al., 2002; Yu et al., 2001; Oda et al., 2000). More recent experimental evidence has indicated that p53 can directly induce MOMP in a transcription-independent fashion by directly activating Bax, Bak or inhibit Bcl2, $BclX_L$ (Mihara et al., 2003;Chipuk et al., 2004). The nuclear histone protein H1.2 released from the nucleus of DNA-damaged cells via a p53-dependent mechanism interacts with Bak to mediate MOMP and cytochrome c translocation to the cytoplasm (Konishi et al., 2003). The BH3 apoptosis activator Bim, normally sequestered in the cytoskeleton by binding to microtubules, is set free leading to induction of MOMP and apoptosis by paclitaxel, a microtubule-active chemotherapeutic drug (Sunters et al., 2003; Tan et al., 2005). Hyperphosphorylation of Bcl2 leading to functional inactivation has been observed in paclitaxel-treated cells and correlated with opening of the PT pores (Srivastava et al., 1998; Ruvolo et al., 2001).

Extrinsic death signaling pathway

Death-inducing membrane receptors provide the link between extracellular death signals and the intracellular apoptosis-inducing signaling cascades. Members of the TNF receptor superfamily include TNF-R1 and TNF-R2, TRAIL receptors DR4, DR5 (and non-functional decoy receptors DcR1, DcR2), FasL receptor Fas (a non-functional decoy receptor DcR3), DR6 and $p75^{NTR}$. Binding of TNF to its receptors results in the formation of the death-inducing signaling complex (DISC) and activation of the caspase cascade in certain susceptible cells. However, in the majority of cases, activated TNF receptors recruits adaptor proteins TRADD, TRAF, RIP to form a different signalosome to activate the NF-kB pathway that mediate release of inflammatory cytokines and anti-apoptosis responses. TNF mediates its anticancer property by targeting tumor microvasculature to induce thrombosis and tumor necrosis rather than directly inducing apoptosis of target cells. On the other hand, bindings of apoptosis-inducing ligand FasL or TRAIL to their cognate receptors result in receptor aggregation, recruitment of FADD and procaspases 8 (and the less physiologically important caspase 10) and formation of the death inducing signaling complex (DISC) and activation of the initiator caspase 8 (Ashkenazi and Dixit, 1999; LeBlanc and Ashkenazi, 2003; Kelley and Ashkenazi, 2004; Nicholson, 1999; Salvesen and Dixit, 1999; Boatright et al., 2003). The DISC-derived activated caspases 8 then cleaves and activates the executioner caspases 3 and 7 leading to apoptosis via a signaling cascade known as the extrinsic pathway, in contrast to the mitochondria-mediated intrinsic death signaling pathway activated following cytotoxic stresses (Kroemer et al., 1998; van Loo et al., 2002). Moreover, activated caspase 8 can also process the BH3-only protein BID to yield truncated BID (tBID) that translocates to the mitochondria and, via interaction with Bak and Bax (Korsmeyer et al., 2000; Scorrano and Korsmeyer, 2003; Ruffolo et al., 2000) on the mitochondrial outer membrane, mediates mitochondrial outer membrane permeabilization (MOMP) and the release of multiple pro-apoptotic proteins including cytochrome c, AIF, SMAC/DIABLO. This links the extrinsic to the intrinsic death signaling pathways (Scaffidi et al., 1998; van Loo et al., 2002; Saelens et al., 2004; Kroemer et al., 1998). Cytosolic cytochrome c, together with Apaf-1, procaspase 9 and ATP, forms the apoptosome which activates caspase 9 (serving as the initiator caspase for the intrinsic death pathway) leading to activation of caspases 3, 7, 6 and induction of apoptosis. Caspase 3-derived activated caspase 6 proteolytically cleaves and activates caspases 2, 8 and 10 thus forming a positive amplification feedback loop to further activate the initiator caspase 8 (Fulda et al., 2002; Almasan and Ashkenazi, 2003). Inhibitors of apoptosis proteins (XIAP, cIAP1/2) can block apoptotic events by inhibiting the catalytic activity of effector caspases 3 and 7 or by blocking the activation of the apopsomal caspase-9 by directly interacting with the active sites of these caspases. XIAP is the most potent inhibitor of caspase activity (Duckett et al., 1996; McEleny et al., 2001). High expression of IAPs in cancer cells can confer resistance to TRAIL-induced apoptosis (Ng and Bonavida, 2002; Ng et al., 2002). The activity of IAPs can be blocked by Smac/Diablo, a mitochondrial protein that is released into the cytosol at some point during the apoptotic cascade, where it promotes cell death by eliminating IAP inhibition of caspases (Du et al., 2000; Verhagen et al., 2000). Death ligand-mediated induction of apoptosis is further classified into type I or type II depending on the involvement of the intrinsic (mitochondria-mediated) death signaling cascade in the effective execution of apoptosis (Scaffidi et al., 1998). Type I cells undergo death receptor-mediated apoptosis independent of mitochondria (and thus not sensitive to $Bcl2/BclX_L$) while type II cells rely on the intrinsic pathway for efficient apoptosis (and apoptosis is abrogated by Bcl2, $BclX_L$ or by the selective caspase 9 inhibitor Z-LEHD-fmk) (Ozoren and el Deiry, 2002). In the extrinsic death signaling pathway, death receptors occupy the apical position in the caspase activation cascade with recruitment of the intrinsic pathway as the secondary step to amplify and to perpetuate the initial death signal to achieve efficient and complete induction of apoptosis. Mitochondria are thus the gateway of death for cells receiving signals to die (extrinsic pathway) or receiving cytotoxic chemotherapeutics (intrinsic pathway).

Induction of cell death by cytotoxic chemotherapeutic drugs: The role of the mitochondria

Anticancer chemotherapeutic drugs are primarily discovered by systematic high throughput screening of natural and

synthetic chemicals followed by elucidation of their mechanism of action and synthesis of more potent drugs or analogs. Standard chemotherapeutics are broadly categorized into DNA-damaging agents, microtubule-active agents and antimetabolites. These drugs have been used for years mostly in multiple drug combinations to treat a wide variety of cancers with well defined track records of objective responses and cure rates. More novel "molecularly targeted" agents (gene therapy, histone deacetylase inhibitors, growth factor receptor antagonists, and mitochondrial toxins to name a few), are being systematically developed and tested in clinical trials for their anticancer efficacy and toxicity as well as pharamacokinetics/pharmacodynamics properties. The role of the intrinsic death signaling pathway in mediating cell death by representative chemotherapeutics e.g. cisplatin, paclitaxel and histone deacetylase inhibitors are briefly reviewed below.

Cisplatin (*cis*-diammine-dicholoro-platinum - CDDP), an antineoplastic agent discovered more than 3 decades ago, is still one of the most commonly used chemotherapeutic drugs to treat a wide variety of solid malignancies. Its primary mode of action is thought to be the formation of DNA-Protein and DNA-DNA adducts, resulting in DNA damage and cell death. Whether cancer cells undergo cell cycle arrest or cell death following CDDP exposure depends on the duration and magnitude of DNA damages. Intrastrand DNA cross-links are the primary lesion seen in CDDP-damaged DNA, and a correlation exists between the degree of lesions and cytotoxicity (Stathopoulos et al., 2006). Conventionally it is believed that cell death primarily ensues via a p53 dependent mechanism and up-regulation of pro-apoptotic factors, many of which converge on the mitochondrial pathway of cell death (Siddik, 2003). More recently, however, there is evidence that in addition to the p53 dependent pathway, cisplatin may act directly on the mitochondria, promoting cell death.

DNA damage results in cell cycle arrest at G2/M at which point this damage can either be repaired, or if overwhelming, result in cell death (Demarcq et al., 1994). The tumor suppressor protein p53 is thought to be activated by DNA damage sensing proteins, which are likely the same proteins involved in the repair machinery, though this is yet to be fully elucidated (Siddik, 2003). In addition to DNA damaged induced activation of p53, there is considerable evidence that stress induced kinases such as SAPK/MAPK, ATM (ataxia telangeiectasia and mutated protein) and ATR (ATM and Rad3-related protein), are activated after exposure to CDDP. These can directly phosphorylate and activate p53 as well as act on p53 down-stream targets such as Bax and Bak (Ihrlund et al., 2006). Some authors have argued that cisplatin induced stress kinase activation is essential to the p53 pathway, as resistance to the drug is seen in kinase deficient cells despite DNA damage induced by the drug (Yeh et al., 2002). Active p53 functions to trans-activate a host of pro-apoptotic pathway that engage and form the mitochondrial machinery of apoptosis, or intrinsic pathway (Hopkins-Donaldson et al., 2006). The Bcl-2 family proteins are most prominently affected by p53 leading to alterations of the expression levels of pro-apoptotic Bak and Bax exceeding the levels of anti-apoptotic proteins Bcl-2 and Bcl-X_L (Del Bello et al., 2001). In addition to suppression of Bcl-2, p53 also activates inhibitors of Bcl-2 such as Bid and Puma, which not only amplify the pro-apoptotic signal but function as points of cross-talk between intrinsic and extrinsic apoptotic signals (Jiang et al., 2006; Devarajan et al., 2002). Certainly there is tremendous evidence that in CDDP-treated cells p53 exerts its apoptotic function via the Bcl-2 family genes, which ultimately act on the mitochondria. Over-expression of Bcl-2 abrogates the cytotoxic effect in many systems, as does knock out of Bax or Bak (Du et al., 2006; Fox et al., 2005; Bauer et al., 2005; Eliopoulos et al., 1995). Furthermore, inhibitors of apoptosis (IAP's) such as survivin that can act downstream of cytochrome c release can curtail the effect of CDDP (Tirro et al., 2006). Bcl-2 family proteins can also be the direct targets of stress induced kinases as well as the protein kinase C (PKC) pathway (Lasfer et al., 2006). This pathway may be critical in induction of apoptosis in p53 dysfunctional tumors. Taken together, CDDP induced DNA damage and stress induced signaling result in ultimate engagement of the mitochondrial death machinery instrumental in cell death.

In addition to the nuclear effects of CDDP, the drug directly affects the mitochondria. It has been shown that CDDP preferentially accumulates in the mitochondria, and consumes cellular glutathione which may potentiate oxidative cellular damage (Dzamitika et al., 2006). It has also been reported that mitochondrial DNA (mtDNA) is severely damaged by CDDP, and this appears to be repaired markedly less efficiently than nuclear elements, though whether this directly correlates to cytotoxicity is yet to be elucidated. Depletion of mtDNA renders cells resistant to CDDP, suggesting mtDNA damage may play a direct role in cytotoxicity (Liang and Ullyatt, 1998). Mitochondrial density also correlates with degree of toxicity of cisplatin and the generation of oxidative injury. Expression of superoxide dismutase protects against this damage and CDDP toxicity, further linking mitochondrial damage to the CDDP effect (Nishikawa et al., 2001). In cell free evaluation, mitochondria appeared to be more sensitive to CDDP exposure, with ultra-structural changes occurring at earlier time points than nuclear changes. This could be to the direct binding of CDDP to mitochondrial proteins such as VDAC a core component of the PT pore complex, which can function to permeabilize the outer membrane independent of Bax (Yang et al., 2006). These findings could be supported by the occasional observation of cisplatin sensitivity in p53 deficient cells.

Paclitaxel (Taxol®) is a cytotoxic agent also used clinically in a wide variety of malignancies. Its primary mode of action is to stabilize microtubules and to prevent microtubule

disassembly, leading to mitotic arrest and apoptosis. There is also evidence that microtubule dysfunction also affects other cellular processes (Jordan et al., 1993). Since paclitaxel does not directly damage DNA as other commonly used chemotherapeutics, its cytotoxicity does not rely heavily on the p53 regulated pathway of cell death. This is confirmed by the observation that many p53 deficient tumors retain sensitivity to paclitaxel (Bacus et al., 2001). Nevertheless, this drug has been shown to influence many effector molecules that function in the intrinsic death signaling pathway that converge on the mitochondria for the execution of cell death.

While not heavily dependent on functional p53 to exert its cytotoxic effect, paclitaxel has been shown to modulate downstream targets of p53. Bcl-2 phosphorylation and degradation appear to be closely related to paclitaxel induced cell death, and the induction of MAPK and JNK is thought to be associated with this phenomenon (Brichese et al., 2004). Bax and Bak upregulation has also been noted in many cancer cell lines regardless of their p53 status and this has been correlated with cytotoxicity (Thomadaki et al., 2006). Bcl-2 overexpression, which does not alter the microtubular effect of paclitaxel does abrogate the induction of DNA fragmentation and apoptosis (Ibrado et al., 1996). Most interestingly, paclitaxel has been shown to have a direct correlation with the effect of the BH-3 only protein Bim (Li et al., 2005). This molecule is found in normal cells associated with the microtubule-dyenin complex. Upon microtular disruption, Bim is thought to be released and target the mitochondria where it inhibits Bcl-2 (Li et al., 2005). Moreover, paclitaxel has also been observed to induce caspase independent cell death. Several apoptosis like phenomenon are observed, such as cytochrome c release and engagement of cell surface death initiators e.g., FADD (Fas-Associated Death Domain) and cleavage of Bid which initiates the mitochondrial pathway. Overexpression of dominant negative caspase 8 or overexpression of Bcl-2 only partially abrogates cell death in certain studies (Huisman et al., 2002). Nevertheless, the mitochondria appear to be targeted irrespective of caspase activation in the conventional sense of apoptosis.

In cell free systems, naked mitochondria appear to be architecturally distorted after exposure to taxol, and permeabilize independent of the presence of Bax. Taxol appears to open the adenine nucleotide-cyclophilin complex, which may serve to release cytochorome c earlier than Bcl-2 family protein involvement (Varbiro et al., 2001). The formation of oxygen free radicals was also increased in this system. This observation was confirmed in the cellular system, where mitochondria structural and functional changes were seen within hours of taxol exposure, but caspase activation was not seen until 24 hours (Andre et al., 2002).

Histone deacetylase inhibitors (HDACIs) are structurally diverse chemical compounds that share common biological properties of inducing core histone hyper-acetylation leading to gene expression and of mediating potent antitumor effects (Rosato and Grant, 2004; Schrump and Nguyen, 2005; Jaboin et al., 2002). Some HDACIs are either naturally occurring compounds like sodium butyrate (a fatty acid metabolite found in high concentration in the lumen of the large intestine) or pharmacologic compounds such as valproic acid (a commonly prescribed anti-epileptic drug). Still others are complex chemicals isolated from culture broths of microorganisms (Depsipeptide, Apicidin or Trichostatin A) or synthetic derivatives (MS-275, CI-994). HDACIs are subdivided into 4 fundamental groups: short-chain fatty acids (sodium butyrate, phenylbutyrate, valproic acid), synthetic benzanides derivatives (MS-275, CI-994), cyclic tetrapeptides (Depsipeptide, Trapoxin, Apicidin), and hydroxamic acids (Trichostatin A, Suberoylanilide Hydroxamic acid – SAHA, LAQ8240) (Rosato and Grant, 2004). HDACIs induce differentiation, cell cycle arrest and/or apoptosis of cancer cells in culture and in vivo animal models (Rosato and Grant, 2004; Schrump and Nguyen, 2005; Jaboin et al., 2002). Multiple HDACIs (SAHA, Depsipeptide, MS275) have been shown to have anticancer properties in phase I and II clinical trials (Rosato and Grant, 2004; Schrump et al., 2002; Kelly et al., 2003; Piekarz and Bates, 2004). The antitumor activity of HDACIs has been attributed to both their ability to inhibit deacetylases (leading to accumulation of hyper-acetylated histones and alteration of gene transcription) and their ability to suppress mitogenic signal transduction pathways via downregulation of oncoproteins expression (Yu et al., 2002) as well as their effect on the phenotypic expression of Bax, Bak, Bcl2, $BclX_L$ leading to a net increase of the ratio of pro-versus anti-apoptotic proteins of the Bcl2 superfamily and the apoptogenicity of the mitochondria (Ruefli et al., 2001; Marks and Jiang, 2005; Garcia-Morales et al., 2005; Doi et al., 2004; Dunant et al., 2005; Dunant et al., 2005). In addition to inducing hyperacetylation of histone proteins, HDACIs also mediate hyperacetylation of other non-histone proteins including p53 leading to an increase of its function in cell cycle arrest and induction of apoptosis (Marks and Jiang, 2005). Exposure of cancer cells to TSA or SAHA results in activation of caspases 2,3,7, 8 and 9, cleavage of Bid and apoptosis, all of which are strictly dependent on the catalytic processing of caspase 9 due to activation of MOMP as none of these occurs in the absence of caspase 9 or in the presence of Bcl2 overexpression (Henderson et al., 2003). Similar experimental findings have been reported to implicate the essential roles of the intrinsic death signaling pathways in mediating HDACI-induced apoptosis in a variety of cancer cell lines, involving different HDACIs and treatment conditions (Lucas et al., 2004; Jaboin et al., 2002; Ruefli et al., 2001; Rosato and Grant, 2004; Shao et al., 2004;Yeow et al., 2006; Nguyen et al., 2004; Nguyen et al., 2003; Yeow et al., 2006; Maxhimer et al., 2005).

Receptor-mediated induction of apoptosis: Apo2L/TRAIL and the mitochondria

Tumor-necrosis factor receptor apoptosis inducing ligand (TRAIL), a member of the TNF superfamily, is a naturally occurring ligand normally found on the surface of activated immune effector cells and thought to play important roles in immunosurveillance against virally infected cells or malignant transformation (Almasan and Ashkenazi, 2003; Yagita et al., 2004). TRAIL interacts with its receptors (2 functional receptors DR4 and DR5 and two nonfunctional decoy receptors DcR1, DCR2) to mediate intracellular signal transduction pathways leading to the induction of caspase-dependent apoptosis via pathways outlined in Fig. 1. Activation of DR4/DR5 either by recombinant agonistic human anti-receptor monoclonal antibody (Pukac et al., 2005) or by recombinant protein ligand (such as Apo2L/TRAIL) (Ashkenazi et al., 1999) selectively induces apoptosis of cancer cells and abnormal cells (particularly cultured human hepatocytes), thus making targeting DR4/DR5 a very attractive therapeutic opportunity (Kelley et al., 2001; Ashkenazi et al., 1999; Pukac et al., 2005). Molecular analysis of ligand-mediated induction of apoptosis of cancer cells indicated the recruitment of the mitochondria-dependent caspase activation cascade via the Bid/tBid linkage of the extrinsic to the intrinsic death pathways that serve to amplify caspase activation via positive feedback loop to ensure efficient execution of the cell death program (Scaffidi et al., 1998; van Loo et al., 2002; Saelens et al., 2004; Kroemer et al., 1998). Despite expressing adequate levels of functional receptors, a significant proportion of cultured cancer cells are refractory to the cytotoxic effect of Apo2L/TRAIL or antiDR monoclonal antibodies. The molecular basis of such intrinsic resistance is diverse, incompletely understood and cell-line dependent (Zhang and Fang, 2005). One common feature consistently described in the published literature is that combining cytotoxic chemotherapeutic drugs at subtherapeutic/sublethal treatment conditions with Apo2L/TRAIL or antiDR monoclonal antibodies results in profound and synergistic enhancement of cell death in the majority of instances (Muhlethaler-Mottet et al., 2004; Lacour et al., 2003; Kondo et al., 2005; Evdokiou et al., 2002; Liu et al., 2001; Sayers et al., 2003; Inoue et al., 2004; Guo et al., 2004; Fulda and Debatin, 2005; Zhou et al., 2000; Shankar and Srivastava, 2004). Invariably, substantial activation of the caspases 8, 9 and 3 as well as caspase-dependent induction of apoptosis are observed in combination-treated cells. The molecular basis of chemotherapy-induced potentiation of receptor-mediated cytotoxicity is multifactorial and treatment- as well as cell type-dependent (Shankar and Srivastava, 2004; LeBlanc et al., 2002; Kelley and Ashkenazi, 2004; Muhlethaler-Mottet et al., 2004; Lacour et al., 2003; Arizono et al., 2003). Upregulation of death receptors, functional increase of DISC activation leading to stronger death signals at the membrane level, reduced expression of anti-apoptotic proteins, activation of the mitochondria pro-apoptotic property have all been cited as the molecular basis of chemotherapy-induced potentiation of Apo2L/TRAIL cytotoxicity.

Robust caspase 8 activation in cancer cells treated with chemotherapy + Apo2L/TRAIL is thus frequently attributed to DISC activation (Vanoosten et al., 2005; Chopin et al., 2004; Kim et al., 2004; Nakata et al., 2004; Guo et al., 2004;

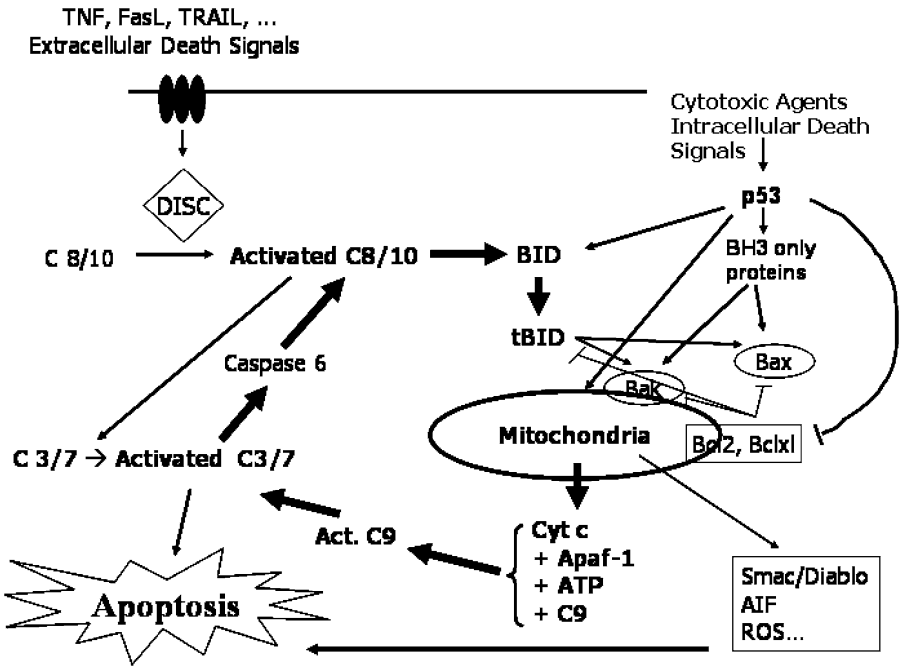

Fig. 1 Schematic representation of the interaction between the receptor-mediated (extrinsic) and the mitochondria-dependent (intrinsic) apoptosis-inducing signaling cascades. The pathway involving mitochondria/caspase 9, caspase 3, caspase 6, caspase 8 and BID form the amplification feedback loop. C8: caspase 8; C3/7 caspases 3/7. The master regulator of apoptosis protein p53 exerts its function by either regulating directly the mitochondria or indirectly through BH3 only proteins

Singh et al., 2005; Inoue et al., 2004). We recently demonstrated that profound activation of caspases 8, 9 and 3 is observed in lung or esophageal or mesothelioma cancer cells treated with chemotherapy (CDDP, paclitaxel or HDACIs) in combination with Apo2L/TRAIL (Nguyen et al., 2006; Ziauddin et al., 2006, Reddy et al, 2007). Overexpression of Bcl2 (blocking the mitochomndria function) or selective caspase 9 inhibitor (abrogation of the cytochrome c-mediated activation of the apical caspase of the intrinsic pathway) completely suppressed treatment-induced caspase 8 activation and subsequent cell death (Nguyen et al., 2006; Ziauddin et al., 2006). These data imply that, at least in our experimental conditions, the robust caspase 8 activation is the result of the mitochondria-dependent pathway and the positive feedback loop and not primarily mediated by the DISC function. In recent series of publications, our group has demonstrated the importance of the intrinsic death signaling cascade in mediating cytotoxicity in cancer cells treated with combinations of a variety of chemotherapeutic drugs and Apo2L/TRAIL. We have observed that 10 of 15 cultured thoracic cancer cells (cell lines derived from primary cancers of the lung, the esophagus or the pleura) are intrinsically resistant to the cytotoxic effect of Apo2L/TRAIL (Nguyen et al., 2006; Ziauddin et al., 2006). Treating these cells with sublethal concentrations of CDDP, paclitaxel or TSA results in significant sensitization of these cells to this death-inducing ligand, as indicated by 2- to more than 20-fold reduction of Apo2L/TRAIL IC_{50} values (concentrations of Apo2L/TRAIL that mediate 50% reduction of cell viability). More importantly, while less than 10% of cancer cells are killed by either chemotherapy alone or Apo2L/TRAIL alone, 60% to more than 90% of combination-treated cells undergo apoptosis. Substantial activation of caspase 8 (apical caspase of the extrinsic death pathway), caspase 9 (apical caspase of the intrinsic pathway) and caspase 3 (executioner caspase) is only observed in cells treated with drug combinations. Either overexpression of Bcl2 or the selective caspase 9 inhibitor LEHD-fmk completely abrogates combination-induced induction of apoptosis indicating that the intrinsic pathway is essential for combination-induced cell death. Moreover, either of these maneuvers aimed at blocking the apoptogenicity of the mitochondria (as evident by complete silencing of caspase 9 activation) also completely inhibits the activation of the capases 8 and 3. Complete abrogation of caspase 3 activity in these cells indicates the exclusive role of the mitochondrial pathway in activating this downstream executioner caspase, and inhibition of caspase 8 implies activation of this apical caspase is downstream of caspase 9 and a result of the amplification feedback loop mediated by the mitochondrial-dependent caspase activation cascade. Increased caspase 8 in combination-treated cells was, therefore, secondary to the amplification feedback loop and not DISC-mediated. As such, the next logical step in the development of efficient TRAIL-based combination therapy for cancer is to directly target the mitochondria to stimulate its apoptosis-inducing property. Suppression of anti-apoptotic protein expression by anti-sense or small interfering RNA techniques has been shown to sensitize cancer cells to TRAIL (Zhu et al., 2005) but this approach, while valuable in providing proof of concept, has limited clinical application mainly because of the redundancy of the Bcl2 superfamily antiapoptotic members, the long half life of Bcl2/$BclX_L$ proteins and the inefficient delivery of ribonucleic acid sequences to every cancer cells *in vivo*. The anti-apoptotic proteins Bcl-2 or Bcl-X_L sequester the pro-apoptotic proteins Bad, Bid, Bim, Bax and Bak by selective interaction between the BH3 domains of these proteins with their own BH3-binding pockets to prevent their translocation to the mitochondria where they perform their death-inducing function (Kuwana et al., 2005). Intense research and development of small molecule inhibitors of Bcl2/$BclX_L$ have identified multiple compounds that can interact with the BH3 binding pockets of these proteins to inhibit their anti-apoptotic function (Reed and Pellecchia, 2005). These drugs are commonly referred to as BH3-mimetics. Some of these drugs are naturally occurring chemicals identified to be BH3-mimetics by fluorescence polarization assay screening or computational chemistry (Fantin and Leder, 2006; Zhai et al., 2006) while others are specifically developed using structure-activity relationship based on nuclear magnetic resonance (Oltersdorf et al., 2005). Moreover, BH3-peptidomimetics have been designed with ingenious modifications for improved solubility and stability to act as novel mitochondria-targeted pharmaceuticals (Fantin and Leder, 2006). BH3-mimetics have been shown to enhance the anticancer activity of cytotoxic chemotherapy or radiotherapy (Oliver et al., 2005; Xu et al., 2005; Oliver et al., 2004). Moreover, these compounds (synthetic BH3I-2′ or natural compound Gossypol) also synergize with soluble TRAIL to induce massive apoptosis via mitochondrial-dependent pathways (Soderling, 1999; Hao et al., 2004 and Yeow et al., 2006).

Concluding remarks

The ultimate goal of drug development for cancer therapy is to achieve optimal cytotoxic efficiency and tissue selectivity. Recent understanding and appreciation of cancer biology as well as the molecular basis of carcinogenesis make these goals attainable. The field of apoptosis research has exponentially expanded with new landmark discoveries and updated models of how apoptosis proceeds at the cellular and molecular levels occurring almost every month. This is coupled with new drug discovery and more innovative therapeutic strategies. The molecular markers of apoptosis are well described

and relatively easy to quantify, especially for *in vitro* preclinical studies to determine the mechanism of drug-induced cell death. One has to be cognizant that other modes of cell death such as mitotic catastrophy, necrosis or autophagy do occur following chemotherapy (either standard cytotoxic or molecularly targeted agents) or radiotherapy. These types of death are less convenient to quantify using standard bioassays. It is particularly difficult to molecularly track treatment-induced apoptosis in patients undergoing standard cytotoxic chemotherapy (Brown and Wilson, 2003). The next challenge in the field of experimental therapeutics is the development of reliable markers of treatment-induced cytotoxicity that would collectively detect different mode of cell deaths. Such development would enable investigators to track and evaluate treatment responses at the molecular levels.

Acknowledgement This research was supported by the Intramural Research Program of the National Cancer Institute, NIH.

Bibliography

Almasan A, Ashkenazi A (2003) Cytokine Growth Factor Rev 14:337–348
Andre N, Carre M, Brasseur G et al (2002) FEBS Lett 532:256–260
Arizono Y, Yoshikawa H, Naganuma et al (2003) Br J Cancer 88:298–306
Ashkenazi A, Dixit VM (1999) Curr Opin Cell Biol 11:255–260
Ashkenazi A, Pai RC, Fong S et al (1999) J Clin Invest 104:155–162
Bacus SS, Gudkov AV, Lowe M et al (2001) Oncogene 20:147–155
Bauer JA, Trask DK, Kumar B et al (2005) Mol Cancer Ther 4:1096–1104
Boatright KM, Renatus M, Scott FL et al (2003) Mol Cell 11:529–541
Brichese L, Cazettes G, Valette A (2004) Cell Cycle 3:1312–1319
Brown JM, Wilson G (2003) Cancer Biol Ther 2:477–490
Certo M, Del GMV, Nishino M et al (2006) Cancer Cell 9:351–365
Cheng EH, Sheiko TV, Fisher JK et al (2003) Science 301:513–517
Cheng EH, Wei MC, Weiler S et al (2001) Mol Cell 8:705–711
Chipuk JE, Bouchier-Hayes L, Kuwana T et al (2005) Science 309:1732–1735
Chipuk JE, Kuwana T, Bouchier-Hayes L et al (2004) Science 303:1010–1014
Chopin V, Slomianny C, Hondermarck H et al (2004) Exp Cell Res 298:560–573
Del Bello B, Valentini MA, Zunino F et al (2001) Oncogene 20:4591–4595
Demarcq C, Bunch RT, Creswell D, Eastman A (1994) Cell Growth Differ 5:983–993
Devarajan P, Savoca M, Castaneda MP et al (2002) Hear Res 174:45–54
Doi S, Soda H, Oka M et al (2004) Mol Cancer Ther 3:1397–1402
Du C, Fang M, Li Y et al (2000) Cell 102:33–42
Du Y, Yin F, Liu C et al (2006) Biochem Biophys Res Commun 345:1092–1098
Duckett CS, Nava VE, Gedrich RW et al (1996) EMBO J 15:2685–2694
Dunant A, Pignon JP, Le Chevalier T (2005) Adjuvant chemotherapy for non-small cell lung cancer: contribution of the International Adjuvant Lung Trial. Clin Cancer Res 11:5017s–5021s
Dzamitika S, Salerno M, Pereira-Maia E et al (2006). J Bioenerg Biomembr 38:11–21
Eliopoulos AG, Kerr DJ, Herod J et al (1995) Oncogene 11:1217–1228
Evdokiou A, Bouralexis S, Atkins GJ et al (2002) Int J Cancer 99:491–504
Fantin VR, Leder P (2006) Oncogene 25:4787–4797
Fox SA, Kusmiaty, Loh SS, Dharmarajan AM, Garlepp MJ (2005) Biochem Biophys Res Commun 337:983–991
Fulda S, Debatin KM (2005) Eur J Cancer 41:786–798
Fulda S, Debatin KM (2006) Oncogene 25:4798–4811
Fulda S, Meyer E, Debatin KM (2002) Oncogene 21:2283–2294
Galluzzi L, Larochette N, Zamzami N, Kroemer G (2006) Oncogene 25:4812–4830
Garcia-Morales P, Gomez-Martinez A, Carrato A et al (2005) Mol Cancer Ther 4:1222–1230
Green DR, Kroemer G (2004) Science 305:626–629
Guo F, Sigua C, Tao J, Bali P et al (2004) Cancer Res 64:2580–2589
Hao JH, Yu M, Liu FT et al (2004) Cancer Res 64:3607–3616
Henderson C, Mizzau M, Paroni G et al (2003) J Biol Chem 278:12579–12589
Hengartner MO (2000) Nature 407:770–776
Hopkins-Donaldson S, Belyanskaya LL, Simoes-Wust AP et al (2006) Neoplasia 8:551–559
Huisman C, Ferreira CG, Broker LE et al (2002) Clin Cancer Res 8:596–606
Ibrado AM, Huang Y, Fang G, Bhalla K (1996) Cell Growth Differ 7:1087–1094
Ihrlund LS, Hernlund E, Viktorsson K et al (2006) Exp Cell Res 312:1581–1589
Inoue S, MacFarlane M, Harper N et al (2004) Cell Death Differ 11 Suppl 2:S193–S206
Jaboin J, Wild J, Hamidi H, Khanna C et al (2002) Cancer Res 62:6108–6115
Jiang M, Wei Q, Wang J, Du Q et al (2006) Oncogene 25:4056–4066
Johnson JR, Bross P, Cohen M et al (2003) Clin Cancer Res 9:1972–1979
Jordan MA, Toso RJ, Thrower D, Wilson L (1993) Proc Natl Acad Sci USA 90:9552–9556
Kelley SK, Ashkenazi A (2004) Curr Opin Pharmacol 4:333–339
Kelley SK, Harris LA, Xie D et al (2001) J Pharmacol Exp Ther 299:31–38
Kelly WK, Richon VM, O'Connor O et al (2003) Clin Cancer Res 9:3578–3588
Kim YH, Park JW, Lee JY, Kwon TK (2004) Carcinogenesis 25:1813–1820
Kondo K, Yamasaki S, Sugie T et al (2005) Int J Cancer
Konishi A, Shimizu S, Hirota J et al (2003) Cell% 19 114:673–688
Korsmeyer SJ, Wei MC, Saito M et al (2000) Cell Death Differ 7:1166–1173
Kroemer G, Dallaporta B, Resche-Rigon M (1998) Annu Rev Physiol 60:619–642
Kuwana T, Bouchier-Hayes L, Chipuk JE et al (2005) Mol Cell 17:525–535
Lacour S, Micheau O, Hammann AD et al (2003) Oncogene 22:1807–1816
Lasfer M, Davenne L, Vadrot N et al (2006) FEBS Lett 580:2547–2552
LeBlanc H, Lawrence D, Varfolomeev E et al (2002) Nat Med 8:274–281
LeBlanc HN, Ashkenazi A (2003) Cell Death Differ 10:66–75
Letai A (2005) J Clin Invest 115:2648–2655
Li R, Moudgil T, Ross HJ, Hu HM (2005) Cell Death Differ 12:292–303
Liang BC, Ullyatt E (1998) Cell Death Differ 5:694–701
Liu W, Bodle E, Chen JY et al (2001) Am J Respir Cell Mol Biol 25:111–118
Lucas DM, Davis ME, Parthun MR et al (2004) Leukemia 18:1207–1214

Marks PA, Jiang X (2005) Cell Cycle 4
Maxhimer JB, Reddy RM, Zuo J et al (2005) J Thorac Cardiovasc Surg 129:53–63
McEleny KR, Watson RW, Fitzpatrick JM (2001) Prostate Cancer Prostatic Dis 4:28–32
Mihara M, Erster S, Zaika A et al (2003) Mol Cell 11:577–590
Muhlethaler-Mottet A, Bourloud KB, Auderset K et al (2004) Oncogene 23:5415–5425
Nakata S, Yoshida T, Horinaka M et al (2004) Oncogene 23:6261–6271
Ng CP, Bonavida B (2002) Mol Cancer Ther 1:1051–1058
Ng CP, Zisman A, Bonavida B (2002) Prostate 53:286–299
Nguyen DM, Schrump WD, Chen GA et al (2004) Clin Cancer Res 10:1813–1825
Nguyen DM, Schrump WD, Tsai WS et al (2003) J Thorac Cardiovasc Surg 125:1132–1142
Nguyen DM, Yeow WS, Ziauddin MF et al (2006) T Cancer J 12:257–273
Nicholson DW (1999) Cell Death Differ 6:1028–1042
Nishikawa M, Nagatomi H, Nishijima M et al (2001) Cancer Lett 171:133–138
Oda E, Ohki R, Murasawa H et al (2000) Science 288:1053–1058
Oliver CL, Bauer JA, Wolter KG et al (2004) Clin Cancer Res 10:7757–7763
Oliver CL, Miranda MB, Shangary S et al (2005) Mol Cancer Ther 4:23–31
Oltersdorf T, Elmore SW, Shoemaker AR et al (2005) Nature 435:677–681
Ozoren N, el Deiry WS (2002) Neoplasia 4:551–557
Piekarz R, Bates S (2004) Curr Pharm Des 10:2289–2298
Pukac L, Kanakaraj P, Humphreys R et al (2005) Br J Cancer 92:1430–1441
Reed JC, Pellecchia M (2005) Blood 106:408–418
Rosato RR, Grant S (2004) Expert Opin Investig Drugs 13:21–38
Rishindra MR, Yeow WS, Ziauddin MF et al (2007) Apoptosis 12:55–71
Ruefli AA, Ausserlechner MJ, Bernhard D et al (2001) Proc Natl Acad Sci USA 98: 10833–10838
Ruffolo SC, Breckenridge DG, Nguyen M et al (2000) Cell Death Differ 7:1101–1108
Ruvolo PP, Deng X, May WS (2001) P Leukemia 15:515–522
Saelens X, Festjens N, Vande WL et al (2004) Oncogene 23:2861–2874
Salvesen GS, Dixit VM (1999) C Proc Natl Acad Sci USA 96:10964–10967
Sax JK, Fei P, Murphy ME, Bernhard E et al (2002) Nat Cell Biol 4:842–849
Sayers TJ, Brooks AD, Koh CY et al (2003) Blood 102:303–310
Scaffidi C, Fulda S, Srinivasan A et al (1998) EMBO J 17:1675–1687
Schrump, DS and Nguyen, DM (2005) Semin Oncol 92:257–61
Schrump DS, Nguyen DM, Kunst TE et al (2002) Clin Lung Cancer 4:186–192
Scorrano L, Korsmeyer SJ (2003) Biochem Biophys Res Commun 304:437–444
Shankar S, Srivastava RK (2004) Drug Resist Updat 7:139–156
Shao Y, Gao Z, Marks PA, Jiang X (2004) Proc Natl Acad Sci USA 101:18030–18035
Shepherd FA, Rodrigues PJ, Ciuleanu T et al (2005) N Engl J Med 353:123–132
Siddik ZH (2003) Oncogene 22:7265–7279
Singh TR, Shankar S, Srivastava RK (2005) Oncogene
Soderling TR (1999) Trends Biochem Sci 24:232–236
Srivastava RK, Srivastava AR, Korsmeyer SJ et al (1998) Mol Cell Biol 18:3509–3517
Stathopoulos GP, Katis C, Tsavdaridis D et al (2006) Cancer Chemother Pharmacol 58:555–560
Sunters A, Fernandez dM, Stahl M et al (2003) J Biol Chem 278:49795–49805
Tan TT, Degenhardt K, Nelson DA et al (2005) Cancer Cell 7:227–238
Thomadaki H, Talieri M, Scorilas A (2006) Biol Chem 387:1081–1086
Tirro E, Consoli ML, Massimino M et al (2006) Cancer Res 66:4263–4272
Tsao MS, Sakurada A, Cutz JC et al (2005) N Engl J Med 353:133–144
van Loo G, Saelens X, van Gurp M et al (2002) Cell Death Differ 9:1031–1042
Vanoosten RL, Moore JM, Ludwig AT, Griffith TS (2005) Mol Ther 11:542–552
Varbiro G, Veres B, Gallyas F Jr, Sumegi B (2001) Free Radic Biol Med 31:548–558
Verhagen AM, Ekert PG, Pakusch M et al (2000) Cell 102:43–53
Xu L, Yang D, Wang ST et al (2005) Mol Cancer Ther 4:197–205
Yagita H, Takeda K, Hayakawa Y et al (2004) T Cancer Sci 95:777–783
Yang Z, Schumaker LM, Egorin MJ et al (2006) Clin Cancer Res 12:5817–5825
Yeh PY, Chuang SE, Yeh KH et al (2002) Biochem Pharmacol 63:1423–1430
Yeow WS, Ziauddin MF, Maxhimer JB et al (2006) Br J Cancer 94:1436–1445
Yeow WS, Baras A, Chua A et al (2006). Journal of Thorac Cardiovasc Surgery 132:1356–62
Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B (2001) Mol Cell 7:673–682
Yu X, Guo ZS, Marcu MG, Neckers L et al (2002) J Natl Cancer Inst 94:504–513
Zhai D, Jin C, Satterthwait AC, Reed JC (2006) Cell Death Differ 13:1419–1421
Zhang L, Fang B (2005) Cancer Gene Ther 12:228–237
Zhou Q, Fukushima P, DeGraff W et al (2000) Cancer Res 60:2611–2615
Zhu H, Guo W, Zhang L, Davis JJ et al (2005) Cancer Biol Ther 4:393–397
Ziauddin MF, Yeow WS, Maxhimer JB et al (2006) Neoplasia 8:446–457