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The mitochondrial death pathway: a promising therapeutic target in diseases

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Abstract

The mitochondrial pathway to apoptosis is a major pathway of physiological cell death in vertebrates. The mitochondrial cell death pathway commences when apoptogenic molecules present between the outer and inner mitochondrial membranes are released into the cytosol by mitochondrial outer membrane permeabilization (MOMP). BCL-2 family members are the sentinels of MOMP in the mitochondrial apoptotic pathway; the pro-apoptotic B cell lymphoma (BCL)-2 proteins, BCL-2 associated *x* protein and BCL-2 antagonist killer 1 induce MOMP whereas the anti-apoptotic BCL-2 proteins, BCL-2, BCL-x_L and myeloid cell leukaemia 1 prevent MOMP from occurring. The release of pro-apoptotic factors such as cytochrome *c* from mitochondria leads to formation of a multimeric complex known as the apoptosome and initiates caspase activation cascades. These pathways are important for normal cellular homeostasis and play key roles in the pathogenesis of many diseases. In this review, we will provide a brief overview of the mitochondrial death pathway and focus on a selection of diseases whose pathogenesis involves the mitochondrial death pathway and we will examine the various pharmacological approaches that target this pathway.

Keywords: mitochondrial permeability transition • apoptosis • Bcl-2 • caspases • mitochondrial outer membrane permeabilization

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Introduction

The integrity of mitochondrial function is fundamental to cell life. Mitochondria are involved in many processes essential for cell survival, such as energy production, redox control, calcium homeostasis and a number of metabolic and biosynthetic pathways. Mitochondrial calcium uptake plays a major role in influencing cell signalling and in the regulation of mitochondrial function, while excessive mitochondrial calcium accumulation has been implicated in disease. In addition, mitochondria often play an essential role in cell life and cell death decisions. The mitochondrial genome is prone to damage due to increased exposure to reactive oxygen species (ROS) and inadequate repair mechanisms, and therefore genetic damage can lead to impaired electron transport capability and increased free radical generation. These have been linked to the development of neurodegenerative and cardiovascular diseases. Dysfunctional mitochondrial cell death pathways are central players in a wide range of pathological conditions as diverse as cancer, diabetes, obesity, ischemia/reperfusion injury and neurodegenerative disorders such as Parkinson's (PD) and Alzheimer's diseases. It is a long and growing list. Hence, it is not surprising that unravelling the mitochondrial pathway of cell death is one of the actively pursued research frontiers of biomedicine. As there are already many reviews on the mitochondrial death pathways, we will discuss some of the fundamental attributes of mitochondrial death pathways and focus on the pharmacological approaches that can be used to target these pathways.

Mitochondria and cell death

The mitochondrial 'intrinsic' pathway and the death receptor 'extrinsic' pathway are the two principal pathways leading to apoptosis, both of which converge on caspase activation (Fig. 1) [1–3]. Caspases are a family of cysteine proteases that, upon activation, cleave specific substrates, leading to the demise of the cell. Based on the order of activation in cell death pathways, caspases are divided into two major groups: initiator caspases and executioner caspases [4]. The subset of caspases that cleave substrates to produce the typical biochemical changes associated with apoptosis are known as executioner caspases, which in mammals are caspases-3, -6 and -7 [5]. Executioner caspases have a short pro-domain and are in turn activated by apical initiator caspases (caspases-2, -8, -9 and -10). Initiator caspases possess long prodomains that contain one of the two characteristic protein–protein interaction motifs: the death effector domain or the caspase recruitment domain (CARD) and are involved in interacting with the upstream adapter molecules. The initiator caspases are activated by prodomain-mediated dimerization of the zymogens followed by autocatalytic processing [6]. The initiator caspase for the mitochondrial pathway is caspase-9, whereas the initiator caspases for the death receptor pathway are caspases-8 and -10 [7]. Both pathways share the effector caspases (caspases-3, -6 and -7) which cleave cellular substrates leading to apoptotic cell death. Furthermore, caspase-2 is a long prodomain containing initiator caspase involved in stress-induced apoptosis [8]. A protein complex, named PIDDosome has been shown to mediate the activation of caspase-2 in response to genotoxic stimuli [9]. In addition to caspase-2, the PIDDosome contains the p53-induced protein with a death domain (PIDD) and an adapter protein, called RAIDD. Caspase-2 has also been suggested to induce mitochondrial cytochrome *c* release through a mechanism not yet understood [10, 11]. Caspases-1, -4, -5 and -11 function primarily in the processing of inflammatory cytokines through another proteolytic platform called the inflammosome [12].

Death signals originating from cellular stress, including radiation, oxidative stress, genotoxic stress and chemotherapeutic drugs, activate an intrinsic apoptotic pathway that is mediated largely by the mitochondria. Mitochondrial release of cytochrome *c* into the cytoplasm induces the formation of a multiprotein complex called the apoptosome. Apoptosome contains among others cytochrome *c*, pro-caspase-9 and the adaptor protein Apaf-1 [13] and, supports the activation of caspase-9 through enforced multimerization, which in turn cleaves and activates the effector caspase-3 resulting in the subsequent degradation of cellular death substrates (Fig. 1) [14]. In addition to cytochrome *c*, other mitochondrial proteins released during apoptosis have been identified over the past decade and these include second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP) binding protein with a low pI, (Smac/DIABLO), endonuclease G (Endo G), apoptosis-inducing factor (AIF), HtrA2/Omi [15], as well as Hsp60, Hsp10 and adenylate kinase [16] (Fig. 1). Some intermembrane space (IMS) proteins have essential survival functions in mitochondria and a well-established lethal function in the cytosol.

In the extrinsic pathway, ligation of death receptors (a subset of the tumour necrosis factor [TNF] receptor [TNFR] family, including TNFR-1, CD95, TNF-related apoptosis-inducing ligand receptors-1 and -2 [TRAIL-R1 and -R2] and DR3/TRAMP) causes the recruitment and oligomerization of the adapter molecule Fas-associated death domain (FADD) within the death-inducing signalling complex (DISC) [17]. The oligomerized FADD binds pro-caspases-8 and -10, causing their homodimerization and activation (Fig. 1) [18–22]. Depending on the cell type, activated caspase-8 induces apoptosis by two different signalling pathways [23]. In type I cells, large amounts of active caspase-8 formed at the DISC directly induces activation of pro-caspase-3 independently of mitochondria [24]. In type II cells, probably only a low amount of active caspase-8 is generated that is unable to activate sufficient amounts of pro-caspase-3 directly to a level that would be sufficient to execute apoptosis. In these cells, caspase-8 cleaves the 'Bcl-2 homology (BH) 3-only protein' Bid, generating an active fragment (tBid) that activates the mitochondrial death pathway (Fig. 1) [25, 26]. In this manner, the death signal may be amplified through formation and activation of the apoptosome which contributes to effector caspase activation.

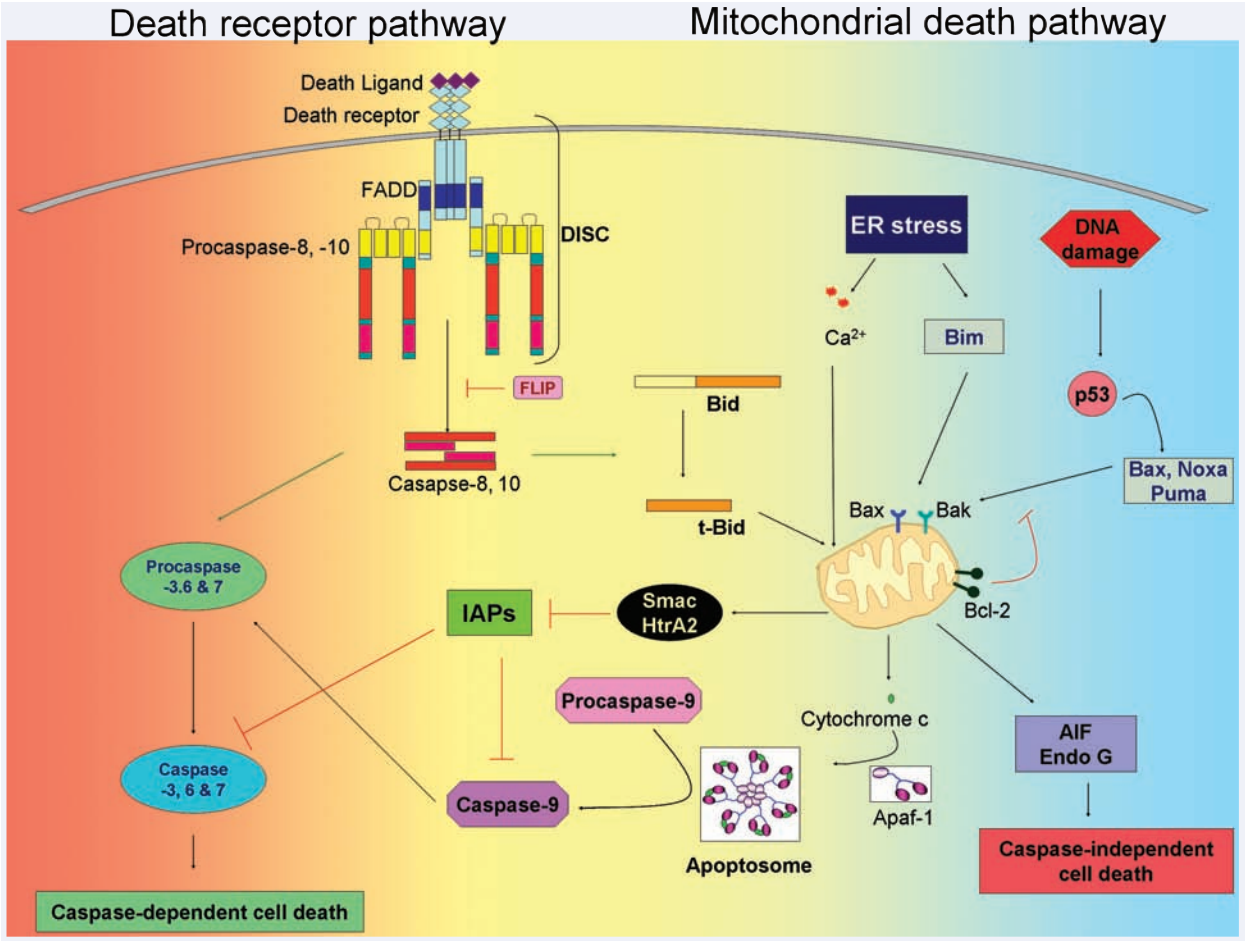

Fig. 1 Schematic representation of the extrinsic and intrinsic apoptotic pathway. In the extrinsic pathway, ligation of receptor to death receptor causes DISC formation leading to caspase-8 activation. In type I cells caspase-8 directly cleaves caspase-3, which starts the death cascade. In type II cells an additional amplification loop is required, which involves tBid-mediated cytochrome c release from mitochondria followed by apoptosome formation. In the intrinsic pathway, stress signals from a variety of insults are sensed by BH3-only pro-apoptotic proteins and communicated to multidomain pro-apoptotic and anti-apoptotic BCL-2 proteins. The functional interplay of the proteins ultimately results in the activation of BAX and BAK at target organelles such as mitochondria and ER, which participate in apoptosis by releasing apoptogenic factors. Cytosolic cytochrome c triggers the formation of apoptosome, followed by activation of caspases-9 and -3. Function of caspase can be modulated on several levels. Activation of caspases at the DISC is inhibited by c-FLIP proteins; activation of effector caspases is inhibited by IAPs (see text for details). Smac/DIABLO and HtrA2/Omi neutralize the inhibition of caspases by IAPs. Smac/DIABLO, HtrA2/Omi, AIF and endo G may also initiate a caspase-independent cell death pathway. Abbreviations: FADD, Fas-associated death domain; DISC, death-inducing signalling complex; BAK, BCL-2 antagonist/killer; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2 protein; IAPs; inhibitor of apoptosis proteins; Apaf-1; apoptosis protease activating factor1 and AIF, apoptosis-inducing factor.

Mitochondrial outer membrane permeabilization (MOMP): point of no return

During apoptosis in vertebrate cells, the process of MOMP appears to represent a point-of-no-return for many cell types as it appears to commit a cell to death regardless of caspase activity [27]. Why, and how, does MOMP commit a cell to die? MOMP is lethal because it leads to the release of caspase-activating molecules, caspase-independent death effectors and metabolic failure in the mitochondria. Majority of cells appear to be committed to die following MOMP because it leads not only to the activation of the well-established caspase-mediated apoptotic pathway, but, should there be a failure of its execution through insufficient caspase activation, a parallel, caspase-independent cell death pathway is set in motion that is controlled by HtrA2/Omi, AIF and Endo G. However, sympathetic neurons that are induced to undergo

apoptosis by withdrawal of nerve growth factors (NGF) can recover upon addition of NGF, even after the release of cytochrome *c*, provided that caspase activation is blocked [28, 29]. This is due to the fact that neuronal cells express low levels of Apaf-1 and, sufficient levels of endogenous caspase inhibitors such as X-linked IAP (XIAP) to block the ability of cytochrome *c* to induce apoptosis [30]. The other factors that contribute to cell demise following MOMP are general decline in mitochondrial function. The most important function of mitochondria is the generation of ATP through the process of oxidative phosphorylation. Dissipation of the $\Delta\Psi$m is a general feature of apoptosis, irrespective of cell type and of the apoptotic stimuli (for a review, see [31]). It has been demonstrated that a reduction in $\Delta\Psi$m follows within minutes after the release of cytochrome *c*, but in the absence of caspase activity, mitochondria can regenerate $\Delta\Psi$m and maintain ATP generation [32]. However, loss of mitochondrial energy production will lead to cell death unless another source of energy is available to the cell. If we are to target the mitochondrial death pathway for disorders connected with apoptosis dysregulation, it will be essential to have a detailed understanding of MOMP and its regulation. The mechanisms of MOMP have been controversial, and there are two principal hypotheses: in the first, MOMP is regulated by the BCL-2 family of proteins, and in the second, by the permeability transition pore (PTP) (Fig. 2) [33].

MOMP by BCL-2 family proteins

The first model considers MOMP as a process that is essentially intrinsic to the outer membrane and controlled by members of the BCL-2 family of proteins to promote or prevent the formation of large, protein permeable pores (Fig. 2). The BCL-2 family of proteins is divided into three groups, based on the presence of BCL-2 homology domains (BH1–4 domains) [34]. The multidomain, anti-apoptotic BCL-2 proteins (*e.g.* BCL-2, BCL-w, BCL-x_L [BCL-2 related gene, long isoform], A1 and MCL-1 [myeloid cell leukaemia 1]] contain BH domains 1–4 and are generally localized to various intracellular membranes such as outer mitochondrial membrane, endoplasmic reticulum (ER) membrane and nuclear membrane [35]. These proteins are thought to function within the apoptotic pathway by directly binding and inhibiting the pro-apoptotic BCL-2 proteins.

The pro-apoptotic members of the family are divided into two groups: the multidomain pro-apoptotic molecules and the BH3-only proteins. The multidomain molecules (*e.g.* BAK [BCL-2 antagonist killer 1] and BAX [BCL-2 associated *x* protein]) contain BH 1–3 domains and are believed to permeabilize the outer mitochondrial membrane by forming oligomeric pores (megachannels) that allow the release of apoptogenic molecules from the intermembrane space [36]. The BH3-only proteins (*e.g.* BAD [BCL-2 antagonist of cell death], BID [BCL-2 interacting domain death agonist], BIK [BCL-2 interacting killer], BIM [BCL-2 interacting mediator of cell death], BMF [BCL-2 modifying factor], bNIP3 [BCL-2/adenovirus E1B 19-KD protein interacting protein 3], HRK [Harakiri], NOXA and PUMA [p53 up-regulated modulator of apoptosis]), function by physical interactions with the other BCL-2 family members either resulting in inhibition of the anti-apoptotic members, or activation of the pro-apoptotic multidomain members [36]. The BH3-only pro-apoptotic proteins are sentinels that sense apoptotic signals and communicate with the multidomain anti-apoptotic and pro-apoptotic molecules to shift their balance towards promotion of death. In what is generally referred to as the 'rheostat' model, cell survival is determined by the balance among the anti-apoptotic BCL-2 family proteins such as MCL-1, BCL-x_L or BCL-2, and pro-apoptotic members [33]. Activation of BAX and BAK during apoptosis involves multiple conformational changes that are accompanied by homo-oligomerization and insertion into the membrane. Oligomerization of BAX and BAK at the outer mitochondrial membrane is a crucial step in MOMP [37]. Indeed, structural and biophysical studies using synthetic lipid bilayers and vesicles support the intrinsic pore-forming capacity of several BCL-2 family proteins, including BAX, BCL-2 and BCL-x_L [38–41]. Studies using vesicles formed from isolated mitochondrial outer membrane (MOM) have shown that BCL-2–family proteins can regulate the permeability of the MOM in the absence of interior structures of the mitochondria; moreover, many features of this process of membrane permeabilization can be reproduced using defined liposomes and recombinant BCL-2–family proteins [38, 39]. Although several BCL-2–family proteins possess ion channel activity in lipid bilayers, only the multidomain pro-apoptotic proteins BAX and BAK can render membranes permeable to cytochrome c or larger macromolecules However, these cell-free systems do not recapitulate all the complexity of the permeabilization process as it occurs in the cell; other proteins of the MOM could modulate or potentiate the function of BAX and BAK. Furthermore, the nature and consequence of protein–protein interactions among members of the BCL-2 family are still not clearly understood. BAX and BAK are thought to homo-oligomerize to induce MOM permeabilization. The BH3-only proteins regulate MOM permeabilization by a combination of release of BAX/BAK from their inhibitory complexes with anti-apoptotic counterparts including BCL-2 and MCL-1 as well as direct activation of BAX/BAK to promote their conformational change, membrane insertion and oligomerization. For a detailed account of regulation of MOMP by BCL-2 family members refer to the recent review by Green and colleagues [33].

MOMP by permeability transition pore

The second prominent model for MOMP is based on a phenomenon known as the mitochondrial permeability transition (MPT) (Fig. 2). PT involves the permeabilization of the inner mitochondrial membrane (IMM) to solutes with a molecular mass of less than 1500 Da, this results in the loss of the mitochondrial membrane potential ($\Delta\Psi$m), mitochondrial swelling, and as observed *in vitro*, rupture of the outer mitochondrial membrane [42]. The pore allowing the release of the matrix solutes is the PT-pore (PTP) which is thought to be composed of at least three proteins, the voltage-dependent anion channel (VDAC) located in the outer

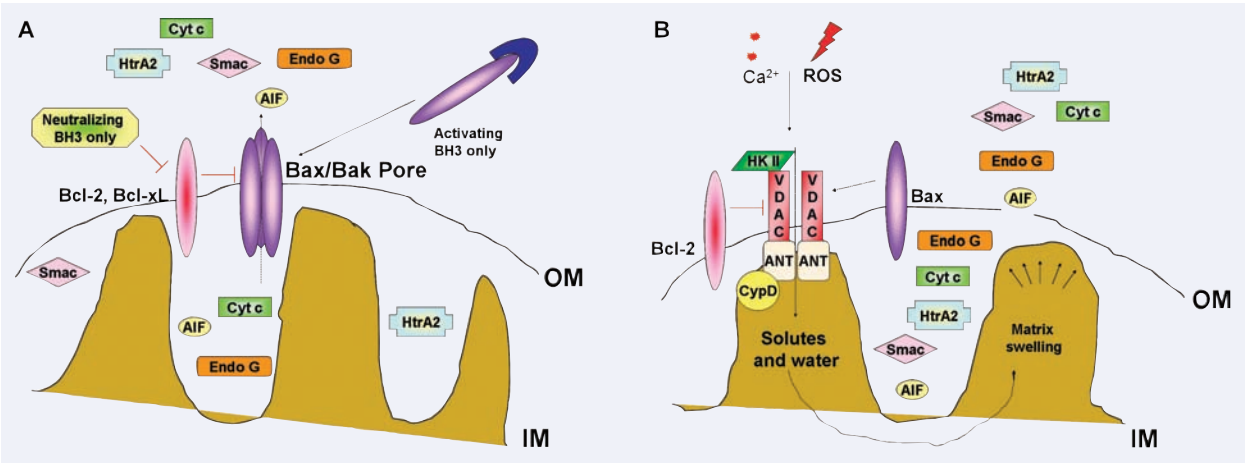

Fig. 2 Molecular mechanisms of MOMP. (**A**) According to first model, the pro-apoptotic members of BCL-2 family BAX and BAK form a multimeric pore across the outer mitochondrial membrane upon activation by BH3-only proteins. This channel mediates the release of apoptogenic factors from IMS. (**B**) According to second model, opening of voltage-gated channel results in mitochondrial matrix swelling and rupture of MOM, releasing IMS proteins in the cytosol. Abbreviations: ANT, adenine nucleotide translocator; BAK, BCL-2 antagonist/killer; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2 protein; BH3, BCL-2 homology domain 3; CypD, cyclophilin D; HK, hexokinase; IM, mitochondrial inner membrane; OM, mitochondrial outer membrane and VDAC, voltage-dependent anion channel.

mitochondrial membrane, the adenine nucleotide translocator (ANT), a specific ATP/ADP transporter located in the IMM and cyclophilin D (CypD), a chaperone with peptidylprolyl isomerase (PPIase) activity. CypD is directly associated with ANT in the matrix of mitochondria [43]. The PTP complex is thought to span the contact sites between the inner and outer mitochondrial membranes.

It has long been thought that VDAC, ANT and CypD play an essential role in PT, but convincing evidence is still lacking to conclude that these are both necessary and sufficient to induce PT. There is evidence to suggest that VDAC is a component of the PT pore [44, 45]. A direct role of the VDAC in PT has been demonstrated in studies using specific anti-VDAC antibodies. Two polyclonal anti-VDAC antibodies, which recognize different VDAC epitopes and inhibit its activity in liposomes, have been shown to inhibit the Ca^{2+}-induced PT, supporting a crucial role for VDAC in this process [46]. However, mitochondria isolated from VDAC1-deficient cells undergo PT normally, suggesting that VDAC1 is not important for this process [47]. However, this result could have been due to compensation for VDAC1 deficiency by other isoforms, including VDAC2 and VDAC3. This question was recently addressed by Baines and colleagues by using fibroblasts from a triple knockout mouse lacking *Vdac1*, *Vdac2* and *Vdac3*. They demonstrated that mitochondria from *Vdac1–Vdac3*-null mice exhibited a Ca^{2+}- and oxidative stress-induced PT that was indistinguishable from wild-type mitochondria. Furthermore, wild-type and *Vdac1–Vdac3* deficient mitochondria and cells exhibited equivalent cytochrome *c* release, caspase cleavage and cell death in response to the pro-death BCL-2 family members BAX and BID [48]. These results suggest that VDACs are dispensable for both PT and BCL-2 family member-driven cell death. Regarding the role of ANT in PT, biochemical isolation and reconstitution of the PTP in liposomes suggested that these protein complexes consist of ANT in the inner membrane and VDAC in the outer membrane [49, 50]. However, liver mitochondria from mice lacking both ANT1 and ANT2 still underwent PT, [51]. This finding suggests that ANT1/2 only play a limited role, if any, in PT or it is possible that deficiency of ANT1/2 was compensated by other channel protein(s). The lack of an important role for ANT in PT corroborates the observation that mitochondria isolated from yeast lacking ANT can undergo PT-like changes, including loss of membrane potential and swelling in response to ethanol [52]. If ANT is not involved in PT, modulation of PT by ANT ligands such as bongkrekic acid or atractyloside suggests a role for a yet unidentified, ANT-like inner membrane channel(s) in PT. The role of CypD in PT was initially suggested by the finding that PT is blocked by cyclosporine A (CsA) a known inhibitor of the PPIase activity of cyclophilins. It has been demonstrated that CypD-deficient mitochondria isolated from the livers of CypD deficient mice do not undergo the CsA-sensitive PT in response to a variety of inducers, including Ca^{2+}, atractyloside and H_2O_2 [53, 54]. However, cells isolated from CypD-deficient mice, such as thymocytes, mouse embryonic fibroblasts (MEFs) and hepatocytes, undergo apoptosis normally in response to various stimuli, including etoposide, staurosporine and TNF-α [53, 55]. The inhibitory effect of CsA on apoptosis might need to be re-evaluated because the concentration of CsA used in these experiments was relatively high which could have had a secondary effect on apoptosis. It is also possible that BAX/BAK megachannels were responsible for the induction of apoptosis in the CypD deficient cells. More studies are needed to elucidate the molecular nature of PT pore complex, especially the

role of the accessory proteins that have been detected associated with the PTP such as hexokinases-I and -II (HK) [56]. This protein interacts directly with VDAC in the outer membrane of mitochondria [57]. Its displacement from VDAC is necessary for BAX binding and cell death induction [58, 59]. Hence, BAX as well as other BCL-2 family members can, depending of the physiological state of the cell, interact with PTP components in the MOM. Finally, there is a strong possibility that the PT pore complex may not be a single entity but that multiple proteins can adopt a pore function. For instance, recent work has implicated the mitochondrial phosphate carrier as a component as well as a potential independent pore involved in PT [60].

In normal conditions the PTP exists in a state of low conductance that may be subject to transient flicker during inositol 1, 3, 4-trisphosphate-mediated Ca^{2+} mobilization from nearby ER sacs. However, when excessive amounts of Ca^{2+} are released from the ER that overloads the mitochondria, the pore transitions to a high-conductance state [61]. This passage from low to high conductance is irreversible and strictly depends on the saturation of the calcium-binding sites of the PTP [61]. The high-conductance conformation allows free diffusion of water and ions between the cytosol and the matrix, causing collapse of $\Delta\Psi m$, uncoupling of oxidative phosphorylation and swelling of the mitochondrial matrix [42, 43]. This may lead to rupture of the MOM and consequent release of IMS proteins (Fig. 2). Despite the lack of evidence for PT as a mechanism of apoptotic death in general, it may be an important therapeutic target for inducing PT-mediated cell death selectively in certain cell types. Regardless of the mechanism, MOMP is a crucial step for many pathways that induce apoptosis and disruption of this event is likely to affect cell fate.

Regulation of MOMP: many ways to skin the cat

Many death signals originating from cellular stress activate an intrinsic pathway of apoptosis which is mediated by the mitochondria. MOMP is an important step in the intrinsic pathway. Multiple distinct signalling pathways converge on MOMP.

Role of calcium in MOMP

Ca^{2+} is one of the key regulators of not only cell survival but also cell death in response to a variety of cellular signals. The pro-apoptotic effects of Ca^{2+} are mediated by a diverse range of Ca^{2+}-sensitive factors that are compartmentalized in various intracellular organelles including the ER and mitochondria [62]. The ER is a complex organelle composed of membrane sheets that enclose the nuclear envelope and an elaborate interconnected tubular network in the cytosol. The ER can be an initiator of apoptosis when accumulation of unfolded proteins or inhibition of the ER-Golgi transport results in the ER stress response [63]. Ca^{2+}-dependent stimuli at the ER induce apoptosis through a mitochondrial pathway including mitochondrial dysfunction, cytochrome *c* release and caspase activation [64]. Both BCL-2 and BAX can localize to the ER and they can modulate Ca^{2+} fluxes [65]. Overexpressed BCL-2 reduces resting ER Ca^{2+} concentration and the extent of capacitative Ca^{2+} entry, pointing to a specific role of BCL-2 at the ER in the control of cell death or setting the threshold of sensitivity to pro-apoptotic stimuli [66]. When large quantities of Ca^{2+} accumulate in the mitochondrial matrix, Ca^{2+} interacts with CypD and other components of the PTP to induce opening of the pore [61, 67]. Furthermore, the rise in mitochondrial Ca^{2+} stimulates the generation of ROS and free fatty acids that also promote the opening of the PTP [68, 69]. Recent studies have demonstrated that the regulated process of mitochondrial fusion and fission controls the spatiotemporal properties of mitochondrial Ca^{2+} responses and thus, the physiological and pathological consequences of increased Ca^{2+} concentration in the cytosol and Ca^{2+} taken up by mitochondria [70]. Two proteins involved in the mitochondrial fission machinery, Drp1 and hFis1, have an antagonistic effect on BCL-2 [70]. Drp1, with the assistance of hFis1, sensitizes cells to PT by reducing mitochondrial Ca^{2+} retention.

ROS-induced MOMP

All mammals use O_2 for energy production. Oxidation is the loss of an electron by a substance. Under normal metabolic conditions, the electron-transporting complexes I, II, III and IV of the mitochondrial respiratory chain plus the non-redox H^+-translocating complex, the ATP synthase (also called complex V, F_0F_1-ATP synthase) together with co-enzyme Q and cytochrome *c* carry out the process of terminal oxidation. The respiratory enzyme complexes transfer electrons from the reducing equivalents NADH or $FADH_2$ to O_2, while transporting protons across the IMM. The total proton-motive force across the IMM is the sum of the mitochondrial membrane electrical potential and the H^+-concentration gradient (ΔpH^+). This proton-motive force is used to drive protons from the intermembrane space into the matrix through the ATP synthase. The ATP synthase uses the energy of the H^+ flow to synthesize ATP from ADP and Pi. The energy of the electrochemical proton gradient is also used to import proteins into the mitochondria and to regulate metabolite transport across the mitochondrial membrane. A small percentage of the total O_2 consumed by terminal oxidation even in healthy tissues becomes ROS, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) [71]. This ROS production occurs primarily in complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome *c* reductase) and the accumulation of ROS has been shown to occur predominantly in the IMS [72, 73]. ROS can attack DNA, proteins, lipids and carbohydrates and cause DNA strand breaks, protein oxidation and lipid peroxidation. Polyunsaturated fatty acid residues in phospholipids are especially sensitive to oxidation [74]. Mitochondrial lipid peroxidation products can impair the barrier function of membranes by either directly interacting with the protein and/or indirectly interacting with the lipid moieties in the membrane [75]. Oxidative damage to DNA causes modification of the purine and pyrimidine bases and can result single or double strand–breaks [76]. mtDNA is especially susceptible to damage by ROS owing to its close proximity to the electron transport chain, the major locus for free-radical production and the lack of protective histones. The amino acids tyrosine, histidine, arginine, lysine

and proline are particularly vulnerable to ROS modification, which can lead to gain or loss of receptor activity, enzyme function and signal transduction pathways [77, 78]. ROS can lead to oxidative damage and inactivation of the iron-sulphur (Fe-S) proteins in the mitochondria such as aconitases, complex I NADH dehydrogenase and succinate dehydrogenase [79]. Oxidative stress has been shown to markedly sensitize mitochondria toward MOMP [80]. Oxidative stress is a key factor in many diseases, including neurodegenerative diseases such as PD. For example, in models of PD, inhibition of complex I with MPP^+ induces cell death and 6-hydroxydopamine causes oxidative stress that is linked to MOMP and release of IMS proteins including cytochrome *c* and Smac/DIABLO [81, 82]. Similarly, cardiolipin, an anionic phospholipid located in the IMM and therefore exposed to the oxidizing environment of the IMS [73], can be readily oxidized, which reduces its capacity to bind cytochrome *c* and increases the level of soluble cytochrome *c* within the IMS [83, 84].

Role of caspases in MOMP

Previous studies have shown that incubation of isolated mitochondria with recombinant human caspases promotes MOM permeabilization and release of cytochrome *c* and Smac/DIABLO into the cytosol [85, 86]. Pro-caspase-2 efficiently inserts into the mitochondrial membranes and triggers the release of cytochrome *c* bound to cardiolipin [87]. One study has shown that active caspase-3 can enter the mitochondria and cleave NDUF1, a component of complex I of the respiratory chain. Cleavage of NDUF1 by caspase-3 reduced electron transport by complexes I and II by up to 88% and 94%, respectively [88]. Mutation of the caspase-3 cleavage site in NDUF1 could preserve mitochondrial functions during apoptosis and delay plasma membrane events associated with caspase activation, including loss of plasma membrane integrity and externalization of phosphatidylserine. Interestingly, treatment with zVAD-fmk, a pan-caspase inhibitor, preserved electron transport chain functionality but failed to inhibit cytochrome *c* release. Additional studies on intact cells and isolated mitochondria have shown that zVAD-fmk was able to inhibit the release of Smac/DIABLO, HtrA2/Omi, AIF and Endo G, but could not inhibit the release of cytochrome *c* [89]. Embryonic fibroblasts and thymocytes derived from mice lacking both caspases-3 and -7 exhibited resistance to drugs that induce the intrinsic (mitochondrial) and extrinsic (membrane death receptor) pathways to apoptosis [90]. In all conditions studied, the cells displayed a pronounced delay in cytochrome *c* release and translocation of BAX to the outer membrane. The mitochondrial membrane potential was unaffected. Overall, the results of this study suggest that caspases-3 and -7 are important mediators for mitochondrial events in apoptosis [90]. However, it remains unclear how a cytosolic protease can cross both mitochondrial membranes to cleave a matrix-exposed subunit embedded within IMM.

Other regulators of MOMP

The tumour suppressor p53 acts, in part, to induce apoptosis by inducing expression of the BH3-only protein, PUMA and PUMA-deficient cells display a resistance to p53 mediated apoptosis. However, p53 can trigger MOMP and apoptosis in the absence of transcription, and this can occur through direct activation of BAX or BAK or through sequestration of BCL-2 and BCL-x_L to block their activity [91]. Resolving the role of p53 at the mitochondria *versus* its role in the nucleus as a transcription factor will be important in understanding the apoptotic function of p53. An emerging theme is one of nuclear proteins and nuclear factors functioning in the cytosol through direct interactions with BCL-2 family proteins. Ku70, involved in DNA repair, can inhibit BAX [92]. Another nuclear protein, TR3, binds BCL-2 and perhaps promotes MOMP through this interaction [93]. Histone 1.2 released from the nucleus upon X-ray–induced DNA damage can trigger MOMP perhaps through an interaction with BCL-2 family members [94]. Finally, ADP-ribose polymers which are formed extensively in response to DNA damage and are released from the nucleus have been shown to mediate BAX-dependent MOMP [95].

Mitochondrial IMS: poison cabinet

Irrespective of its mechanisms, MOMP can seal the point of no return for the cell by the release of several apoptogenic molecules such as cytochrome *c*, Smac/DIABLO, Endo G, AIF and HtrA2/Omi [15]. Some of these proteins have cytotoxic activities due to caspase-dependent and -independent processes.

The release of cytochrome *c* into the cytoplasm, in the presence of dATP induces the formation of the Apaf-1-containing macromolecular platform called the apoptosome that activates caspase-9 [13]. Mature caspase-9 remains bound to the apoptosome, recruiting and activating executioner caspase-3 and/or caspase-7 [14]. The release of cytochrome *c* has often been considered the point of no return in cell death since cytochrome *c* participates in the mitochondrial electron-transport chain, using its haem group as a redox intermediate to shuttle electrons between complex III and complex IV and is responsible for the generation of the $\Delta\Psi m$ [96]. The loss of mitochondrial cytochrome *c* has also been associated with enhanced ROS formation [97]. Cytochrome *c* has two distinct functions in the cell: (*i*) under normal physiological conditions it is required within mitochondria for maintenance of mitochondrial electron transport chain and (*ii*) under apoptotic conditions it is released from mitochondria and has an apoptotic role in the cytoplasm. Thus, the release of cytochrome *c* has a strong impact on cell fate determination because in addition to the activation of the caspase cascade, in the absence of cytochrome *c*, mitochondrial respiration and the control of ROS formation are impaired [98].

The absence of the apoptosome inhibits the execution of the apoptotic process in many systems. Genetic studies have confirmed the importance of the apoptosome in the intrinsic apoptotic pathway. Apaf-1 null and caspase-9 null mice display brain malformation due to impaired neuronal apoptosis [99–102]. Lys72 of cytochrome *c* is essential for the stability of the interaction

between cytochrome *c* and Apaf-1. Lys72Ala knock-in mice recapitulate the embryonic lethality and brain developmental defects of Apaf-1 knockout and caspase-9 knockout mice [103]. Apaf-1 failed to oligomerize in Lys72Ala-cytochrome *c*-mutant cells following an apoptotic stimulus. Mouse embryonic fibroblasts from Lys72Ala-mutant mice failed to activate caspases-3 and -9 and thus were resistant to several apoptotic stimuli [103]. The phenotypic similarity between the Lys72Ala knock-in mice and Apaf-1- and caspase-9-knockout mice suggests that these molecules are equally important for the apoptotic function of mitochondria in cytochrome *c* release, apoptosome formation and caspase-9 activation. Several studies based on cryo-electron microscopy have been carried out to identify the apoptosome structure. They have revealed a wheel-like complex made up of seven Apaf-1 molecules [104–106]. In the apoptosome, the CARD domains are located at the central hub, where pro-caspase-9 binds, whereas the WD40 repeats form Y-shaped tails at the end of the spokes.

Many factors are involved in apoptosome formation and regulation [107]. Heat shock protein 90 and 70, induced by several toxic stimuli; prevent apoptosome formation by binding to Apaf-1 and inhibiting its oligomerization [108, 109]. Inhibition of cytochrome c release, *e.g.* by Hsp27, prevents induction of apoptosis in a model of PD [81]. Tumour up-regulated CARD-containing antagonist of caspase-9 binds pro-caspase-9 by its CARD domain, thereby preventing its interaction with Apaf-1 [110, 111]. Phosphorylation of caspase-9 by extracellular signal-regulated kinase (ERK) 1/2 at Threonine-125 prevents proteolytic processing and activation of pro-caspase-9 without affecting its recruitment to apoptosome [112]. The oncoprotein, prothymosin-A, inhibits the formation of the apoptosome, while tumour suppressor putative HLA-DR-associated proteins facilitate apoptosome-mediated pro-caspase-9 activation [113].

IAPs are involved in the regulation of apoptosome function and are characterized by the presence of the baculoviral IAP repeat (BIR) domain [114]. XIAP regulates activity of initiator and effector caspases through different mechanisms [115]. An active effector caspase, such as caspase-7, exists as a homodimer and contains two active sites, one on each monomer. The active site of caspase-7 can be tightly bound by a short peptide sequence in the linker region preceding the BIR2 domain of XIAP [116]. This binding blocks substrate entry resulting in the inhibition of caspase-7 [116]. An initiator caspase, such as caspase-9 exists as monomer and BIR3 of XIAP interacts with caspase-9 monomer, thereby trapping caspase-9 in its monomeric state [117]. XIAP-mediated steric hindrance thereby prevents homodimerization-induced activation of caspase-9 and retains a caspase-9 monomer in its inactive state [117]. The Ring domain which has an E3 ubiquitin ligase activity promotes ubiquitination and subsequent degradation of pro-caspases-3 and -9 [118, 119]. However, recent studies have revealed that unlike XIAP, other IAPs are unable to inhibit caspases at physiological concentrations. In particular, cIAP1 and cIAP2 are able to bind, but not to inhibit caspases, probably because of the lack of critical amino acids required for caspase inhibition [120]. However, the two mitochondrial IMS proteins namely Smac/DIABLO and HtrA2/OMI appear to interact with and inhibit IAPs [121–125]. All IAP binding proteins share a conserved four-residue IAP binding motif (IBM) at their N-terminus that allows them to bind IAPs. Smac/DIABLO and HtrA2/Omi are nuclear encoded and synthesized as precursor proteins of 239 and 458 amino acid residues containing an N-terminal mitochondrial localization signal (MLS) [122–125]. The amino acid residues from 1–55 in Smac/DIABLO and 1–133 in HtrA2/Omi comprise the MLS. Upon mitochondrial import, the MLS is removed by proteolysis, exposing the IBM at the N-terminus of mature Smac/DIABLO and HtrA2/Omi [122–125]. Smac/DIABLO knockout mice are viable, grow normally into adulthood and do not exhibit any histological abnormalities [126]. HtrA2/Omi, has an important role within the mitochondria and the proteolytic activity of HtrA2/Omi is required to maintain mitochondrial function. The binding of Smac/DIABLO and HtrA2/Omi to IAPs can promote cell death by releasing the inhibitory activity of IAPs on caspase activation and caspase activities. Unlike Smac/DIABLO, HtrA2/Omi possesses serine protease in addition to IAP binding property and it can promote cell death independent of the cellular caspase activity. Indeed expression of cytosolic protease active HtrA2/Omi has been shown to induce cell death in Apaf $^{-/-}$ and caspase-9$^{-/-}$ mouse embryo fibroblasts [123]. Further inhibition of cellular caspases with zVAD-FMK, XIAP, XIAP-BIR3, or dominant negative caspase-9 does not affect the ability of cytosolic protease active HtrA2/Omi to kill the cells [123]. c-IAP1 and c-IAP2 have been shown to interact with (TNF-α receptor associated factors) TRAF molecules, the proteins that mediate signal transduction pathway induced by TNF receptor-like proteins [127, 128]. It remains to be elucidated whether Smac/DIABLO and HtrA2/Omi could regulate TNF signalling by removing IAPs from TRAFs.

The neurodegenerative phenotype of mice lacking HtrA2/Omi or expressing the enzymatically inactive protein as in Mnd2 mutant mice indicates that the protease activity of HtrA2/Omi has a protective role in the mitochondria of neuronal cells [129, 130]. The phenotype of HtrA2/Omi deficient mice and Mnd2 mutant mice resemble the clinical manifestations of PD. Moreover, single nucleotide polymorphisms in the HtrA2/Omi gene that cause missense mutations (A141S and G399S) and affect the enzymatic activity of the protease have been associated with the development of PD in human beings [131]. The protease activity of HtrA2/Omi is up-regulated in the presence of peptides corresponding to carboxy terminus of presenilin-1 that bind to their PDZ domains suggesting a link between HtrA2/Omi and Alzheimer's disease [132].

During apoptotic conditions following OMM permeabilization, Endo G and AIF are released from mitochondria. However, release of Endo G and AIF is compromised in Apaf-1 deficient cells and can also be inhibited by broad caspase inhibitor zVAD-fmk. These observations suggest that release of Endo G and AIF into the cytosol requires caspase activation downstream of MOMP [89]. Once in the cytosol they translocate to the nucleus and affect chromatin in a caspase-independent way [15]. Endo G was identified by mass spectrometry in the supernatant fraction of tBid-treated mouse liver mitochondria, as a protein that induces caspase-independent DNA fragmentation in purified HeLa nuclei [133]. Although early reports suggested that the knock-out of Endo G is

embryonic lethal, it has been recently demonstrated that this phenotype was due to the disruption of an adjacent gene, and that Endo G knock-out mice can develop to adulthood without obvious abnormalities [134, 135]. AIF is an NADH oxidase with a local redox activity that is required for the correct assembly and/or function of the respiratory chain. Upon MMP, AIF is released into the cytosol and translocates to the nucleus, where it promotes chromatin condensation and DNA degradation independently of caspases [136, 137]. Like HtrA2/Omi, AIF also plays an important role in mitochondrial homeostasis in healthy cells. Harlequin mutant mice, that express only 20% of the AIF levels of their WT counterpart due to a retroviral insertion in the first intron of the AIF gene develop neurodegeneration (with ataxia owing to cerebellar atrophia) and blindness because of retinal degeneration [138]. Muscle-specific knockout of AIF leads to severe mitochondrial dysfunction, skeletal muscle atrophy and dilated cardiomyopathy [139]. However, as with AIF null mice, distinguishing between the true apoptotic role and the vital mitochondrial function of AIF remains a challenge.

Mitochondrial pathway of cell death and disease pathogenesis

It is evident that defects in the apoptotic machinery or aberrations in apoptotic responses to death signals can contribute to various human diseases. MOMP-dependent apoptosis is involved in major pathologies, with far-reaching medical and pharmaceutical implications. Mitochondria are involved in several known human diseases, including ischemia-reperfusion injury of the heart, ischemic and traumatic brain damage, muscular dystrophy caused by collagen VI deficiency, amyotrophic lateral sclerosis (ALS), acetaminophen-induced hepatotoxicity, hepatocarcinogenesis induced by 2-acetylaminofluorene and death receptor induced hepatitis [140].

Ischemia/reperfusion

Ischemia is the process whereby the blood supply of an organ is interrupted and results in cell death, most likely due to disruption of cellular energy metabolism such as loss of ATP. Injury following reoxygenation may be due in part to the formation of ROS [141] and mitochondrial calcium overload both of which, have the capacity to induce opening of the MTP pore (permeability transition pore complex [PTPC]). PTPC opening has been observed in many models of ischemia/reperfusion [142], and cytochrome c release has been observed following reperfusion of the ischemic brain [143]. In many cases, the cell death is preventable by agents that act at the level of the mitochondria. For example, treatments that are known to prevent PTPC opening seem to protect tissue from damage [144]. The majority of available evidence concerns the immunosuppressive compound CsA, thought to act by binding to the CypD component of the PTPC (discussed later). Therefore, mitochondrial apoptosis pathway may a play an important role in reperfusion injury. By preventing mitochondrial cell death, the damage can be minimized and greater organ function retained.

Neurodegenerative disorders

Apoptotic pathways and specifically MOMP is involved in the pathophysiology of widespread and devastating neurodegenerative disorders (reviewed in [145]). Imaging studies of postmortem brain tissue have revealed apoptotic nuclei in patients with Alzheimer's disease [146], ALS [147], Huntington's disease (HD) [148] and PD [149].

Alzheimer's disease is characterized by a general decrease in cognitive ability, especially short-term memory [150]. In addition to postmortem nuclei suggestive of apoptosis, certain proteins with apoptotic potential were modulated in the brains of Alzheimer's patients [150]. BCL-2 expression was decreased and BAX was up-regulated in neurons with neurofibrillary tangles [151]. Reduced complexes II, III and IV activity is seen postmortem in AD brains [152]. Presenilins are genes that were originally isolated due to their ability to induce an early-onset form of familial AD. Mutations in presenilin-1 (PS-1) increase neuronal sensitivity to ischemia, hyperosmotic shock and calcium overload [153]. PS-1 and PS-2 have been demonstrated to bind anti-apoptotic BCL-x_L and at least PS-2 can modulate mitochondrial apoptosis, presumably through inactivation of anti-apoptotic BCL-2 family members [154].

PD is characterized clinically by bradykinesia, rigidity and tremor, which correlates histologically with a loss of dopaminergic neurons in the substantia nigra pars compacta [155]. Impairment of complex I and subsequent oxidative stress have been widely demonstrated in experimental models of PD and in postmortem PD samples [156]. In neuronal culture, dopamine (DA) has been shown to result in apoptotic cell death in a dose-dependent manner [157]. Moreover, BCL-2 overexpression, seen *in vivo* in surviving DA neurons in the substantia nigra [158], is also able *in vitro* to abrogate DA-induced cell death, suggesting mitochondrial involvement in the death process [157].

HD is a dominantly inherited neuromotor disease characterized by involuntary, hyperkinetic movements, retardation of voluntary movements and cognitive impairment [159]. The disease is caused by expanded CAG repeats within the huntingtin gene, and degeneration of neurons in specific brain regions [159]. The importance of apoptosis in HD was suggested by experiments wherein expression of full length human huntingtin containing 48 or 89 CAG repeats: (a) caused JNK activation and apoptosis in a rat hippocampal neuronal cell line (HN33) and (b) demonstrated clinical disease in transgenic mice [160]. Mitochondrial depolarization and apoptosis was blocked by CsA treatment, implicating involvement of the ANT subunit of the PTPC. Further implicating mitochondrial involvement in HD, a primate model of HD is generated by 3-nitropropionic acid, a respiratory chain complex II poison [161].

ALS is a disease characterized by selective and progressive degeneration of upper and lower motor neurons, progressive muscle weakness and paralysis [162]. The pathophysiology of

~20% of familial ALS is known to result from mutations of the superoxide dismutase-1 gene, whose overexpression in neuronal cells is sufficient to trigger apoptosis, and is inhibited by BCL-2 and caspase inhibitors [163, 164]. A proportion of SOD1 has been shown to localized to mitochondrial intermembrane space [165, 166] and matrix [167] These findings support the hypothesis that mutant SOD1 may damage mitochondrial function and integrity directly, from inside the mitochondria. Caspase inhibition and BCL-2 expression are known to delay the onset and mortality of disease in mouse models of ALS [168, 169].

Cancer

Tumorigenesis can be aided by defects in apoptotic pathways enhancing cell survival and transformation, by giving the cells more time to accumulate genetic alterations that deregulate proliferation and provide growth advantage. The cancerous cells must evade or blunt their apoptotic response to survive and form tumours. Genes encoding important players of mitochondrial apoptosis are involved in the development of cancer. The two best examples are the p53 tumour suppressor and members of the BCL-2 protein family. The p53 tumour suppressor gene is mutated in the majority of human cancers [170], possibly owing to the diverse roles it serves in the cell. As 'guardian' of genome integrity, p53 is capable of inducing cell cycle arrest and senescence as well as apoptosis [171]. $p53^{-/-}$ mice were predisposed to tumour development [172], and there was less evidence of apoptosis seen in situ in developed tumours. p53 is capable of inducing apoptosis through a variety of mechanisms, at least three of which involve the mitochondrial pathway [173]. First, the transcription of several pro-apoptotic genes, including the BCL-2 family members BAX, PUMA and NOXA, have been shown to be induced by p53 following genetic damage [174–176]. Second, there are reports suggesting that p53 induces the production of ROS that can stimulate mitochondrial apoptosis [177, 178]. Third, p53 can act through the permeabilization of the mitochondrial outer membrane, and the release of intermembrane proteins [91, 179].

Mitochondrial encephalomyopathies

Mitochondrial encephalomyopathies are a set of clinically diverse diseases that result from either inherited or spontaneous mutations in mtDNA which lead to altered function of the proteins or RNA molecules that normally reside in mitochondria [180]. Most of these mutations disrupt members of the electron transport chain, thus leading to defects in oxidative phosphorylation. The varied clinical presentations evident in those with the same mutation, and the varying mutations that present with the same clinical syndrome suggest that both genetic background and extra-genetic factors [181, 182] play a role in disease pathology. In addition, different mitochondria within the same cell can genetically complement one another, and thus the phenotype resulting from mtDNA mutations depends also on the percentage of mitochondria in a given cell that carry the mutation [182]. A recent study indicated that apoptotic cell death was dramatically increased in muscle biopsies from patients carrying mtDNA mutations in genes encoding bioenergetic proteins relative to those carrying mutations in structural genes [183]. Defects in oxidative phosphorylation have been demonstrated in many other neurodegenerative diseases, suggesting a mitochondrial role. These include: (*i*) Leber's optic atrophy [184] and (*ii*) idiopathic dystonia [185], both with defects in respiratory chain complex I and (*iii*) some forms of hereditary spastic paraplegia, caused by the mitochondrial metalloproteinase paraplegin [186].

Others

Many viruses have acquired the capacity to intercept or to activate the principal signal transduction pathways leading to cell death [187, 188]. For example, certain viruses kill the host cell by inducing MOMP, while others prevent MOMP to allow propagation of the virus. The HIV protein, viral accessory protein R (Vpr) induces MOMP. The amino acids 52–96 of Vpr directly interacts with ANT and VDAC, thereby triggering MMP associated with $\Delta\Psi_m$ loss, IMS proteins release, and caspase cascade activation [189]. When added *in vitro* to purified mouse liver mitochondria, a synthetic Vpr-derived peptide ($Vpr_{52–96}$) induced large amplitude swelling. This effect could be prevented by BCL-2 as well as by pharmacological agents targeting ANT or VDAC [189]. Importantly, mutation in one of the arginine residues (R77Q) that is required for the interaction of Vpr with ANT [190] is associated with a reduced risk of developing AIDS. In contrast, the cytomegalovirus encodes several proteins that subvert host cell functions in order to favour viral propagation [191]. One of the best characterized among these factors is viral mitochondria-localized inhibitor of apoptosis (vMIA). vMIA has been shown to inhibit apoptosis triggered by different stimuli, including ligation of death receptors and exposure to cytotoxic agents. vMIA exerts its anti-apoptotic activity predominantly by inhibiting MMP at the level of mitochondria [191]. Several oncogenic viruses encode MOMP inhibitory proteins and in human beings, such proteins may contribute to the formation of virally-induced lymphomas or Kaposi's sarcoma. Open reading frame (ORF)16 of human herpesvirus 8 encodes the so-called Kaposi sarcoma–associated BCL-2, a polypeptide of 175 residues that shares limited (15–20%) overall sequence identity with other BCL-2 family proteins [192]. HVS ORF16 has been shown to interact with BAX and BAK to inhibit virus-induced apoptosis [193].

Therapeutic strategies that promote MOMP and cell death

There are a number of drugs that are aimed to induce apoptosis by targeting components of the mitochondrial pathway to induce MOMP. A summary of the agents that promote MOMP and cell death can be found in Table 1.

Table 1 Drugs promoting the mitochondrial membrane permeabilization

Target	Drug	Type of compound	Remarks
Anti-apoptotic BCL-2 proteins	Oblimersen Sodium	Antisense BCL-2 oligonucleotide	Down-regulation of BCL-2 protein level phase III clinical trial in chronic lymphocytic leukaemia and melanoma
	Antimicyn A	BH3 mimetic	Pan inhibitor of anti-apoptotic BCL-2 proteins
	Gossypol	BH3 mimetic	Pan inhibitor of anti-apoptotic BCL-2 proteins phase I/II clinical trials in metastatic breast cancer and glioblastome multiforme
	Porpullogallin	BH3 mimetic	Pan inhibitor of anti-apoptotic BCL-2 proteins
	Chelerythrine	BH3 mimetic	Inhibits BCL-x_L by binding at the BH groove
	Sanguinarine	BH3 mimetic	Inhibits BCL-x_L by binding at the BH1 region
	ABT-737	BH3 mimetic	Inhibits BCL-2, BCL-x_L and BCL-w
Mitochondria	TEAM-VP	Chimeric peptide	Induces MMP *via* VDAC and ANT interaction
	Mastoparan	Peptide	Induces MMP *via* VDAC interaction
	pCoxIV, 3-22aa	Peptide	Induces MMP *via* CoxIV interaction
	(KLAKLAK)2	Peptide	Direct inducer of MMP
	HXK2VBD	Peptide	Sensitizes to BAX-dependent apoptotis *via* inhibition of Hexokinase (HK) II/VDAC interaction
	2-chloro-2′- deoxyadenosine; 2-chloro-2′-ara-fluorodeoxyadenosine	Deoxyadenosine analogue	Induces opening MPT pores
	Jasmonates	Jasmonic acid	Induces swelling in mitochondria in a PTPC-mediated manner
	Peripheral-type benzodiazepine receptor ligands	Small molecules	Induces MMP
	Lonidamine	Indazole-carboxylic acid	Inducer of MMP through ANT conformational change phase II/III clinical trials for metastatic breast, non-small cell lung, ovarian cancer and glioblastoma multiforme
	Lamellarins	Marine pyrrole alkaloids	Disruption of the inner mitochondrial transmembrane potential in a MPT-dependent manner
	Arsenite	Small molecule	Inducer of MPT pores phase II clinical trials for multiple myeloma
	Avicins	Triterpenoid saponins	Inducers of MMP
	Pyridinium derivative F16	Cationic lipophilic	Accumultes in mitochordria; inducer of MMP
	MKT-077	Cationic lipophilic	Accumulates in mitochondira; inducer of MMP; effects on mitochondrial DNA
	Verteporfm	Photo-activable agent	Inducer of MMP
	CM oro methyl-X-ro s amine	Photo-activable agent	Inducer of mitochondrial depolarization and swelling
	Hypericin	Photo-activable agent	Induces detachment of HKs from mitochondria
	Pc4	Photo-activable agent	Induces degradation of BCL-2

VIMP: mitochondrial membane permeabilization and MPT: mitochondrial permeability transition.

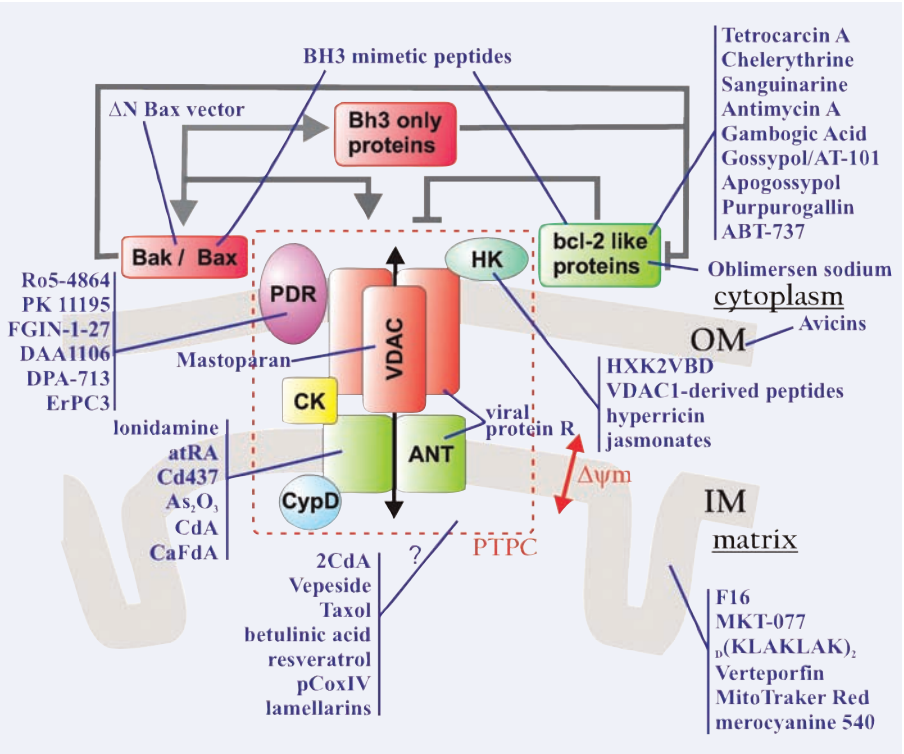

Fig. 3 Therapeutic agents acting on mitochondria to promote cell death. Pharmacological inducers of cell death and their target molecules are shown. Please refer to the text for additional detail. Abbreviations: ANT, adenine nucleotide translocator; BAK, BCL-2 antagonist/killer; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2 protein; BH3, BCL-2 homology domain 3; CK, creatine kinase; CypD, cyclophilin D; HK, hexokinase; IM, mitochondrial inner membrane; OM, mitochondrial outer membrane; PBR, peripheral-type benzodiazepine receptor; PTPC, permeability transition pore complex and VDAC, voltage-dependent anion channel.

Targeting the BCL-2 family

The ratio of the levels of pro-survival and pro-apoptotic members of the BCL-2 protein family is thought to be an important regulatory factor for determining mitochondrial integrity and regulates the sensitivity of mammalian cells to apoptotic stimuli. This information has, in the early days of apoptosis research, led to the consideration of BCL-2 family members as possible therapeutic targets for diseases with deregulated apoptosis (Fig. 3) [194–197]. The following section describes various approaches developed around the members of BCL-2 family.

BCL-2 antisense-based strategies

BCL-2 (B-cell lymphoma 2) is an oncoprotein, which was originally identified as the t(14;18) chromosomal translocation found in the majority of human follicular lymphomas [194]. BCL-2 plays a critical role in inhibiting mitochondria-dependent apoptotic cell death. Its pathologic over-expression observed in many tumour types identified BCL-2 as a possible drug target in the early 1990s. Because BCL-2 is an intracellular protein lacking intrinsic catalytic function, its inhibition by neutralizing antibodies or small molecule drugs are not viable options. On the other hand, in several preclinical and clinical studies antisense BCL-2 therapy combined with chemotherapy has proven to be beneficial in various tumour types. Antisense BCL-2 (AS BCL-2; G3139, oblimersen sodium, Genasense, Genta, Berkeley Heights, NJ, UDA) is an 18-bp phosphorothioate oligonucleotide targeting the first six codons of BCL-2 mRNA. In preclinical studies, the treatment with antisense BCL-2 in combination with an anticancer drug decreased BCL-2 expression and enhanced the mitochondria-dependent apoptosis pathway leading to cell death [198–200]. Oblimersen sodium has advanced through clinical trials, including phase III with tolerable side effects [201] (www.clinicaltrial.com). However, the demonstration of efficacy of antisense BCL-2 in those trials has been variable. In chronic lymphocytic leukaemia, combined oblimersen, fludarabine and cyclophosphamide showed improved major responses in patients, whereas oblimersen and dacarbazine combination therapy in patients with metastatic melanoma or oblimersen and dexamethasone combination therapy in patient with myeloma provided no significant benefit in overall survival. In addition, in these clinical trials the down-regulation of BCL-2 was not observed with any high frequency in tumour cells [201]. In fact, in addition to its antisense effect, several non-antisense effects need to be considered when analyzing the therapeutic efficacy of antisense BCL-2 observed in clinical trials. Production of ROS, interferon (IFN)-γ production and immunostimulatory action through a cytosine-phosphate-guanosine motif in the antisense oligodeoxynucleotides might contribute to the antitumour effects [200, 202]. Thus, the efficacy of BCL-2 antisense strategies has not been overwhelming, and approval of oblimersen as an anticancer agent remains in doubt.

BAX-delivery vector

BAX is one of the pro-apoptotic factors that belong to the BCL-2 family, and its overexpression leads to apoptosis in a wide variety of mammalian cells [203]. BAX, which normally resides in the cytoplasm, translocates to mitochondria in response to apoptotic stimuli, promotes MOMP and elicits the release of pro-apoptotic factors from the intermembrane space [33]. Adenovirus-mediated BAX overexpression is capable of inducing cell death *in vitro* and *in vivo* by engaging the mitochondrial pathway [204–206]. Through its BH3 domain, BAX forms homodimers to promote apoptosis but also forms heterodimers with BCL-2 and BCL-x_L, which silences its function. Likewise, adenovirus-mediated gene delivery of an amino-terminal truncated version of BAX (ΔN BAX: corresponding to amino acid 112–192 of full-length BAX), that cannot be suppressed by the anti-apoptotic BCL-2 family members has been generated. Interestingly, ΔN BAX exhibited a significantly stronger suppression of tumour growth than full-length BAX, suggesting that the truncated version of BAX may provide a better alternative for gene therapy trials [207].

BH3 mimetic peptides

Pro-apoptotic multidomain members of the BCL-2 family (BAK, BAX) are activated by BH3-only proteins (such as BAD, BID, BIM, PUMA, NOXA) to induce mitochondrial apoptotic death events, and BH3 domains are necessary and sufficient for this effect [208]. The interaction between these BCL-2 family members is primarily mediated through the amphipathic α-helices of their BH3 domains [209]. BH3-only proteins act in two ways, either by inactivation of the anti-apoptotic BCL-2 proteins and displacement of BAX (as seen with BAD) or by direct activation of BAX and BAK (as observed with BID and BIM). BH3 mimetic peptides derived not only from BH3-only proteins, such as BAD and BID but also from the multidomain BAX and BAK have been generated [210]. BH3 peptides longer than 14 amino acids can retain an α-helical structure and some biological activities [209]. For example, BH3 mimetic peptides induce oligomerization of BAX and BAK, permeabilization of MOM, and release of cytochrome *c* [211, 212].

In principle, peptides containing BH3 domain sequences should be explored as pharmaceutical lead molecules. However, their use as therapeutic agents is limited by their unfavourable pharmacological properties, including poor cellular permeability, bioavailability, solubility and metabolic stability *in vivo*. Several methods have been tried to overcome these limitations. BH3 peptides have been tagged with peptide transduction domains from *Drosophila* antennapedia protein, human immunodeficiency virus-1 trans-activating (TAT) protein or an arginine homopolymer (R8) transduction domain [211, 212]. These different approaches enhanced the intracellular uptake of BH3 peptides. A chemical strategy, termed hydrocarbon stapling, was also explored, and resulted in maintenance of the α-helical conformation, increased stability, cell-permeability, increased affinity to multidomain BCL-2 member pockets and improved pharmacological properties [209].

Natural and synthetic BH3 mimetic drugs

Natural compounds such as tetrocarcin A, a second metabolite derived from *Actinomyces spp*. [213], chelerythrine [214, 215] and sanguinarine which are plant benzophenanthridine alkaloids, antimycin A, a *Streptomyces*-derived inhibitor of ubiquinone–cytochrome *c* oxidoreductase at the mitochondrial respiration chain [216], gambogic acid derived from the gamboges resin of the tree *Garcinia hanburyi* [217], and certain polyphenols such as gossypol, apogossypol (compounds from cotton seed extracts) [218] and purpurogallin (a natural compound extracted from *Quercus sp. nutgall*) [218] promote death by binding BCL-2 and BCL-x_L and inhibiting their anti-apoptotic functions (Fig. 3).

Computational molecular docking analysis predicted that antimycin A targets the BH3-binding pocket of the anti-apoptotic BCL-2 family molecules [216]. NMR binding studies with BCL-x_L revealed that gossypol and purpurogallin also compete for the BH3-binding pocket [218]. Whereas some of these compounds act as 'true BH3 mimetics', others appear to inhibit anti-apoptotic BCL-2 family members without targeting the BH3-binding pocket per se. Indeed, chelerythrine and sanguinarine bind separately at the BH groove and BH1 region of BCL-x_L respectively, as opposed to the BH3 binding cleft which is targeted by other known inhibitors of BCL-x_L [215].

At the moment, of all the above mentioned natural BH3 mimetic small molecules, only gossypol, in an oral form (AT-101) has advanced into clinical trials (phase I/II) for the treatment of patients with refractory metastatic breast cancer [219] and for the treatment of patients with Glioblastoma Multiforme in combination with the alkylating agent temozolomide with or without radiation therapy (www.clinicaltrial.gov).

A recent highlight in the field is the development of ABT-737. ABT-737 is a cell permeating, synthetic BH3 mimetic that was designed by Oltersdorf *et al*. [220] using a NMR structure-based approach to target the BH3-binding groove on BCL-x_L. It binds with high affinity in the subnanomolar range to BCL-2 and BCL-w. However, despite its high affinity for BCL-2, BCL-x_L and BCL-w many cell types proved refractory to ABT-737. It appeared that the resistance reflects ABT-737's inability to target another pro-survival BCL-2 relative, MCL-1. Subsequently, down-regulation of Mcl-1 by several strategies was shown to confer sensitivity to ABT-737 [221]. Numerous studies have evaluated the merit of ABT-737 in triggering apoptosis *via* the mitochondrial pathways in cancer cell lines and mouse xenograft models [222]. Collectively these studies indicate that ABT-737 as a single agent shows strong potency against a variety of tumour types such as lymphoma, leukaemia, multiple myeloma and small-cell lung cancer and that when used in combination therapy it may help to overcome drug resistance phenotypes in additional tumour types [200, 220, 223]. The BH3 mimetic has also recently been shown to sensitise cancer cells to TRAIL [224] and to induce IMM permeabilization and mitochondrial swelling reminiscent of MTP in chronic leukaemia cells [225]. Even if the preclinical data strongly support a rationale for clinical trials with ABT-737, the compound has not yet entered clinical trials [226].

Targeting mitochondria directly: mitochondriotoxic compounds inducing mitochondrial membrane permeabilization

Shortly after the discovery that MOMP is a 'point of no return' [227] and that once it occurs cells die, mitochondria have become an attractive target to induce apoptosis (Fig. 3). In addition, the rich repertoire of mitochondrial proteins and the essential requirement of the membrane permeability barrier for proper organelle function offer an array of drug development opportunities. To date more than 20 mitochondriotoxic compounds acting directly on the mitochondria to induce cell death have been described and some of them have already been validated pre-clinically and entered clinical trials. These compounds can be classified according to their chemical nature into three main groups: peptide derivatives, small molecules and cationic lipophilic agents.

Peptide derivatives

Peptides derived from viral proteins [228], from proteins of the PTP and natural as well as synthetic peptides have been shown to be able to kill mammalian cells by triggering MOMP. The human immunodeficiency virus, HIV-1 encoded apoptogenic protein Vpr induces MMP *via* interactions with VDAC and ANT [190]. The chimeric peptide TEAM-VP using the MMP-inducing sequence derived from Vpr and a tumour blood vessel RGD-like 'homing' motif has been engineered. This virus-derived mitochondriotoxic compound targets mitochondria of angiogenic endothelial cells to induce MOMP and the release of mitochondrial apoptogenic molecules resulting in apoptosis [229].

Mastoparan, a peptide isolated from wasp venom (as well as its derivative mitoparan) has an α-helical structure and possesses positive charges clustered on one side of the helix. Mastoparan is the first peptide known to induce MOMP *via* interaction with VDAC in a CsA-regulated mechanism [230, 231]. A second amphipathic peptide, the signal sequence of cytochrome oxidase subunit IV from Neurospora crassa (pCoxIV, amino acids 3–22), which targets subunit IV to its mitochondrial location has also been shown to increase the permeability of isolated mitochondria [232].

A peptide structurally similar to Mastoparan, $_{D}$KLAKLAKKLAK-LAK or (KLAKLAK)2 (K = lysine, L = alanine and A = leucine) has recently been shown to disrupt mitochondrial membranes and directly permeabilize this organelle [233]. Moreover, when fused to targeting peptides that interact with surface receptors expressed on angiogenic endothelial cells in tumours (with the tumour blood vessel RGD-like 'homing' motif) [233] or on prostate vasculature (with the prostate-homing phage, SMSIARL) [234], or even with a peptide that inhibits the ErbB-2 receptor kinase [235], these chimeric peptides are translocated to the mitochondria where they induce MOMP especially in the targeted cell population. The ErbB-2 receptor kinase inhibiting peptide also exhibited ability to reduce tumour growth in HER-2-overexpressing human mammary xenografts established in SCID mice [235]. These studies provide a proof of concept for the strategy of targeting mitochondriotoxic hybrid molecules to cancer cells, primarily *via* the recognition by various surface receptors.

For over 70 years, it has been known that tumour cells exhibit a high rate of glycolysis. The high glycolytic rate is now known to be due in part to the greatly increased expression of HKs in transformed cells. HK isoforms I and II bind to VDAC and by this means interfere with the ability of BAX to interact with mitochondria and thereby cell death [58, 236]. A cell-permeable peptide analogue of HK-II VDAC binding domain (HXK2VBD) peptide fused to the internalization sequence of the Antennapedia homeoprotein has been shown to inhibit HK localization to the mitochondrion. HXK2VBD sensitizes cells to a BAX-dependent apoptosis inducer. Therefore, it seems that interference with the binding of HK-I to mitochondria by VDAC1-derived peptides may offer a novel strategy by which to potentiate the efficacy of conventional chemotherapeutic agents [237].

The jasmonates are a group of plant hormones which help regulate plant growth and development. Jasmonates include jasmonic acid and its esters, such as methyl jasmonate (MeJa). MeJa induces death in cancer cells, while being selectively inactive towards non-transformed cells [238]. MeJa acts directly on mitochondria derived from cancer cells in a PTPC-mediated manner [239]. MeJa binds to human HK isoforms I and disrupt its interaction with VDAC, causing the inhibition of glycolysis and the induction of MOMP [240]. MeJa has already been shown to have selective anticancer activity in preclinical studies [241–243], and this finding may stimulate the development of a novel class of small anticancer compounds that inhibit the HK-VDAC interaction.

Small molecules

Conventional chemotherapeutic agents, such as 2-chloro-2′-deoxyadenosine (2CdA) [244], topoisomerase II inhibitor etoposide (Vepeside, VP16) [245] which affects DNA replication, and paclitaxel (Taxol, TM) [246] which disrupts microtubule assembly can disrupt the integrity of mitochondria and induce cell death *via* the opening of the MTP pore. However, the high doses required to obtain this effect raise the question of whether these drugs in a clinical setup can induce cell death *via* MOMP.

Synthetic ligands of the peripheral-type benzodiazepine receptor (PBR) such as the benzodiazepines (Ro5–4864), isoquinoline carboxamides (PK 11195), indoleacetamides (FGIN-1–27), phenoxyphenyl-acetamides (DAA1106), pyrazolopyrimidines (DPA-713) [247] and erucylphosphohomocholine (ErPC3) [248] can induce apoptosis or sensitize cells to apoptosis induction as demonstrated by the drop in the mitochondrial transmembrane potential and an increased mitochondrial release of cytochrome *c* and Smac/DIABLO proteins. These compounds can overcome the cytoprotective effects of BCL-2 or BCL-x_L [249, 250]. Consistently, many experimental studies *in vitro* and also in SCID mice transplanted with human tumour cells, suggest that PBR ligands might be good candidates as chemotherapeutic, or at least chemosensitizing, agents [249, 250]. Whether the apoptogenic or chemosensitizing effects of the above-mentioned agents are truly due to a direct effect on mitochondria, is at least in some cases, a matter

of debate. For instance, the doses of PBR ligands required to obtain cytotoxic effects are several orders of magnitude higher than the K_d of the high-affinity PBR and in certain cases their cytotoxicity even appeared to be unrelated to PBR expression [251] .

A number of agents, for example lonidamine [252, 253], all-trans-retinoic acid [254], the synthetic retinoid RAR-γ ligand 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphtalene carboxylic acid (CD437) [252, 255], arsenic trioxide (As_2O_3) [252], 2CdA Cladribine and 2-choloro-2′-ara-fluorodeoxyadenosine (CaFdA) [244], have been reported to induce a conformational change in the ANT leading to mitochondrial channel formation. Lonidamine enhances the apoptotic response to cisplatin, cyclophosphamide, doxorubicin, paclitaxel, melphalan and γ-irradiation both *in vivo* and *in vitro* [253]. Lonidamine kills a wide range of tumour cells *in vitro* and in animal models. This drug in combination therapy is currently being tested in phase II/III trials for metastatic breast [256], non-small cell lung cancer [257], ovarian cancer [258] and glioblastoma [259] with encouraging results so far. Arsenite, the trivalent inorganic salt formed from arsenic trioxide, which is used to treat acute promyelocytic leukaemia [260] and has entered phase II clinical trials for multiple myeloma [261], causes glutathione depletion, induces PTP opening and its effect is prevented by BCL-2. 2CdA and 2-choloro-2′-ara-fluorodeoxyadenosine (CaFdA) drugs are clinically used for the treatment of indolent lymphoproliferative diseases, they disrupt the integrity of mitochondria and induce the release of pro-apoptotic mitochondrial proteins from these organelles [244, 262].

A number of agents that act on mitochondria possess a steroid-like core structure. For instance, this applies to the above mentioned ANT ligands CD437 [252, 255], all-trans-retinoic acid [254] as well avicins [263], betulinic acid [264] and resveratrol [265]. Betulinic acid, a naturally occurring pentacyclic triterpenoid, induces apoptosis in tumour cells through the mitochondrial pathway. Combined treatment with betulinic acid and anticancer drugs acted in concert to induce loss of mitochondrial membrane potential and the release of cytochrome *c* and Smac from mitochondria. Isolated mitochondria from different cell types are permeabilized by betulinic acid, and this effect is prevented by CsA, the ANT ligand bongkrekate, as well as by BCL-2 overexpression [264]. It is unclear, however, through which receptor (if any) betulinic acid acts on mitochondria. Avicins, a novel plant-derived metabolite reduces energy metabolism in tumour cells by targeting the outer mitochondrial membrane and pushing cells towards the apoptotic pathway by permeabilization of the outer mitochondrial membrane [263]. The effect of Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a phytoalexin found in grapes and other plant food, is more complex as it has been shown to be able to both promote [266] and inhibit [267] the mitochondrial death pathways. Resveratrol treatment of isolated mitochondria also led to depolarization, suggesting that the drug may target mitochondria directly [265, 266]. However, as in the case with PBR ligands, it is always difficult to ascertain in an *in vivo* context whether mitochondria are targeted directly by the above drugs or whether mitochondrial damage is an event that is secondary to their interaction with other cellular targets.

Lamellarins are a large family of marine alkaloids, with potential anticancer activities, isolated from diverse marine organisms, mainly ascidians and sponges. The best known member in the series is lamellarin D (lam D), first regarded as a conventional topoisomerase I poison [268], it has been shown promote an MPT-dependent release of cytochrome *c* and AIF from isolated mitochondria [269, 270]. A few synthetic analogues of lam D have been selected as preclinical drug candidates based on their *in vivo* efficacy against a panel tumour xenograft models and acceptable absorption, distribution, metabolism and excretion profiles. One of the prominent candidates is the amino derivative PM031379 which has revealed little toxicity toward non tumour cells *in vitro* [269] and displays potent anticancer activities *in vivo* in a human colon tumour xenograft model [269].

Cationic lipophilic agents

The lipid composition of the IMM is very different from that of other intracellular membranes. Lipophilic cations can cross cellular membranes and accumulate in mitochondria driven by the mitochondrial membrane potential to induce MOMP (Fig. 3). Because $\Delta\Psi m$ is often higher in malignant cells, lipophilic cations may selectively accumulate in their mitochondria, sparing the organelles of normal cells [271]. Several types of cancer cells have been described to accumulate such agents, *e.g.* rhodamine 123, to a higher level than normal cells [272]. Attempts have been made to use cationic lipophilic toxins as mitochondriotoxic agents. For instance, the pyridinium derivative F16 accumulates in the mitochondria and inhibits growth of human breast cancer cell lines [273]. MKT-077, a cationic rhodacyanine dye, is selectively toxic to cancer cells *in vitro* and *in vivo* [274]. MKT-077 activity has been associated with an effect on mitochondrial membranes and mitochondrial DNA [275] The antitumour effect of MKT-077 in xenografted mice models along with its selective accumulation in tumour mitochondria prompted its evaluation in the clinic. Renal toxicity encountered during the phase I trial, stopped further development of MKT-077 [276]. Another cationic ampholyte is the previously mentioned α-helical peptide $_D(KLAKLAK)_2$ which can directly permeabilize mitochondria. Furthermore, lipophilic cations may be employed as specific carriers, to selectively deliver toxins to mitochondria of cancer cells. An attempt was made in the early 1980s to complex platinum(II) tetrachlorodianion to rhodamine 123. The platinum–rhodamine 123 complex displayed some degree of selectivity towards transformed cells [277].

Finally, a promising class of photoactivatable antitumour agents is being developed for mitochondrion-targeted chemotherapy. Photodynamic therapy involves the treatment of tumours in which visible light is used to activate a photosensitizer. Mitochondrial membranes have been identified as an important intracellular target for singlet oxygen produced during the photochemical pathway. Verteporfin, a porphyrin-derived photosensitizer, similarly causes mitochondrial membrane permeabilization irrespective of BCL-2 or BCL-x_L overexpression [278]. The photoproduct of merocyanine 540 triggers cytochrome *c* release from isolated mitochondria and promotes apoptosis [279].

Photoactivation also enhances the mitochondrial toxicity of the cationic rhodacyanine MKT-077 [274]. Another cationic lipophilic dye, chloromethyl-X-rosamine (MitoTraker Red), a mitochondrion-selective fluorescent probe, has a strong photosensitising action. Photo-irradiation of intact cells loaded with MitoTraker Red induces depolarization of the IMM and swelling of mitochondria, subsequently resulting in apoptosis [280]. The photosensitizer hypericin detaches HKs from mitochondria [281], whereas the phthalocyanine photosensitizer Pc4 causes the photochemical destruction of BCL-2 [282, 283].

Bypassing the mitochondria: mitochondrial pro-apoptotic factors as chemotherapeutic agents

MOMP results in the release of a plethora of cell death effectors from the intermembrane space. Such factors include Smac/DIABLO, a protein that exerts much of its pro-apoptotic function by neutralizing IAPs that function as caspase inhibitors. Another pro-apoptotic factor released from permeabilized mitochondria is the AIF, a mitochondrial flavoprotein that translocates to the nucleus where it contributes to chromatin degradation [284, 285].

The binding of Smac/DIABLO to IAPs is mediated by a relatively short stretch of amino acids located in the N-terminus of the protein [286]. Several studies have shown that overexpression of Smac/DIABLO sensitizes neoplastic cells to apoptotic death [287]. These findings prompted the development of peptides derived from the NH_2-terminus of Smac/DIABLO and small molecules that mimic Smac/DIABLO functions which could potentially be used in cancer therapy. NH_2-terminal peptides of Smac/DIABLO fused to *Drosophila* antennapaedia penetrating sequences enhance apoptosis mediated by different anti-neoplastic agents in breast cancer [288, 289] and glioblastoma cell lines [290, 291]. Similarly, a small Smac-mimic compound was able to increase the apoptotic effects of death stimuli [291]. It is interesting to note that this small molecule induces apoptosis by itself in MDA-MB-231 breast cancer cells, which have high expression levels of XIAP and c-IAP1. In contrast, it only sensitizes MDA-MB-453 (human breast cancer cell line) and T47D cells, which have low IAP expression, to apoptotic triggers [289]. The sensitizing effects of Smac peptidomimetics and small Smac-mimic compounds have also been demonstrated *in vivo* using malignant glioblastoma xenograft mouse models [289, 292]. Taken together, these results show that the NH_2-terminal Smac/DIABLO derivatives and small molecules that mimic its function could be useful as adjuvant therapy in tumours and await clinical trials.

An alternative strategy is to exploit the pro-apoptotic properties of AIF. In fact, microinjection of recombinant AIF is sufficient to induce hallmarks of apoptosis [285, 293]. Different AIF delivery strategies are under development. Recently, a chimeric protein containing an ErbB-2-specific antibody and the cytotoxic moiety of AIF has been shown to direct AIF to ErbB-2-expressing cancer cells, causing their demise [294]. BZL101 is a drug derived from Scutellaria barbatae that has been shown to induce the translocation of AIF to the nucleus and the induction of cell death. Recent clinical trials have documented its effectiveness in treating patients with advanced breast cancers [295]. Likewise, the novel cationic amphiphilic compound atiprimod has been shown to exert antimyeloma effects in mouse experiments and its ability to kill mantle cell lymphoma cells has been attributed to the activation of the AIF-dependent pathway [296].

Therapeutic strategies that inhibit MOMP and cell death

Although mitochondria are an important target for drugs designed to kill target cells, especially in cancer chemotherapy, considerable work has also focused on protecting mitochondria in an effort to prevent cells from dying in stress situations such as during ischemia/reperfusion injury, stroke and drug-induced damage. Indeed, a number of pharmacological agents that directly or indirectly protect mitochondria (Table 2) have been discovered and show promising therapeutic applications (Fig. 4).

Cyclosporin A and the inhibition of MPT

Work dating back to the early 1990s showed that CsA alleviated ischemic liver injury *in vivo* [297]. In these early studies, the link between hepatoprotection and the prevention of PT was not investigated, and CypD-independent effects of CsA cannot be ruled out. However, subsequent work in perfused hearts subjected to anoxic injury convincingly demonstrated that the ability of CsA to prevent pore opening was responsible for its protective effect on the heart [298]. Likewise, CsA was reported to protect isolated rat hepatocytes from prooxidant-induced cell death as a result of its pore blocking activity in mitochondria [299]. Subsequent *in vivo* and *in vitro* studies have identified a contribution of the PTP in CD95- and TNFR-1-induced liver injury [300]. These two death receptors are important mediators of inflammatory liver injury during septic shock and immune-dependent drug-induced hepatotoxicity. CsA has been shown in these cases to alleviate the injury, although the cytoprotective effect was generally more consistent under *in vivo* conditions as compared to the more variable cytoprotection achieved in liver cells grown in culture [247]. CsA was also found to significantly protect rats [301] and mice [302, 303] from acetaminophen (paracetamol)-induced liver injury. The protection correlated with a diminution of mitochondrial GSH depletion, cytochrome *c* release and mitochondrial swelling after their isolation [303]. Similarly, studies in liver slices and cultured or freshly isolated hepatocytes have found that the MPT inhibitor CsA (but not FK506, an immunosuppressive drug that does not block MPT) protected from paracetamol-induced loss of viability [301, 304–306], and in two studies [305, 306] the use of calcein acetoxymethyl ester in conjunction with a mitochondrial membrane

Table 2 Drugs inhibiting the mitochondrial membrane permeabilization

Target	Drug	Type of compound	Remarks
MPT pores	Cyclosporin A	Small molecule	Prevents opening of MPT pores *via* cyclophilin D (CypD) interaction. Side toxicity due to interaction with other cyclophilins
	NIM811	Small molecule	Prevents opening of MPT pores *via* CypD interaction. More specific than cyclosporine A (CsA).
	Sanglifehrin A	Small molecule	Prevents opening of MPT pores *via* CypD interaction. Binds to CypD at different sites from CsA
	Adenosine	IPC mimetics	Induces desensitization of MPT by causing IPC
	Bradykinin	IPC mimetics	Induces desensitization of MPT by causing IPC
	Opioids	IPC mimetics	Induces desensitization of MPT by causing IPC
	Diazoxide	IPC mimetics	Mimics IPC, induces desensitization of MPT by opening mitoKATP
	Pinacidil	IPC mimetics	Mimics IPC, induces desensitization of MPT by opening mitoKATP
	Nicoradil	IPC mimetics	Mimics IPC, induces desensitization of MPT by opening mitoKATP
	Isofluorane	Anaesthetic	Induces IPC-like condition (APC), inhibits complex I and glycogen synthase kinase 3 p, preserves mitochondrial functions.
	Minocycline	Semi-synthetic antibiotic	Inhibits MPT by decreasing mitochondrial Ca^{2+} uptake

MPT: mitochondrial permeability transition; IPC: ischemic precondition. mitoK_{ATP}: mitochondrial ATP-sensitive K-channels and APC: anaesthetic precondition.

potential probe, confirmed CsA-sensitive PT pore opening in hepatocytes exposed to paracetamol. CsA has also shown promising neuroprotective effects and is currently under clinical investigation against traumatic brain injury [307]. Indeed, preclinical work has shown that CsA (but not the inactive FK506) reduces cortical damage after traumatic brain injury in mice and rats even when administered after injury [308].

Novel CsA analogues and other inhibitors of the pore

The toxicity of CsA and its non-specificity towards multiple cyclophilins has hampered its wide-spread use to test the role of PT in different pathologies. The search for potent and more specific CsA analogues has led to the identification of NIM811 (N-methyl-4-isoleucine cyclosporine). This cyclic polypeptide is as potent as CsA in blocking the pore but is selective for CypD and has no immunosuppressive properties [309]. NIM811 has been used with success to block PTP opening and prevent cell death in a number of experimental settings, including experimental liver transplantation [310] and spinal cord injury [311]. Another potent inhibitor of the pore is sanglifehrin A. This compound is a macrolide produced by actinomycetes that binds to CypD at a different site from CsA [312] and that has been shown to have cytoprotective effects in the reperfused heart [313]. Another pharmacological inhibitor of PT with potential therapeutic applications is 5-(benzylsulfonyl)-4-bromo-2-methyl-3(2H)-pyridazinone [314]. However, further work is required to ascertain its usefulness as a PT blocker.

Preconditioning of the heart protects by sparing mitochondria

Ischemic preconditioning (IPC) has long been known to provide substantial protection to the myocardium. By exposing the heart to one or more brief cycles of sub-lethal ischemia, this organ is protected from subsequent more prolonged and lethal ischemic insult as first reported by [315]. Mitochondria play a key role in IPC, and they have been shown to respond to IPC with diminished calcium sequestration [316–318], improved respiration [319, 320] and desensitization to PT [313, 321]. Not surprisingly, considerable efforts have been devoted to the study of the role of PT in IPC. The sensitivity of cardiac mitochondria to undergo PT is consistently decreased after IPC [313, 321]. However, this is normally only observed in cells and intact tissues, not with mitochondria isolated from IPC myocardium. This suggests that IPC affects PT indirectly and involves key upstream signalling events that target the mitochondria. The signalling molecules that have been linked to IPC and inhibition of PT include among others protein kinase Cε, nitric oxide, protein kinase G, protein kinase B (Akt), glycogen synthase kinase 3β, AMPK, mitochondrial ATP sensitive K-channels and connexion 43. A full review of their individual contributions to IPC would be beyond the scope of this publication and the reader is referred to an excellent recent review on this

topic [322]. Therefore, we will only focus on the pathways that have been identified as potential targets for pharmacological intervention, with the rationale that pharmacological induction of IPC could be used to protect the heart and possibly other organs such as liver and brain from ischemia/reperfusion damage.

Pharmacological IPC mimetics

A number of physiological ligands to plasma membrane receptors are released during ischemia and these are now known to play a key role in IPC (Fig. 4). These include adenosine, bradykinin and opioids and appear to provide the initial signal for the pathways leading to IPC. A proposed pathway involves receptor-mediated activation of phosphatidylinositol 3-kinase followed by the activation of Akt and endothelial nitric oxide synthase, causing it to produce nitric oxide and downstream activation of protein kinase G, which in turn causes the opening of the mitochondrial ATP-sensitive K-channels (mitoK$_{ATP}$) through a yet to be defined phosphorylation step [323]. In support of this pathway, the pharmacological inhibition of protein kinase c by KT-5823 prevented the desensitization of PT in IPC [324]. There is also evidence for the involvement of mitochondrial protein kinase Cε in the effect of IPC on mitochondria, either through signalling downstream of cell surface receptors or through ROS-mediated activation [325]. A direct physical interaction between protein kinase Cε and mitoK$_{ATP}$ has been proposed [323, 324]. The evidence for mitoK$_{ATP}$ being responsible for the manifestation of IPC stems to a large extent from pharmacological studies. Indeed, agents such as diazoxide and pinacidil have been shown to mimic IPC by opening mitoK$_{ATP}$ [326, 327]. More recently, a similar mechanism of action on mitochondria through mitoK$_{ATP}$ opening was reported with nicorandil [328] and bepridil [329]. The exact link between mitoK$_{ATP}$ opening and desensitization of PT is unclear but may involve a mild uncoupling of the mitochondria leading to decreased mitochondrial Ca^{2+} levels and decreased ROS production. However, one of the key remaining issues is that neither the pharmacological tools that were used to implicate mitoK$_{ATP}$ in IPC are not specific for this channel nor is the exact molecular identity of mitoK$_{ATP}$ known at present.

Volatile anaesthetics such as isoflurane have also been known to induce an IPC-like condition known as anaesthetic preconditioning (APC) [330]. Like IPC, APC involves the preservation of mitochondrial function in the cell following ischemia/reperfusion injury. For example, isoflurane and sevoflurane have been reported to inhibit complex I of the respiratory chain [331] and to lead to mild uncoupling of the mitochondria [332] and the generation of ROS by complex III [333]. The opening of mitoK$_{ATP}$ and mitoK$_{Ca}^{2+}$ by the anaesthetics appears to play an important role in the mechanism of protection and the observed prevention of PT during subsequent conditions of ischemia/reperfusion [334]. Isoflurane has also been shown in the heart to inhibit glycogen synthase kinase 3β [335] which has been shown to play a role in controlling the mitochondrial pathway of apoptosis. Recent phosphoproteomic studies on mitochondria isolated from isoflurane preconditioned hearts have identified Tyr194 as a novel phosphorylation site on ANT1 [336]. Although this report demonstrated a role for this phosphorylation event in mitochondrial bioenergetics in yeast, its role in APC remains to be determined. In the brain, APC induced by isoflurane may additionally involve the up-regulation of the anti-apoptotic BCL-2 protein [337].

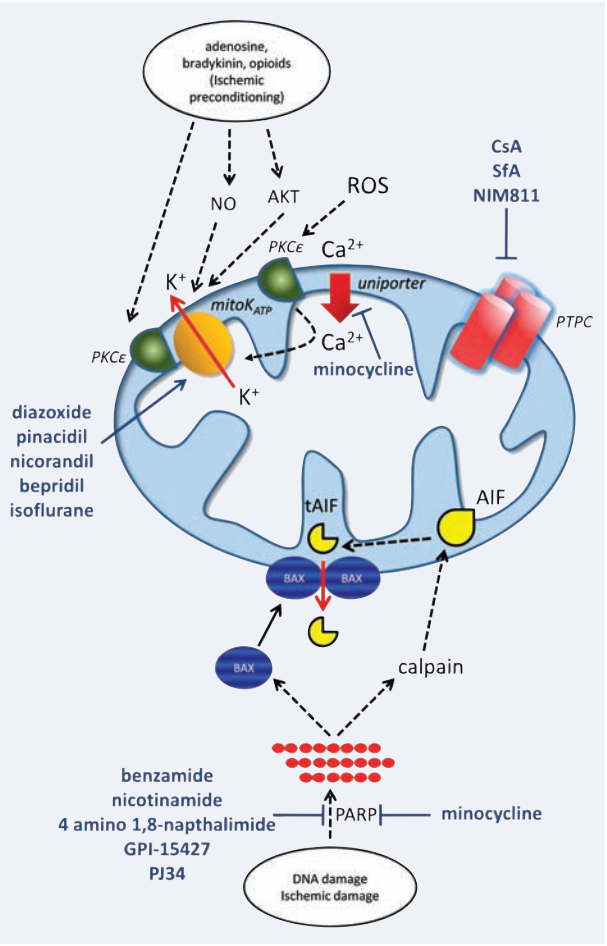

Fig. 4 Therapeutic agents acting on mitochondria to prevent cell death. Pharmacological compounds and their target molecules are shown. Please refer to the text for additional detail. Abbreviations: CsA, cyclosporine A; SfA, sanglifehrin A; PTPC, permeability transition pore complex; AIF, apoptosis inducing factor; tAIF, truncated AIF and ROS, reactive oxygen species.

Minocycline

The semi-synthetic tetracycline antibiotic minocycline has been found in a large number of studies to provide protection from neurodegeneration and ischemic neuronal injury [338], CD95-mediated hepatic injury [131] and hypoxic/ischemic renal and hepatic injury [310, 339]. Different mechanisms involving

mitochondria have been proposed by which minocycline may exert cytoprotection and these include decreased mitochondrial release of pro-apoptotic factors such as cytochrome *c* [340], up-regulation of anti-apoptotic BCL-2 [291] and inhibition of PT [310, 341]. The latter was attributed to minocycline decreasing mitochondrial Ca^{2+} uptake [310, 341]. However, the direct targeting of mitochondria by minocycline and the involvement PT in its protective effect have recently been challenged in preference to its anti-inflammatory properties [342] and effects on poly(ADP-ribose)polymerases (PARP) [343] (see below).

Inhibitors of PARP and the prevention of DNA damage-mediated mitochondrial damage

PARP are nuclear enzymes that are responsible for the formation of poly(ADP-ribose) (PAR) polymers from NAD^+ on nuclear proteins to regulate gene expression, chromatin configuration and metabolism in response to DNA damage, ROS and ischemic injury [344]. Although the mechanism of PARP overactivation-induced cell death was initially attributed to excessive NAD and ATP consumption [345], there is recent evidence that suggests that PAR itself can act as a death signal. This has been observed in response to excitotoxic neuronal damage, N-methyl-N'-nitro-N-nitrosoguanidine-induced DNA damage or hydrogen peroxide treatment [95]. The target for PAR has been identified to be AIF which is released from mitochondria to translocate to the nucleus to induce DNA fragmentation, chromatin condensation and caspase-independent cell death. The release of active AIF from mitochondria occurs in response to PAR-mediated activation of calpain and the proteolytic processing of native AIF to its truncated form [95]. This event also requires BAX translocation to mitochondria and its activation (but not that of BAK) to induce MOMP [95]. The identification of PARP as a key trigger of cell death has prompted a number of preclinical and clinical studies on pharmacological inhibitors of PARP in an effort to prevent neuronal and cardiac cell death. For example, the PARP inhibitor PJ34 has been reported to be neuroprotective in a model of permanent focal cerebral ischemia in mice [346]. Other PARP inhibitors such as benzamide, nicotinamide, GPI-15427 and 4 amino 1, 8-napthalimide has also been shown to be protective in various *in vivo* models of neuronal damage [347–350]. However, further studies are required to confirm that the protection afforded by PARP inhibitors in these studies indeed involves the inhibition of the PARP-PAR-AIF pathway.

Conclusion and future directions

Deregulation of the mitochondrial apoptosis pathway with a causative or contributing role in many diseases has become increasingly evident. Emerging knowledge about molecular mechanisms of apoptosis has revealed a plethora of potential drug discovery targets. Structural analysis of apoptotic proteins and studies of their biochemical mechanisms have suggested strategies for lead generation resulting in numerous novel chemical entities with mechanism-based activities. The mitochondrial apoptosis pathway holds great promise as a target for therapeutic intervention. There is ample evidence showing that apoptosis can be potently activated with the use of compounds targeting key protein components of the mitochondria cell death pathway. Whether or not these routes and targets are suitable to block the proliferation of tumour cells remain to be seen. The relatively low rate of clinical entry associated with these molecules is related to the lack of specificity, low efficacy, or development of drug resistance. These issues are being addressed as our understanding of the field evolves. However, pharmacological control of mitochondria is not without risk for the physiology of normal cells as, mitochondria play a critical role in supplying the cell with the bulk of its ATP needs *via* oxidative phosphorylation and any cell type or tissue with a high aerobic energy requirement is likely to be affected when this organelle is dysfunctional. Therefore, due to the frequent implication of mitochondria in cardiac and neurodegenerative disorders, the impact of mitochondria-targeted anticancer agents on normal physiology of cardiovascular system and central nervous system will need a special attention.

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