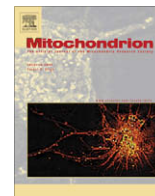




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Exploiting mitochondrial apoptosis for the treatment of cancer

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ABSTRACT

Mitochondria are key regulators of many forms of cell death and often altered in human malignancies. Since the inefficacy of established cancer therapies is, at least to a large extent, the result of oncogenic blockade of cell death pathways, compounds that directly affect mitochondrial functions are considered to present a promising alternative approach to eradicate chemotherapy-resistant cancer cells. Since mitochondria-targeted drugs can directly initiate mitochondrial perturbations independent of upstream signaling events, these agents may induce cell death and overcome drug resistance under circumstances, where conventional drugs fail to act because pathways upstream of mitochondria are frequently disrupted in cancer cells. A better understanding of the fundamental mechanisms that govern the complex processes of mitochondrial apoptosis is expected to open new perspectives for cancer drug development. Hopefully, this knowledge will eventually be translated into medical application for the treatment of human cancer.

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1. Introduction

A characteristic feature of human cancers is their ability to escape the induction of cell death (Fulda, 2009; Hanahan and Weinberg, 2000). Failure to undergo cell death upon appropriate stimuli is also a major cause of treatment failure and presents a yet unsolved problem in oncology (Fulda, 2009; Hanahan and Weinberg, 2000). Therefore, novel therapeutic strategies are required that circumvent resistance to conventional treatment approaches. Since the failure of conventional treatments is often due to oncogenic blockade of cell death programs, drugs that directly trigger cell death cascades have the potential to bypass resistance to established regimens (Fulda and Debatin, 2006).

Mitochondria exert dual functions in the regulation of cell survival and cell death. On one end, they are the powerhouse of the cell for the production of energy and therefore, are vital for the survival of a cell (Kroemer et al., 2007). On the other end, mitochondria are at the gateway of the intrinsic pathway of apoptosis (Kroemer et al., 2007). Accordingly, at the level of the mitochondria, the release of mitochondrial intermembrane space proteins such as cytochrome *c* and Smac/DIABLO into the cytosol presents a key initial step (Kroemer et al., 2007). Initiation of the mitochondrial pathway of apoptosis constitutes a point of no return in many models of apoptosis, which leads to activation of caspases, a family of proteases that act as common death effector molecules (Degte-

rev et al., 2003). A wide variety of apoptotic stimuli directly or indirectly engage signal transduction processes that result in permeabilization of the mitochondrial outer membrane (Kroemer et al., 2007). In light of the critical regulatory role of mitochondria in the control of cell death and in light of the fact that mitochondrial functions are frequently altered in human malignancies (Gogvadze et al., 2008), agents that target mitochondria are considered as promising cancer therapeutics to eliminate tumor cells including those, which are resistant to conventional therapies.

2. Regulation of mitochondrial apoptosis

2.1. Mechanisms of mitochondrial outer membrane permeabilization

Under basal conditions in the absence of apoptotic stimuli, pro-apoptotic members of the BCL-2 family reside in an inactive state (Adams and Cory, 2007). In this scenario, the mitochondrial transmembrane potential is high (Bouchier-Hayes et al., 2008), while the permeability transition pore complex (PTPC) is in its low-conductance state that allows the exchange of ions and small metabolites between the cytosol and the mitochondrial matrix. The PTPC is a multimeric complex that comprises the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), the peripheral benzodiazepine receptor (PBR), hexokinase (HK), and cyclophilin D (CYPD) (Kroemer et al., 2007). The interaction of HK and CYPD, which both regulate mitochondrial membrane permeabilization, occurs predominantly under physiological conditions (Majewski et al., 2004; Nakagawa et al., 2005).

Several mechanisms can contribute to mitochondrial outer membrane permeabilization (MOMP) and thereby promote the

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translocation of mitochondrial intermembrane space proteins including cytochrome *c* and Smac/DIABLO into the cytoplasm.

First, the prolonged opening of the PTPC promotes the loss of the mitochondrial membrane potential accompanied by an osmotic imbalance that causes the swelling of the mitochondrial matrix (Kroemer et al., 2007). These events are called the mitochondrial permeability transition (MPT) (Kroemer et al., 2007). Given that the surface area of the inner mitochondrial membrane is substantially larger than that of the outer mitochondrial membrane, MPT provokes preferentially the physical rupture of the OM (Kroemer et al., 2007). Second, pro-apoptotic members of the BCL-2 family such as BAX and BID translocate upon their activation from the cytosol to the mitochondria, while BAK as sessile mitochondrial protein undergoes conformational change locally at the outer mitochondrial membrane (Chipuk et al., 2006). The resulting homo- and/or hetero-oligomers of BAX and BAK form channels through which mitochondrial intermembrane space proteins can be released into the cytosol (Chipuk et al., 2006). Third, pro-apoptotic stimuli such as the cleaved form of BID, i.e. tBID, can directly stimulate MOMP via the destabilization of mitochondrial lipids, thus permeabilizing the outer membrane to allow the release of intermembrane space proteins (Kuwana et al., 2002). Fourth, inner membrane proteases are involved in the regulation of cytochrome *c* release from mitochondria. To this end, presenilin-associated rhomboid-like (PARL) is an integral protease of the inner mitochondrial membrane that controls the release of cytochrome *c* by cleaving the dynamin-related protein optic atrophy 1 (OPA1), which regulates remodeling of mitochondrial *cristae* independently of mitochondrial fusion (Cipolat et al., 2006; Frezza et al., 2006).

2.2. Metabolic reprogramming

Another hallmark of cancer cells is their increased flow through glycolysis despite high oxygen tension, a phenomenon called the “Warburg effect”, since it was first described by (Warburg et al., 1924). Production of ATP by aerobic glycolysis offers advantages to cancer cells, since they can shuttle glycolytic intermediates toward anabolic processes, e.g. glucose 6-phosphate to glycogen and ribose 5-phosphate synthesis, dihydroxyacetone phosphate to lipid synthesis and pyruvate to the synthesis of alanine and malate (Gatenby and Gillies, 2004). In cancer cells, the two isoforms of hexokinase are more tightly bound to VDAC at the outer membrane than in normal cells, linking residual ATP production/export from mitochondria to the rate-limiting step of glycolysis. In addition, pyruvate is preferentially converted to lactate in the cytosol in cancer cells compared to normal cells due to higher expression of the lactate dehydrogenase isoform M.

3. Targeting mitochondrial apoptosis in cancer

3.1. Targeting anti-apoptotic Bcl-2 family proteins (Table 1)

BH3 mimetics are small molecules, which closely resemble BH3-only proteins both in structure and in function. Most BH3 mimetics that have been developed so far bind to and antagonize pro-survival members of the BCL-2 family of proteins. Depending on the set of anti-apoptotic BCL-2 proteins that they neutralize, BH3 mimetics can exert distinct pro-apoptotic functions. **ABT-737** was developed through rational NMR-based screening and structure-based design and currently presents the most advanced and best characterized BH3 mimetic (Oltersdorf et al., 2005). ABT-737 binds to BCL-2, BCL-X_L and BCL-W, similar to the BH3-only protein BAD (Oltersdorf et al., 2005).

ABT-737 shows single agent activity dependent on expression levels of BCL-2 family proteins. Accordingly, cancer cells with high endogenous BCL-2 levels such as chronic lymphocytic leukemia and small cell lung carcinoma cells proved to be particularly sensitive to ABT-737 (Konopleva et al., 2006; Mason et al., 2008; Oltersdorf et al., 2005). As the anti-apoptotic BCL-2 family protein MCL-1 (Van Delft et al., 2006) is not neutralized by ABT-737, MCL-1 levels have been linked to the resistance of some cancer types towards ABT-737 (Konopleva et al., 2006; Van Delft et al., 2006).

Several ABT-737-based combination therapies have been developed to sensitize cancer cells for apoptosis induction. To this end, ABT-737 has been demonstrated to act in concert with conventional chemo- and radio-therapy in hematological malignancies as well as in different types of solid tumors (Hann et al., 2008; Kang et al., 2007; Kutuk and Letai, 2008; Mason et al., 2008; Tagscherer et al., 2008; Van Delft et al., 2006). Additionally, ABT-737 cooperated with various targeted agents including proteasome inhibitors (Miller et al., 2009; Paoluzzi et al., 2008), histone deacetylase (HDAC) inhibitors (Whitecross et al., 2009), kinase inhibitors (e.g., inhibitors of BCR-ABL, FLT3, EGFR, MEK1/2) (Cragg et al., 2007, 2008; Gong et al., 2007; Kohl et al., 2007; Kuroda et al., 2006, 2007) and also the death receptor ligand TRAIL (Huang and Sinicrope, 2008; Song et al., 2008). In several preclinical mouse models of human cancers, e.g. in SCLC and acute leukemia, ABT-737 has demonstrated potent antitumor activity (Hann et al., 2008; Konopleva et al., 2006; Mason et al., 2008, 2009), both as standalone agent and within combination regimens (Hann et al., 2008; Konopleva et al., 2006; Mason et al., 2008, 2009).

ABT-263 has been developed as an orally available derivative and is currently under clinical evaluation in phase I/II trials for chronic lymphocytic leukemia, lymphoma and SCLC, as monotherapy or in combination with various anticancer drugs depending on the type of cancer or with monoclonal antibodies (i.e., rituximab) (Tse et al., 2008). Of note, clinical studies so far indicate that the major mechanism-based side effect, i.e. BCL-X_L-induced thrombocytopenia, can be handled by appropriate dosing schedules (Mason et al., 2007; Roberts et al., 2009; Zhang et al., 2007).

Obatoclax (GX15-070, from Gemin X) is a small-molecule indole bipyrrrole compound directed against BCL-2, BCL-X_L, BCL-W and MCL-1 (Nguyen et al., 2007; Perez-Galan et al., 2007; Trudel et al., 2007). Accordingly, obatoclax can also overcome the MCL-1-dependent resistance to ABT-737 (Nguyen et al., 2007). In a phase I clinical study in chronic lymphocytic leukemia (CLL), obatoclax showed some single agent activity (O'Brien et al., 2009). Obatoclax alone or in combination regimens is currently evaluated for the therapy of hematological malignancies as well as solid tumors (Schimmer et al., 2008).

Gossypol (AT-101) is a natural compound found in cotton plant (Lynn and Jones, 1979) that inhibits BCL-2, BCL-X_L, BCL-W and also MCL-1 (Azmi and Mohammad, 2009). The derivative apogossypol has been reported to exhibit even better antitumor activity combined with reduced toxicity in comparison with gossypol (Kitada et al., 2008). It is interesting to note that AT-101 showed clinical activity as monotherapy in a phase I trial for the treatment of prostate cancer (Liu et al., 2009) and is currently evaluated as mono- or combination therapy in several malignancies.

3.2. Targeting mitochondrial metabolism (Table 1)

Alterations of the mitochondrial functions lie at the crossroad between cell death regulation and metabolism and represent promising targets for the design of new anticancer strategies. Given that metabolic reprogramming constitutes a hallmark of human cancers, strategies that interfere with the hyperglycolytic state of malignant cells have the potential to preferentially prime cancer cells to cell death.

Table 1
Examples of mitochondria targeting compounds.

Compound	Target/mode of action	References
<i>Modulators of the BCL-2 protein family</i>		
ABT-737, ABT-263	BCL-2, BCL-X _L , BCL-W	Oltersdorf et al. (2005)
GX15-070 (Obatoclox)	BCL-2, BCL-X _L , BCL-W, MCL-1	Nguyen et al. (2007)
AT-101	BCL-2, BCL-X _L , BCL-W, MCL-1	Kitada et al. (2003)
<i>Metabolic inhibitors</i>		
2DG	HK	Simons et al. (2007)
MJ	HK2/VDAC interaction	Goldin et al. (2008)
DCA	PDK inhibitor	Bonnet et al. (2007)
SB-204990	ACL	Hatzivassiliou et al. (2005)
<i>VDAC/ANT targeting agents</i>		
GSAO	ANT crosslinker	Don et al. (2003)
Lonidamine	ANT ligand	Oudard et al. (2003)
Clodronate	ANT inhibitor	Lehenkari et al. (2002)
ATRA	ANT ligand	Notario et al. (2003)
CD437	PTPC	Belzacq et al. (2001) and Marchetti et al. (1999)
PK11195, RO5-4864	PBR ligand	Decaudin et al. (2002)
<i>ROS regulators</i>		
Menadione	ROS production	Costantini et al. (2000)
β -lapachone	ROS production	Bey et al. (2007)
BSO	GSH synthesis inhibitor	Maeda et al. (2004)
Imexon	GSH depletion	Dragovich et al. (2007)
PEITCs	GSH depletion, GPX inhibition	Trachootham et al. (2006)
Mangafodipir	SOD mimic	Alexandre et al. (2006)
2ME	SOD inhibition	Huang et al. (2000)
ATN-224	SOD inhibition	Juarez et al. (2008)
Arsenite trioxide	ANT ligand, ROS production	Belzacq et al. (2001)
<i>Natural compounds</i>		
Betulinic acid	PTPC	Fulda et al. (1997)
Resveratrol	F ₁ -ATPase	Gledhill et al. (2007)
α -TOS	Ubiquinone-binding sites in respiratory complex II	Dong et al. (2008)

Abbreviations: 2DG, 2-deoxy-D-glucose; 2ME, 2-methoxyestradiol; α -TOS, α -tocopherol succinate; ACL, ATP citrate lyase; ANT, adenine nucleotide translocase; ATN-224, tetrathiomolybdate; ATRA, all-trans-retinoic acid; BSO, buthionine sulphoximine; CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; DCA, dichloroacetate; GSAO, 4-(N-(S-glutathionylacetyl)amino) phenylarsenoxide; GX15-070, obatoclox; GPX, glutathione peroxidase; GSH, reduced glutathione; HK, hexokinase; MJ, methyl jasmonate; PBR, peripheral benzodiazepine receptor; PDK, pyruvate dehydrogenase kinase; PEITCs, phenyl ethyl isothiocyanates; PTPC, permeability transition pore complex; ROS, reactive oxygen species; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel.

Glycolysis can be inhibited by the administration of 2-deoxy-D-glucose (2DG), which reduces the stability of the association between HK and VDAC, resulting in increased binding of Bax to VDAC. 2DG has been reported to enhance the cytotoxicity of conventional chemotherapeutics, for example cisplatin, in head and neck cancer cells through the production of reactive oxygen radicals (Simons et al., 2007). Early clinical trials with 2DG in patients with advanced solid tumors or prostate cancer are currently underway.

The plant hormone methyl jasmonate has been reported to detach hexokinase (HK) from mitochondria through direct binding, thereby initiating mitochondrial apoptosis (Goldin et al., 2008). HK is often overexpressed in human tumors and binds tightly to VDAC especially in cancer cells.

Dichloroacetate (DCA), an inhibitor of mitochondrial PDK, can be used to switch the abnormally high rate of glycolysis in cancer cells to glucose oxidation. Indeed, DCA has been demonstrated to decrease the elevated mitochondrial membrane potential of cancer cells, raise the production of mitochondrial ROS and activate K⁺

channels preferentially in malignant cells (Bonnet et al., 2007). As single agent, DCA is currently tested in a phase I study in patients with advanced solid tumors.

Further, inhibition of ATP citrate lyase (ACL), the key enzyme that links glucose metabolism to lipid synthesis by causing the conversion of citrate to cytosolic acetyl-CoA, by the pharmacological inhibitor SB-204990 has been reported to suppress tumor cell proliferation and growth *in vitro* and *in vivo* (Hatzivassiliou et al., 2005).

3.3. PTPC-targeting agents (Table 1)

Components of the PTPC can be targeted by multiple approaches. For example, 4-(N-(S-glutathionylacetyl)amino) phenylarsenoxide (GSAO), a glutathione-coupled trivalent arsenical compound, inhibits the ATP/ADP antiporter activity of ANT by crosslinking the key cysteine residues of ANT at position Cys160 and Cys257, which in turn causes ROS production, ATP depletion, mitochondrial outer membrane permeabilization and apoptosis (Don et al., 2003).

The indazole carboxylate lonidamine is another ANT ligand that initiates mitochondrial apoptosis (Belzacq et al., 2001). Early clinical experience with lonidamine demonstrated that it is well tolerated and holds tumor growth in patients with recurrent glioblastoma multiforme (Oudard et al., 2003).

The bisphosphonate clodronate has been reported to act as competitive ANT inhibitor (Lehenkari et al., 2002), while retinoid-related compounds such as 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) and all-trans-retinoic acid (ATRA) trigger ANT-dependent MPT and subsequently apoptosis independent from their ability to bind to nuclear receptors (Belzacq et al., 2001; Marchetti et al., 1999; Notario et al., 2003). Also, ligands of the PBR such as PK11195, RO5-4864 and diazepam have been demonstrated to exert antitumor effects *in vitro* and *in vivo*, even in some resistant forms of cancer, e.g. secondary to BCL-2 overexpression (Decaudin et al., 2002).

3.4. ROS regulators (Table 1)

During oxidative phosphorylation, the respiratory chain transfers electrons via a series of coupled acceptor systems that leads to a reaction with oxygen. Inhibition of the respiratory chain or its overload favors the production of ROS. For example, the redox centres along the respiratory chain can leak electrons to molecular oxygen resulting in the generation of superoxide anion radical. Compounds that generate ROS trigger mitochondrial outer membrane permeabilization and apoptosis via excessive oxidative damage of mitochondria. Such agents comprise thiol-crosslinking agents that cause thiol-oxidation of ANT such as diazenedicarboxylic acid bis-5 N,N-dimethylamide (diamide), bismaleimido-hexane (BMH) and dithiodipyridine (DTDP) (Costantini et al., 2000; Palmera and Wallace, 1997; Petronilli et al., 1994) as well as compounds that undergo futile redox cycles on the respiratory chain such as 2-methyl-1,4-naphthoquinone (menadione), and β -lapachone (ARQ 501). ROS production can also result from the inhibition of antioxidant systems, which is the case for buthionine sulphoximine (BSO) that inhibits GSH synthesis (Maeda et al., 2004) and imexon that depletes the GSH pool due to its thiol-binding activity (Dragovich et al., 2007). The dietary phenyl ethyl isothiocyanates (PEITCs) have been shown to inhibit the GSH antioxidant system by conjugating GSH and by inhibiting glutathione peroxidase, resulting in ROS generation and oxidative damage-mediated mitochondrial apoptosis (Trachootham et al., 2006; Xiao et al., 2006). Mangafodipir belongs to the class of a superoxide dismutase (SOD) mimics that possesses catalase and glutathione reductase activities and has been reported to exert antioxidant functions in normal cells,

while increasing H₂O₂ levels in cancer cells (Alexandre et al., 2006). Some estrogen derivatives, e.g. 2-methoxyestradiol, or the intracellular copper-chelating agent tetrathiomolybdate (ATN-224) have been demonstrated to kill cancer cells by inhibiting SOD, thereby increasing ROS generation (Huang et al., 2000; Juarez et al., 2008). The anticancer effects of arsenic trioxide have been linked, at least in part, to the generation of oxidative stress secondary to irreversible inhibition of thioredoxin reductase (Lu et al., 2007).

3.5. Natural compounds (Table 1)

Betulinic acid (3b, hydroxy-lup-20(29)-en-28-oic acid) is a naturally occurring pentacyclic triterpenoid, which triggers the mitochondrial pathway of apoptosis preferentially in cancer cells (Fulda et al., 1997, 1998a; Liby et al., 2007). Betulinic acid has been shown to directly initiate mitochondrial outer membrane permeabilization and cytochrome *c* release in a cell-free system using isolated mitochondria (Fulda et al., 1998a). These changes were prevented by inhibition of the permeability transition pore complex or over-expression of BCL-2 or BCL-X_L, while it occurred in a caspase-independent manner (Andre et al., 2002; Fulda et al., 1997, 1998b). Betulinic acid regulates the expression of the BCL-2 family protein MCL-1 in a context-dependent fashion with increased expression of MCL-1 in melanoma cells, while MCL-1 levels remained unchanged in squamous cell carcinoma cells (Selzer et al., 2000, 2002; Thurnher et al., 2003). Betulinic acid has been reported to induce apoptosis in a p53-independent manner (Fulda et al., 1997; Fulda and Debatin, 2000; Meng and El-Deiry, 2001; Salti et al., 2001; Selzer et al., 2000; Wick et al., 1999; Zuco et al., 2002).

Resveratrol is a polyphenolic phytoalexin found in grapes and red wine that inhibits mitochondrial ATP synthesis via binding to a hydrophobic pocket in the γ -subunit of F₁-ATPase (Gledhill et al., 2007). To improve the targeting to mitochondria, resveratrol has been coupled to the lipophilic TPP cation that is membrane-permeant (Biasutto et al., 2008). Mitochondria-targeted resveratrol derivatives, i.e. 4-triphenylphosphoniumbutyl-4'-O-resveratrol iodide, have indeed been demonstrated to accumulate in mitochondria (Biasutto et al., 2008).

Furthermore, vitamin E analogues, i.e. α -tocopheryl succinate (α -TOS), can selectively trigger mitochondrial apoptosis in tumor cells (Constantinou et al., 2008). Recent evidence suggests that α -TOS interacts directly with the proximal and distal ubiquinone-binding sites of the respiratory complex II, which causes the displacement of ubiquinone from complex II and ROS generation (Dong et al., 2008). In addition to cancer cells, α -TOS also targets endothelial cells (Dong et al., 2007). The key role of the intrinsic apoptosis pathway in this model was demonstrated in mtDNA-depleted endothelial cells, which were refractory to α -TOS (Dong et al., 2007).

4. Conclusions

Compounds that directly exert their action on mitochondria can potentially bypass primary or acquired resistance mechanisms that frequently exist or develop towards classical chemotherapeutics. In contrast to many conventional anticancer drugs, which rely on upstream signaling cascades to engage the mitochondrial apoptosis pathway, mitochondria-targeting drugs offer the advantage to act independent of these upstream events that are often blocked in cancers. Thus, such mitochondriotoxic compounds are likely effective in otherwise refractory cancers and may overcome some forms of cancer resistance. The exploitation of the knowledge on mitochondria-mediated cell death in cancer cells is anticipated to provide rewarding opportunities for cancer drug discovery, especially if done in a systematic global screening approach. This may

eventually lead to more effective therapies for patients suffering from cancer.

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