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Review

Recent advances in apoptosis, mitochondria and drug resistance in cancer cells ☆

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ABSTRACT

Defective or inefficient apoptosis is an acquired hallmark of cancer cells. Thus, a thorough understanding of apoptotic signaling pathways and insights into apoptosis resistance mechanisms are imperative to unravel novel drug targets for the design of more effective and target selective therapeutic strategies. This review aims at providing an overview of the recent understanding of apoptotic signaling pathways, the main mechanisms by which cancer cells resist apoptotic insults, and discusses some recent attempts to target the mitochondrion for restoring efficient cell death signaling in cancer cells. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

1. Introduction

Cell death is an essential part of the normal development and maturation cycle [1], and a homeostatic balance between the rates of cell proliferation and cell death is critical for maintaining normal physiological processes. Alterations or defiance against these natural death mechanisms can lead to diseases such as AIDS, diabetes mellitus, neurodegenerative diseases (such as Parkinson's disease) and cancer, many of which are now, also known to be reactive oxygen species (ROS)-mediated.

The progressive transformation of normal human cells to malignant derivatives is a complex multi-step process, which requires dynamic alterations of the genome and successful breaching of intracellular checkpoints [2]. Extensive research in the cancer field has explored numerous pathways in cancer progression to elucidate effective measures to target these cancer cells.

Though cancer therapeutics favor a multi-prong approach where individual treatment varies between surgery, radiation, chemicals, antibodies and/or cells of the immune system, the effectiveness of the treatment differs largely between individuals and the cancer background [3]. In this regard, cancer cells often reside in unique microenvironments armed with a variety of adaptive responses and carry mutations, such as defective apoptotic machinery, that further confer survival advantage. Thus, improving therapeutic efficacy and selectivity and overcoming drug resistance are the major goals in developing anti cancer agents today [4].

To fulfill these goals, a thorough understanding of apoptotic signaling pathways and how tumor cells resist apoptosis is imperative, because they provide directions to unravel novel therapies and key targets to surpass or supplement current cancer treatments. In this regards, one emerging target is the cellular powerhouse, the mitochondrion. Before we discuss the significance of the mitochondria to cancer therapy, we will review the apoptotic signaling pathways and how cancer cells resist apoptotic insults.

Abbreviations: ANT, adenine nucleotide translocase; AIF, apoptosis-inducing factor; Cyt c, BH, Bcl-2 homology; COX, cytochrome oxidase; c, cytochrome; DYm, mitochondrial transmembrane potential; ER, endoplasmic reticulum; IAP, inhibitor of apoptosis; MMP, mitochondrial membrane permeabilization; IM, inner membrane; OM, outer membrane; PTPC, permeability transition pore complex; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel

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1.1. Apoptotic signaling

Apoptosis is characterized by cell shrinkage, blebbing of plasma membrane, maintenance of organelle integrity, condensation and fragmentation of DNA, followed by ordered removal of phagocytes [5]. It works like a "suicide" program and it causes minimal damage to surrounding tissues. Apoptosis has been subclassified into two types of death pathways, namely, the extrinsic pathway and the mitochondria-mediated pathway. These two processes however, are not exclusive and evidence suggests that they can be linked and that molecules in one pathway can influence the other [6]. Moreover, recent evidences support non-apoptotic roles for many effectors of the apoptotic signaling pathways. For instance, caspase 2, the most conserved member of the caspase family, also plays a role in cell cycle regulation, DNA repair, and tumor suppression [7].

1.2. Extrinsic or receptor-mediated pathway

The extrinsic receptor mediated death pathway requires effective engagement between the death receptors found on the surface of the cell membranes and their respective ligands [8] (Fig. 1). The receptor-mediated pathway involves death receptors from the tumor necrosis factor (TNF) superfamily such as TNF, CD95 (Fas) and TNF-related apoptosis inducing ligand (TRAIL) receptors. These receptors have an extracellular domain to engage the ligands and an intracellular cytoplasmic domain that is also referred to as the death domain. This death domain is responsible for transmitting the death signal from the surface to the intracellular signaling pathways [9]. Activation of CD95 or TNF receptors often leads to receptor clustering and intracellular recruitment of proteins into a death-inducing signaling complex (DISC), which then activates an initiator caspase, procaspase 8. Activated caspase 8 then triggers the execution phase of apoptosis via the activation of the downstream effector caspase, caspase 3 [10]. The activated caspases can also induce mitochondrial damage and reinforce the death signal by facilitating the egress of death amplifying proteins from the mitochondrial inter-membranous space [11–14].

1.3. Intrinsic or mitochondria-mediated pathway

The mitochondria pathway of cell death can be activated by a variety of receptor-independent stimuli such as radiation, free radicals, viral infections and serum/growth factor withdrawal (Fig. 1). Initially, it was demonstrated that these triggers invariably result in changes in the inner mitochondrial membrane permeability due to the opening of the mitochondrial permeability transition (MPT) pore. The major consequences of this change of permeability are the loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), the release of pro-apoptotic proteins and the arrest of the bioenergetic function of the organelle. The proteins that are released can be broadly classified into two categories. The first category comprises of proteins that can activate the caspase dependent pathway. This group includes proteins such as cytochrome *c* (cyt *c*) and Smac/DIABLO (second mitochondria-derived activator of caspases). The holocytochrome *c* induces Apaf-1 oligomerization, leading to the activation of caspase 9. This active cyt *c*/Apaf-1/caspase 9 complex forms the apoptosome and activates the executioner caspases 3 and 7 resulting in the dismantling of the cell through nuclear fragmentation[10,15,16]. Smac/DIABLO binds to IAPs (inhibitor of apoptosis proteins) and deactivates them, thus preventing the IAPs from arresting the apoptotic process, permitting apoptotic progression. Overexpression of Bcl-2, the founder oncogene of the Bcl-2 family (see below) has been shown to block apoptosis in certain cancer cells, which have been classified as type II cells, as compared to type I cells in which the execution of apoptosis program is Bcl-2-independent [8].

The second group includes other pro-apoptotic proteins such as apoptosis inducing factor (AIF) and endonuclease G (Endo G). In some models, the release of these proteins is a late event in apoptosis, which occurs once the cells are committed to die. Following the release of AIF, it translocates to the nucleus where it promotes DNA fragmentation. Both AIF and Endo G act in a caspase-independent manner to execute cell death [17]. For example, in an ovarian stem cell model, Endo G was shown to mediate caspase-independent cell death in response to chemotherapeutic agents [18]. Alternatively, AIF has also been recently proposed to participate in a different form of cell death, namely programmed necrosis [19].

In the last decade, increasing evidence demonstrated that pro-apoptotic mitochondrial membrane permeabilization (MMP) could exclusively affect the outer membrane. This would be achieved via the formation of lipidic pores or pores formed by members of the Bcl-2 family (discussed below) and then, independent of permeability transition, affect the inner membrane. It is not known whether the various modes of MMP can co-exist within a single cell, but it is plausible that the choice between permeabilization of one membrane or both depends on the nature of the death stimulus, its intensity and the cell itself.

2. Regulation of apoptosis

2.1. The Bcl-2 family of proteins

The founding member of Bcl-2 family of proteins, bcl-2, was first cloned in neoplastic B cells with the t (14; 18) chromosome translocation [20]. Apoptotic events are highly regulated by this family of proteins, which shares homology in any of the four common Bcl-2 homology (BH) domains. Thus, the Bcl-2 family of proteins has been divided according to the domains they carry. The anti-apoptotic proteins, Bcl-2, Bcl-X_L, and Mcl-1 possess all the four domains (BH1-4). These proteins also contain a hydrophobic carboxy terminal domain that enables them to dock onto the outer mitochondria membrane (OM), the nucleus and ER [21]. It has been shown that Bcl-2 and Bcl-X_L can protect the cells by interacting with mitochondrial proteins such as the adenine nucleotide translocase (ANT) or the voltage dependent anion channel (VDAC), thus preventing them from forming mitochondrial pores, protecting membrane integrity, and inhibiting the release of apoptogenic factors such as cyt *c* [22]. More recent evidence has highlighted novel functional biology of the anti-apoptotic protein Bcl-2 [23]. Overexpression of Bcl-2 was associated with a slight increase in steady state intracellular superoxide (O_2^-) production, which was linked to increases in mitochondrial oxygen consumption and the activity of the rate limiting enzyme in the electron transport chain, cytochrome c oxidase (COX). Interestingly, pharmacological or molecular inhibition of the NADPH oxidase restored apoptosis sensitivity of Bcl-2 overexpressing cells, thus implicating the pro-oxidant activity of Bcl-2 in its anti-apoptotic function.

The second category of Bcl-2 family of proteins contains BH domains 1, 2 and 3. These proteins would include Bax and Bak. Bax is a pro-apoptotic protein that resides in the cytosol under physiological conditions. An apoptotic trigger however, can lead to its translocation to the mitochondria and its subsequent insertion into the OMM. At the mitochondria, Bax can homodimerize or heterodimerize with other pro-apoptotic members such as Bak or truncated Bid, thus disrupting the integrity of the OMM by forming mitochondrial pores and increasing its permeability. These pores can then lead to the release of apoptogenic factors such as cyt *c* [24]. Some reports have also suggested that Bax engages in a close molecular cooperation with proteins from the PTPC, such as ANT and/or VDAC, to induce mitochondria membrane permeabilization (MMP) [25]. Bcl-2 and Bcl-X_L have been shown to antagonize the apoptotic cascade by a direct interaction and sequestration of these pro-apoptotic proteins [26]. Similarly Bak, which is normally inhibited by its interaction with VDAC, can also homodimerize and result in pore formation at the mitochondria when freed.

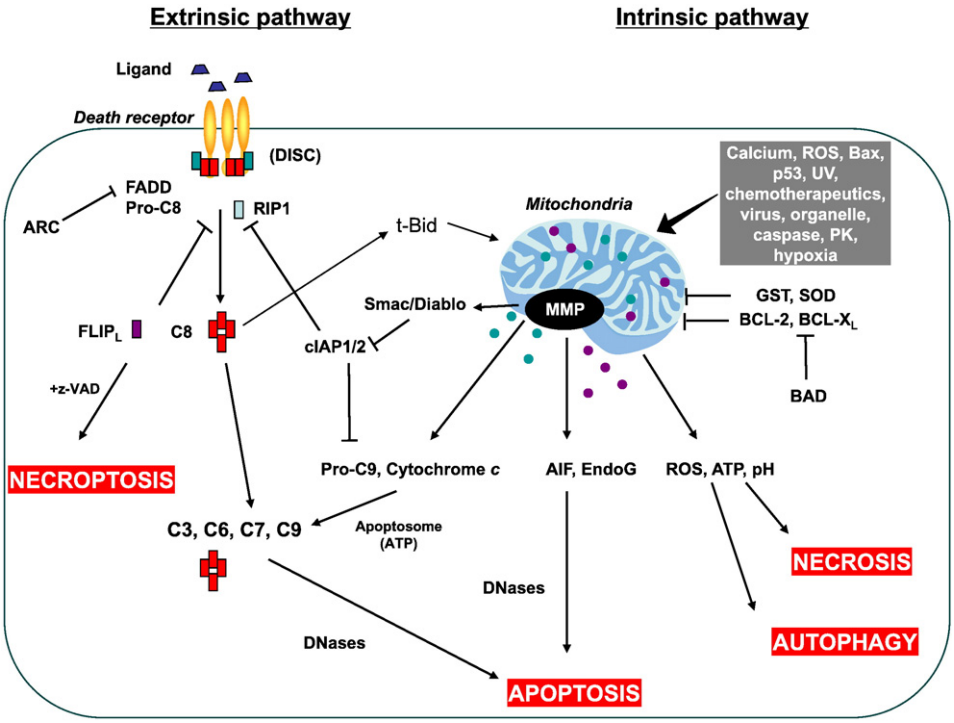

Fig. 1. The extrinsic and intrinsic pathways of apoptosis: Death receptor pathway (left) is initiated by the ligation of the ligands to their respective surface receptors. This leads to receptor oligomerization and DISC formation, which then triggers a caspase activation cascade that result in detrimental modifications at the cytosol, membrane and nucleus. Where caspases cannot be activated, cell death is seen to resemble necrosis and is termed necroptosis. The intrinsic pathway (right) is activated by multiple stimuli (grey box), which converge at the mitochondrion and induce mitochondrial membrane permeabilization (MMP) and subsequently result in the release of pro-apoptotic mitochondrial proteins into the cytosol. This activates caspase-dependent and caspase-independent processes culminating in cell death. MMP hampers mitochondrial function, which triggers a bioenergetic crisis due to loss of ATP, ROS production and pH alterations. Depending on the intensity of the mitochondrial insult, the cell can undergo apoptosis, necrosis and/or autophagic cell death. Both the intrinsic and extrinsic pathways can be inhibited by proteins such as Bcl-2 and cIAP1/2 promoting cell survival. AIF: apoptosis inducing factor; cIAP1/2: inhibitor of apoptosis protein; C, caspase; z-VAD, pan-caspase inhibitor.

The third group of Bcl-2 family of proteins is the BH3-only proteins. The BH3-only family members include Bim, Bad, Bmf, Noxa and Puma. They act by neutralizing the anti-apoptotic proteins [27]. For instance, Bim, Puma, Bad and Bmf heterodimerize with Bcl-2 and Bcl-X_L and sequester them, thereby blocking their anti-apoptotic action at the mitochondria.

2.2. Inhibitors of apoptotic proteins

Cell fate is tightly regulated by the interactions between pro and anti-apoptotic proteins which act to tweak the balance between survival and cell death [27]. In cancers, the crippling of pro-apoptotic pathways or enhancement of the anti-apoptotic pathways via the modulation of the regulatory proteins largely confers survival advantages onto the cells in the face of death triggers.

The inhibitors of apoptosis (IAPs) family were first identified in *baculovirus* and to date, eight mammalian IAPs have been described [28]. This would include neuronal apoptosis inhibitory protein (NAIP), cellular IAP1 and IAP2 (cIAP1 and cIAP2), X-linked inhibitor of apoptosis (XIAP), Survivin, Testis-specific IAP (Ts-IAP), BIR-containing ubiquitin conjugating enzyme (BRUCE) and Livin [29]. IAPs are characterized by the presence of 70–80 amino acid baculoviral IAP repeat (BIR) domain(s), which are important for the binding and inhibition of caspases. They play a critical role in blocking cell death by regulating the caspase cascade, and then, may influence both the intrinsic and extrinsic pathway in the cells.

Of the IAPs, XIAP has been best described, possibly due to the extensive studies on its anti-apoptotic role and the plausible therapeutic benefits in targeting it. XIAPs have been shown to antagonize the apoptotic cascade via the direct inhibition of caspases and via proteasome-dependant degradation of caspases. In addition to these caspase inhibitory roles, it has also been found to activate nuclear factor kappa B (NF-κB) by promoting the nuclear localization of NF-κB.

IAPs have been shown to be regulated by IAP binding proteins such as second mitochondrial activator of caspases (Smac/DIABLO) [30]. Smac/DIABLO normally resides in the mitochondria and upon receiving an apoptotic stimulus via the intrinsic pathway is proteolytically cleaved and released into the cytosol through the Bax/Bak channels or via Bid-induced permeabilization of the outer mitochondrial membrane. Subsequently, Smac/DIABLO associates with IAPs and prevents them from inhibiting caspases, thus promoting apoptosis.

2.3. The tumor suppressor P53 and P63/P73

P53, also known as the “guardian of the genome” [31] has been shown to play a critical role in intrinsic tumor suppression via two mechanisms. This would include cell cycle arrest and induction of apoptosis. A variety of triggers such as DNA damage, oncogene activation, and telomere erosion can lead to the activation of p53.

DNA damage can be due to exposure to radiation or drugs. Subsequently this damage signals the activation of the cellular checkpoint kinases such as ATM (Ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), which then leads to the phosphorylation of p53. P53 is normally sequestered by Mdm2. Phosphorylation of p53 disrupts p53's interaction with Mdm2 and ushers its activation [32,33]. P53 then holds the cell at a checkpoint until the damage is repaired. If the damage is irreversible, apoptosis is triggered. Oncogenes such as MYC, RasV12 and E2F-1 have also been shown to induce apoptosis by indirectly activating p53 via activation of ARF (Alternative Reading Frame) [34]. ARF acts by sequestering Mdm2 thus releasing p53 [35].

P53 has been shown to regulate apoptosis in both a transcription-dependent and -independent manner [36]. In the transcription dependent pathway, p53 activates the expression of several pro-apoptotic proteins such as PUMA, Bax and BID, which are involved in the regulation of the intrinsic cell death pathway as well as upregulates CD95 (Fas/Apo1) and DR5 receptors, which mediate the extrinsic cell death signals. In addition to transcriptional activation of pro-apoptotic proteins, p53 has also been shown to suppress anti-apoptotic proteins such as Survivin [37].

In the transcription-independent pathway, it has been shown that a fraction of p53 localizes to the mitochondria following apoptotic stimuli where it physically interacts with Bcl-2 and/or Bcl-X_L and antagonizes their anti-apoptotic function at the outer mitochondrial membrane. This interaction frees Bax and Bid to elicit their downstream effects. Moreover, p53 has also been shown to interact with Bak thus releasing it from the neutralizing hold of Mcl-1 [38]. In addition to the effects of the mitochondria localized p53, cytosolic p53 has also been reported to induce the activation of Bax in a transcription-independent manner, thereby leading to mitochondrial membrane permeabilization and cyt *c* release [39–42].

Thus, irrespective of being transcription-dependent or -independent p53 activities, they both elicit their effects at the mitochondria. While nuclear p53 via its transcriptional activity induces expression of Bax, PUMA, Noxa [43] and Bid, cytosolic or mitochondrial p53 via transcription-independent mechanisms directly activates Bax/Bak and neutralizes the anti-apoptotic effect of Bcl-2/Bcl-X_L at the mitochondria. Two other members of the p53 family, p63 and p73, play a role in normal development in a tissue specific manner. Though these homologues share a remarkable degree of structural similarity, they display considerable functional diversity [44]. Despite the structural homology, mutations of p63 and p73 are rarely associated with human cancer, however, in tumorigenesis the net effect of these homologues is suggested to be a function of the relative expression of their multiple protein isoforms, regulated by transcriptional and post-translational mechanisms [45,46].

2.4. Influence of ROS on cell death signaling

Even though ROS does not act directly through the death receptors, it can influence the extrinsic death pathway by altering the intracellular milieu, making the environment conducive for effective engagement between receptors and ligands or the execution of the downstream events leading to apoptosis. Receptor upregulation has been observed in several systems with exogenous ROS or ROS inducing agents. Upregulation of CD95 and TRAIL death receptors have been observed in response to hydrogen peroxide (H_2O_2) [47]. In addition, intracellular ROS induced by treatment with cisplatin also promoted surface clustering of CD95 and treatment with antioxidants abolished this clustering [48]. ROS has also been shown to sensitize cancer cells to TRAIL-induced apoptosis [49]. The induction of intracellular ROS following treatment with TRAIL has also been shown to result in caspase activation, which pushes the cell further toward execution [50]. Caspases are critical downstream executioners, which work most effectively under reducing conditions. As such, proteins that can antagonize a surge in cellular ROS levels following exposure to ROS inducing triggers can protect cancer cells from ROS mediated apoptosis. In this regard, it has been recently shown that hTERT overexpression in cancer cells alleviates cellular ROS levels by way of potentiating the cellular anti-oxidant defense systems and altering mitochondrial activity, thus conferring survival advantages onto cancer cells against ROS mediated death stimuli [51,52]. While high levels of ROS have been deemed to be detrimental, exposure of cells to a slight pro-oxidant state has been shown to result in the oxidation of caspases at the catalytic cysteine residues rendering them ineffective and the cells resistant to caspase-dependent apoptotic triggers [53,54]. Thus, ROS can play a dual role with regards to apoptosis by modulating the cellular micro-environment [55–57].

The intrinsic pathway of cell death is especially susceptible to ROS. Previously, it has been shown that exogenous addition of H_2O_2 has an inhibitory effect on Na^+/H^+ exchanger activity resulting in cytosolic acidification, which creates a conducive environment for apoptosis [58,59] and similarly, exposure of cells to the experimental drug C1 has also been shown to trigger intracellular H_2O_2 production, which causes intracellular acidification. This change in cytosolic pH influences the conformational status of Bax, resulting in its activation and oligomerization at the mitochondria, thereby facilitating the release of apoptosis amplification factors from the mitochondrial inter-membranous space [60,61]. The inner mitochondrial protein, ANT, is also a target of ROS modulation by virtue of its redox-sensitive cysteines, providing an additional mechanism by which drug-induced ROS production may activate mitochondrial apoptosis [62]. Moreover, superoxide radical (O_2^-) has also been shown to modulate the function of the OM protein, VDAC, to facilitate Cyt.C release [63]. Finally, ROS can directly modulate protein complexes within the mitochondrial electron transport chain, activate caspases and trigger cell death [64]. Other targets of ROS within mitochondria remain to be identified, but based on the pivotal involvement of the mitochondria in energy supply, lipid biosynthesis, as well as detoxification, it is highly probable that oxidative modification of these targets could affect cell fate.

3. The problem of death resistance

Surgery, radiation and chemotherapy have been the conventional therapies in cancer treatment. Different combinations of these treatments have been used to largely improve disease prognosis and combat cancer. However, the evolution of adaptive mechanisms provides cancer cells with the ability to evade apoptotic execution and bestows upon them a survival advantage.

3.1. Upregulation of pro-survival proteins

Upregulation of the anti-apoptotic family of proteins has been a frequent explanation for the resistance observed in cancer cells. Increased expression of the anti-apoptotic family members such as Bcl-2, Bcl-X_L and Mcl-1 has been often observed in cancer cells [65], where they serve to antagonize mitochondria-mediated cell death pathway. Specifically, Bcl-2/Bcl-X_L upregulation is clearly associated with poor prognosis in cancer [66]. The ability of these proteins to antagonize the pro-apoptotic family of proteins such as Bax and Bak has been the key mechanism by which these cells acquire resistance to apoptosis. As such, measures to target Bcl-2 or Bcl-X_L via anti-sense oligonucleotides have been employed in clinical studies to sensitize cancer cells to apoptotic triggers [67,68]. However, as eluded above there is emerging evidence to support a novel mechanism of death inhibition by Bcl-2 involving its ability to modulate cellular redox status and mitochondrial metabolism.

As described earlier, IAPs are a class of anti-apoptotic proteins upregulated in a variety of human cancers. IAPs inhibit the activity of caspases and hence protect cells from the deleterious effects of active caspases. Smac/DIABLO, a natural antagonist of IAPs, has been shown to sensitize cells to drug- and receptor-induced apoptosis by binding to XIAP and releasing caspase-9 in vitro.

In addition to the upregulation of anti-apoptotic proteins, activation of pro-survival paths such as PI3K-Akt pathway further helps to resist death triggers. Following stimulation by growth factors or cytokines, phosphatidylinositide-3-OH kinase (PI3K) is activated and phosphorylates phosphoinositides. Phosphatidylinositol 4, 5-bisphosphate 3 activates the kinase 3-phosphoinositide-dependent protein kinase-1, which in turn activates the kinase Akt by phosphorylation at two key regulatory sites. Once activated, Akt phosphorylates its target substrates

and initiates several pro-survival pathways. Akt phosphorylates IκB, which results in NFκB activation that promotes survival and Bad phosphorylation resulting in Bad inactivation and blocking of the apoptotic signal. In addition, phosphorylation of caspase 9 also blocks the induction of apoptosis [69].

Increased resistance to drugs that activate the extrinsic death pathway has also been observed with upregulation of proteins such as c-Flip and decoy receptors. c-Flip inhibits the autoproteolytic cleavage of pro-caspase 8, which is involved in DISC formation, and hence, the downstream transduction of the death signals via the extrinsic death pathway [70]. Decoy receptors can compete with death receptors for the ligands such as TRAIL and CD95, thus inhibiting/abolishing death signal transduction (reviewed in [71]). Thus cells with higher expression of decoy receptors or with mutant death receptors that retain their ability to bind the death ligand while lacking the ability to engage the downstream processes such as DISC formation are more resistant to the extrinsic apoptotic triggers.

3.2. Suppression of pro-apoptotic proteins

In addition to the upregulation of pro-survival factors, suppression of pro-apoptotic proteins also contributes to resistance against apoptosis-inducing therapeutic regimens. Thus, Bax is one key pro-apoptotic member that is frequently suppressed or mutated in cancers. The mutations include frameshifts and/or mutations at the BH domains, which lead to a loss of function. Indeed tumors with reduced Bax expression have been found to have a poorer prognosis [72,73]. In addition to Bax deficiencies, it has also been reported that Bak deficiencies can also lead to substantial inhibition of mitochondria-mediated apoptotic cell death. The activation of Bax and Bak is mediated via caspase 8-induced cleavage of the BH3 only protein Bid to tBid. Activation and oligomerization of BAX or BAK have been proposed to result in the formation of a VDAC-containing pore and permeabilization of mitochondrial membranes. This leads to the release of cyt *c* and the subsequent engagement of the Apaf-1-caspase-9 apoptosome complex, which activates downstream effector caspases. In this regard, suppression of components which act downstream of the mitochondria such as cyt *c*, Apaf-1 and caspases can protect the cells from apoptotic insults. Accordingly, the expression levels of several pro-apoptotic members of Bcl-2 family, such as Bim and Puma have been shown to correlate with colon carcinoma susceptibility to chemotherapy [74].

Silencing death receptors is another common strategy employed by cancer cells to protect them from death receptor-induced pathways (CD95 and TRAIL). Besides downregulation, death receptors can also be mutated such that even if the ligands can bind [77], they may still be unable to engage the intracellular apoptotic signaling pathway. The mutations include point mutations in the cytoplasmic death domain of CD95 and/or deletion that leads to a truncated form of the death receptor. Moreover, deletions and mutations of the death receptors TRAIL-R1 and TRAIL-R2 have also been observed in tumors [75,76].

3.3. Defects in p53 signaling pathways

P53 is the most commonly mutated tumor suppressor gene in human cancers. In addition to the loss of tumor suppression function and exertion of dominant-negative effects over the remaining wild-type protein, several p53 mutants can also actively regulate cancer development and progression [77]. Similarly, modulation of the regulators of p53 can also contribute to genome instability and apoptosis resistance. One such modulator is p19 ARF, which when activated by oncogenic stress facilitates p53 stabilization. As such, besides defects in p53, deficiencies in ARF have been shown to result in protection for tumors with wild-type p53 [78]. Additionally, upregulation of the p53 inhibitors MDM2 and/or MDM4 also leads to p53 degradation, thus offering further protection from cytotoxic insults [79].

4. Targeting the mitochondria as a novel strategy for cancer therapy

Though targeting the inherent adaptive anti-apoptotic strategies enhance sensitization of cancer cells to therapeutic regimes, the rise of resistance in cancer cells poses a challenge for scientists and physicians. In this regard, numerous anticancer agents target pathways that lie upstream of the mitochondria, which then converge onto the intrinsic death pathway. However, the deregulation of several master switch proteins such as HIF1, HKII, c-Myc and P53, leads to resistance in these cells. Thus, directly targeting the mitochondria may allow us to overcome the problem of resistance that arises from engaging the upstream pathways [4].

The mitochondria are the cellular powerhouses and hence the primary source of energy for the cells. Since cancer cells generally display a higher energy demand, targeting the cellular energy source seems to be an attractive solution. ATP is the energy currency of cells and a high ratio of ATP/ADP is produced primarily by the oxidation of glucose. Oxidation of glucose is a two stage process namely, glycolysis, which is the first stage, that occurs at the cytosol and oxidative phosphorylation, the second stage that occurs at the mitochondria, where most of the ATP is produced. In addition to the pertinent role in cell survival, the mitochondria are critical regulators of the intrinsic pathway of apoptosis [80]. They control several death effector cascades through the release of pro-apoptotic proteins such as Apaf-1, cyt *c* and Smac/DIABLO, which normally reside in the inter-membranous space. Thus, the convergence of these life and death regulatory roles at the mitochondria has made it a highly favorable target for therapeutic manipulation.

4.1. Cancer cell mitochondria

Therapeutic targeting of cancer cells have often relied on the inherent differences between normal cells and transformed cells, which allow for manipulation, better selection, and eradication of these cancer cells. In this regard, one of the main traits of cancer cells is their ability to proliferate rapidly and survive well under hypoxic conditions. This differential behavior has been reported as an outcome of altered metabolic efficiencies and/or mitochondrial functions, which will be discussed further in the sections below.

4.2. Altered bioenergetics of cancer cells

Rapidly proliferating cancer cells have a higher energy demand that needs to be met amidst an oxygen-deprived environment. The search to find the mechanisms by which cancer cells meet their energy requirement led to the findings by Otto Warburg [81] in 1926, and subsequently to the Warburg hypothesis that cancer cells derive most of their energy from glycolysis even under aerobic conditions, and that cancer is an outcome of defective mitochondrial energy metabolism. Since then, many theories have risen to challenge this hypothesis and question if the impaired mitochondrial metabolism is, indeed, the cause or an effect of transformation, given the other pertinent factors involved in the process of carcinogenesis [82,83]. In this regard, it is possible that upregulation of glycolysis could be an adaptive response of cancer cells to increase ATP production in an oxygen deprived environment. This shift in bioenergetics has also been suggested to confer tumor cells with additional mechanism of resistance as it transforms the intracellular milieu into a more acidic environment, conducive for invasion of surrounding tissues [84] (Fig. 2). Additional causes contributing to promote the resistance of mitochondria toward pro-apoptotic permeabilization reside in the downregulation of pro-apoptotic factors (e.g. ions, proteins, ROS) and

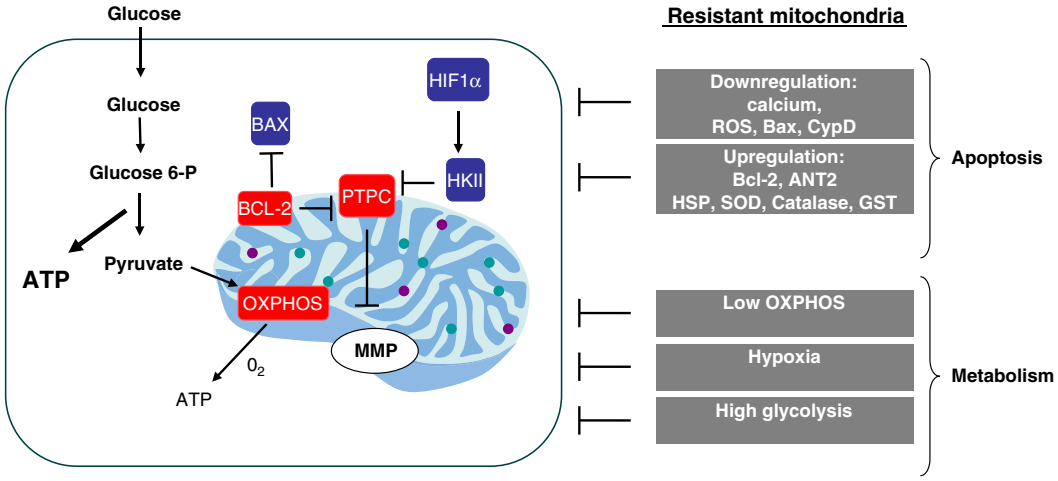

Fig. 2. Apoptosis-resistant mitochondria and cancer. Resistance to mitochondrial membrane permeabilization (MMP) is a common phenomenon observed in cancer cells. It often arises from blocking the permeability transition pore complex (PTPC) opening or due to the failure of pro-apoptotic BAX/BAK activation by anti-apoptotic BCL-2 family members. As a result, the release of pro-apoptotic proteins from the mitochondrial intermembrane space is inhibited. Resistance also arises from the down regulation of pro-apoptotic factors (proteins or second messengers such as calcium and reactive oxygen species (ROS)) and up-regulation of anti-apoptotic proteins, thus tilting the balance toward survival. Moreover, alterations in mitochondrial bioenergetics and hence the switch in energy supply from oxidative phosphorylation (OXPHOS) to glycolysis, along with conditions such as hypoxia can also contribute to MMP resistance. O_2: oxygen.

the upregulation of anti-apoptotic factors (e.g. Bcl-2, ANT2, chaperones, anti-oxidant enzymes …) (Fig. 2).

Though the reasons behind the glycolytic shift and the glucose addiction has yet to be fully unfolded, it is now known that this observation transcends across all malignancies, a trademark that is being utilized today in cancer diagnostics such as PET scans [85]. Positron emission tomography (PET) utilizes radioactive glucose molecules and provides a dynamic image of the body's interior based on the rates of glucose metabolism; a trait that is distinctly amplified in cancer cells. In the years following the postulation of the Warburg's hypothesis, the discovery of numerous critical cancer regulators, such as the p53 protein and other tumor suppressors or oncogenes have shifted focus away from the mitochondria. In the last decade however, a renewed interest has returned to the role of mitochondria and energy metabolism in cancer.

In addition to the increased glycolysis, the altered bioenergetics observed in many human carcinomas has been highly correlated with the downregulation of the catalytic subunit of the mitochondrial H + ATP synthase (β-F1-ATPase) [86]. Analysis of mitochondrial and glycolytic phenotypes in cancer cells and normal cells has also resulted in a bioenergetic signature, consistent across several types of human carcinomas including breast, colon and stomach. In this regard, Cuezva et al. defined a bioenergetic index of the cell (BEC) based on the levels of a bioenergetic marker of mitochondria relative to a cellular glycolytic marker [87]. The relative levels were assessed in tissue biopsies via immunohistochemical staining or immunoblotting. Their observations demonstrated that BEC index served as a reliable indicator along with the expression of β-F1-ATPase in breast, gastric, lung and esophageal cancers.

Another important cause of resistance of cancer cells to apoptosis, linked to the metabolic reprogramming of cancer cells, is the enhanced association of hexokinase with mitochondrial porin, VDAC. This has been suspected to affect VDAC pore opening and to limit the association of pro-apoptotic members of the Bcl-2 family (e.g. Bax, t-Bid) with VDAC and hamper mitochondrial permeabilization and cyt *c* release [88] (Fig. 2).

4.3. Perturbation of the mitochondrial DNA in cancer cells

In aerobic cells, mitochondrial respiration is one of the major sources of ROS. In the mitochondrial electron transport chain (ETC), there is a sequential transfer of electrons (e-) with cytochrome oxidase (COX) being the terminal acceptor, which reduces bound O_2 to water [89]. During this process of electron transfer, some electrons may leak onto oxygen and lead to the formation of ROS. Indeed, it has been estimated that at physiological oxygen levels, 1–4% of oxygen might be incompletely reduced to $O_2^{\bullet-}$ [90,91]. One key target of ROS is mitochondrial DNA (mtDNA), which encodes critical proteins of the ETC. The lack of protective histones around the mtDNA, limited DNA repair mechanisms found in the mitochondria, and the close proximity to the ROS generating ETC, makes the mtDNA more easily prone to ROS-induced DNA damage.

Defective mtDNA that translate into defective mitochondrial components can further aggravate mitochondrial dysfunction by accelerating aberrant ROS production and inefficient ATP generation. This can then result in oxidative damage of cellular DNA and proteins thus setting in motion, a vicious cycle of events [92]. As such, the mutations incurred due to oxidative damage either sensitize the cells to death signaling or confer survival advantage by altering cellular response to apoptotic stimuli, and the expansion of these surviving populations could give rise to resistant clones.

4.4. Morphological and physiological differences

The morphological differences observed in the mitochondria vary between tumor cell lines in terms of size, shape and count. It has been noted that the mitochondria in rapidly growing tumors tend to be fewer in number and smaller and have fewer cristae then slow growing tumors, normal cells or well differentiated tumors [93]. In addition, differences in the IM composition have also been observed between normal and transformed cells [94]. Interestingly, studies have also shown that the mitochondrial membrane potential is approximately 60 mV higher in carcinomas as compared to their normal controls [95].

5. Riding on mitochondrial dysfunctions to target cancer cells

The mitochondria are at the crossroads of life sustaining and death inducing paths. As such, it is an attractive option to be targeted to restore normalcy in defective cells and abrogate progression to malignancy. Alternatively, given the stark differences the mitochondria present in cancer cells, riding on its dysfunctions could help to select and sever the lifeline to these cancer cells.

5.1. Mutant mitochondrial DNA

Mitochondrial DNA mutations are often found in a variety of cancers including lung, ovarian, thyroid, colon, gastric, brain, bladder, head and neck and breast. Interestingly, the mutations are homoplasmic in nature and this has often been attributed to the clonal selection of cancer cells, and alternatively to unbiased mtDNA replication and sorting during cell division. As an outcome all the mtDNA in a cell and in a tumor express the same mtDNA mutations [96]. These homoplasmic mutations have much diagnostic value, as they are easily detectable in body fluids such as blood, saliva and urine. Due to its abundance and dominance in cancer cells, it may also be a possible option worth exploring as a molecular marker of cancer [97]. Given the immense impact mtDNA mutations has in determining cellular transformation coupled with the lack of efficient repair mechanisms at the mitochondria, research in the areas of DNA repair and maintenance at the mitochondria might provide valuable tools, which could help repair and/or protect mitochondrial genome from impending damage [98,99].

5.2. The switch in energy metabolism

Though maintenance of the intracellular pH at an optimal level is critical for the functioning of many enzymes and cellular processes, the high metabolic activity and glycolysis in cancer cells results in elevated levels of lactate production and the change in the intracellular pH activates membrane pH regulatory pumps, such as the Na+/H+ antiporter (NHE), to actively extrude H+ ions leading to an acidic extracellular and an alkaline intracellular milieu. To that end, a number of reports have demonstrated increased activity of the NHE-1 exchanger in cancer cells, which correlated with cellular sensitivity to apoptosis. In addition, the cellular uptake of chemotherapeutic drugs may also be altered by the changes in the pH gradient [100,101]. As such, drugs that may perturb the intracellular pH gradient could potentially modify cellular responses to the chemotherapeutic agents. In this regard, treatment with proton pump inhibitors (PPI), which acts by irreversibly binding to the proton pump and inhibiting proton translocation and acidification of the extracellular environment has been shown to reverse drug resistance in chemoresistant melanoma cancer cells and sensitize multi-drug resistant cells to cytotoxic drugs [102,103].

5.3. The energy supply

Since cancer cells display a high energy demand, drugs that can directly perturb mitochondrial respiration and glycolysis could lead to an extensive depletion of ATP, sensitizing cancer cells to death. In this regard, Sahra et al. demonstrated that treatment with 2-deoxyglucose (2DG), a potent inhibitor of glucose metabolism via the inhibition of hexokinase and metformin, a widely used anti diabetic agent that inhibits oxidative phosphorylation, induced massive reduction in cell viability in LNCaP prostate cancer cells as compared to normal prostate epithelial cells by depletion of cellular ATP [104].

Another drug that has been shown to block aerobic glycolysis in cancer cells, possibly by inhibiting hexokinase, is lonidamine (LND), which is derived from indazole-3-carboxylic acid. LND disrupts energy metabolism in cells resulting in ATP depletion, and in addition, leads to an accumulation of lactate in the cells [105,106]. This drug is now being used in combination therapy to sensitize cancer cells to conventional chemotherapeutic drugs such as cisplatin at lower doses [97,98,107,108]. Its prospects are also being reviewed in Phase III clinical trials [109].

High metabolic rates and mitochondrial dysfunction has been shown to lead to elevated intracellular ROS levels in cancer cells. Thus anti-cancer compounds that push the cellular ROS levels past the threshold for cancer cells are potent inducers of apoptosis. Pelicano et al. adopted a combinatorial treatment strategy involving 2-methoxyestradiol (2-ME), which inhibits SOD and results in O_2^- accumulation, with arsenic trioxide (As_2O_3) resulting in inhibition of mitochondrial respiration and increased O_2^- production in leukemia [110]. Similar death sensitizing effects of drug-induced ROS production have been reported with small molecule compounds LY30 [111,112], C1 [113], and resveratrol [114]. Along similar lines, it was recently shown that the small molecule orphan drug, dichloroacetate (DCA), reversed the metabolic reprogramming observed in the *ex vivo* glioblastoma model by targeting pyruvate dehydrogenase kinase II. DCA triggered cell death in cancer cells by inducing mitochondrial membrane depolarization and increased mitochondrial ROS production [115].

5.4. Mitochondria membrane permeabilization

The mitochondria is enveloped in two membranes commonly referred to as IM and the OM [116]. The IM surrounds the inner mitochondrial matrix and is folded into the numerous cristae, while the outer mitochondrial membrane surrounds the inner membrane, thus creating the intermembrane space. The intermembrane space is where many of the pro-apoptotic proteins such as Cyt. C, Smac/DIABLO and AIF are localized. In healthy cells, the IM is impermeable to ions [117], which allows the ETC to actively build up the proton gradient across the IM. The $\Delta\Psi_m$ results from the difference in electrical potential generated by the electrochemical gradient across the IM. This proton gradient is then used to drive ATP synthesis via ATP synthase that is located at the IM. As such, the maintenance of the proton gradient is imperative for survival and is a tightly regulated mechanism [117–119].

Of interest, the mitochondrial membrane potential is often found to be elevated in cancer cells. This negative transmembrane potential found on the inside of the mitochondria allows the mitochondria to be targeted using positively charged ions such as the delocalized lipophilic cations (DLCs), which accumulate in the mitochondria. DLCs are toxic to the mitochondria at higher concentrations. Thus their selective accumulation in the mitochondria of carcinomas, which display an increased mitochondrial membrane potential, allows for targeted elimination of these cancer cells [95,120]. One such compound is MKT-077, which accumulates in the mitochondria in a potential-dependent manner to inhibit respiration and growth [121]. However, the development of this compound was discontinued after results of phase I clinical trials indicated renal toxicity.

5.5. The permeability transition pore

The PTPC is a high conductance channel formed by the association of several mitochondrial proteins. Its composition is still unknown and could vary depending on the tissues. Previously, PTPC composition has been proposed to include VDAC, ANT and cyclophilin D (Fig. 3). ([122,123]. Under physiological states, this complex is kept closed and can open in a voltage-dependent and dynamic manner. The additional PTPC interacting proteins would include peripheral benzodiazepine receptor that is localized at the outer mitochondria and kinases such as hexokinase and creatine kinase [117,123,124].

Physiological factors such as ROS, changes in pH and association with proteins such as Bax induce a conformational change resulting in the opening of the pore and the sudden increase in the permeability of the IMM brings about the dissipation of $\Delta\Psi_m$. This then leads to a rapid swelling of the matrix that has a high solute content and the subsequent rupture of the OMM and release of the intermembrane space proteins [118,125].

Thus drugs that can disturb PTPC would allow for direct targeting of the mitochondria and help circumvent the problem of apoptosis resistance upstream of the intrinsic mitochondrial pathway. Moreover, differences in the composition of PTPC and the critical interacting proteins within the complex have been reported between

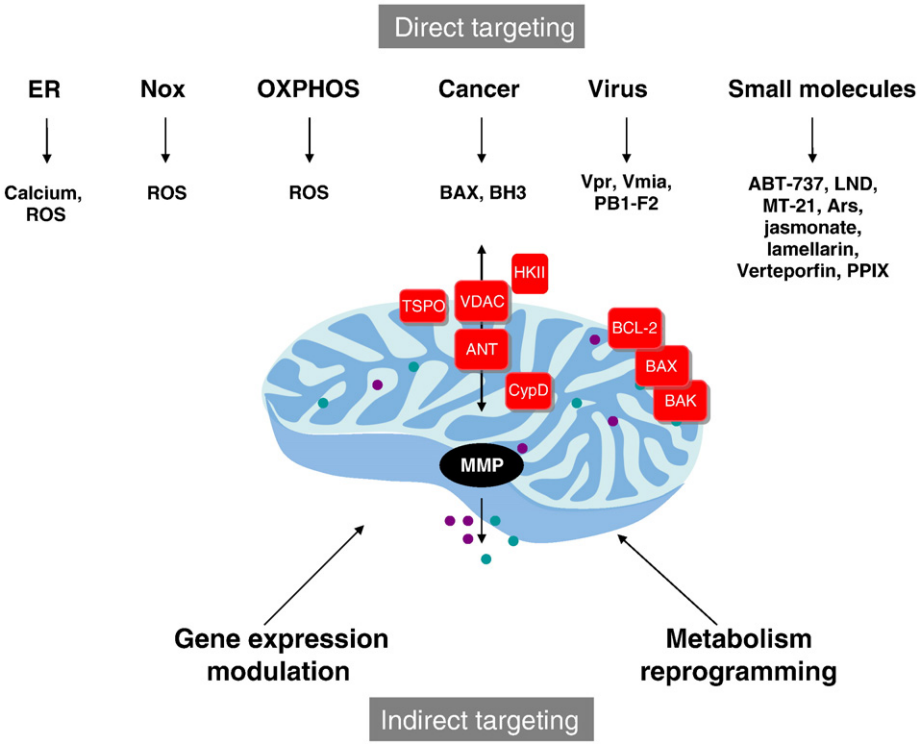

Fig. 3. Mitochondria: promising targets for cancer chemotherapy. Permeability transition pore complex (PTPC) and Bcl-2 family members can be directly targeted by (1) second messengers such as calcium and ROS originating from the endoplasmic reticulum (ER), plasma membrane (NADPH oxidase; NOX) and oxidative phosphorylation (OXPHOS); (2) endogenous tumor suppressor proteins or derived peptides, (3) viral proteins or derived peptides and (4) small molecules. Indirectly, MMP could be favored by modulation of gene expression or metabolic reprogramming to activate OXPHOS and decrease glycolysis. ANT: adenine nucleotide translocase; VDAC: voltage-dependent anion channel; CypD: cyclophilin D; TSPO: Translocator protein; HKII: hexokinase II.

normal and cancerous cells. For instance, studies on the patterns of ANT showed that the transcript levels were shown to be different between the tumor and transformed cell lines. In particular, ANT2 gene was found to be upregulated in oncocytomas and renal tumors [126]. Apart from ANT, the levels of peripheral benzodiazepine receptor (PBR), hexokinase II and mitochondrial creatine kinase have also been found upregulated in some tumors [127,128]. In an attempt to target the ANT, several ANT ligands have been used to induce or prevent mitochondrial apoptosis [129]. These ANT-based drugs will largely aim to convert the ANT into non-specific pores [22] or to inhibit its ADP/ATP exchange function [130]. Other drugs include PK11195, an antagonist of the PBR. PK11195 displays high binding affinity to PBR and has been shown to contribute to a decrease in $\Delta\Psi_m$ and cyt *c* release. Its anti-tumor effects have now been tested in combination with a variety of cytotoxic and chemotherapeutic agents in various human tumor cell lines [131].

5.6. The members of the Bcl-2 family

As described in the earlier sections of this review, the Bcl-2 family of proteins plays a critical role in switching the balance between survival and death largely by regulating MMP. In that light, Bax and Bak have been shown to regulate the ANT and VDAC channels, altering the mitochondrial functions such as causing ANT to open and VDAC to close resulting in matrix swelling and outer mitochondrial membrane disruption [22,132]. The anti-apoptotic proteins Bcl-2 and Bcl-X_L on the other hand have been shown to directly interact with VDAC and elicit a protective role at the mitochondria [133]. Bcl-xl binds with VDAC1 and prevents the closure of the channel, hence maintaining the ATP/ADP exchange, preventing mitochondrial hyperpolarization, matrix swelling and rupture [134].

The altered ratios favoring the pro-survival proteins such as Bcl-X_L and Bcl-2 over the pro-apoptotic proteins such as Bak and Bax have been shown to lead to chemoresistance in cancer cells. The need to drive the ratio toward death in cancer cells has led to the development of anti-cancer compounds that can either act to suppress the pro-survival proteins such as Bcl-2 and Bcl-X_L, or mimic the pro-death BH3 only proteins such as Bid, Bim, Bad, Bmf, Noxa and PUMA to promote cell death. In this regard, HA14-1 was one of the first small-molecule Bcl-2 inhibitors to be reported. This synthetic chromene molecule works by displacing Bax and hence releasing it from Bcl-2's inhibition, resulting in apoptosis. Of value, it has been shown to overcome radioresistance and chemoresistance in several cancer models including glioblastoma, prostate cancer and leukemia [135,136]. Another attractive drug target is the naturally occurring Bcl-2 inhibitor gossypol, which is derived from cottonseeds [137]. Gossypol, like HA14-1 was also shown to bind to the BH3 domain of Bcl-2. The anti-apoptotic effects of Gossypol have been demonstrated in head and neck cancer, colon, prostate, pancreatic and leukemia cell lines [138–140]. Unfortunately, the general toxicity and undesirable pharmacological properties it presented halted its progress in clinical development. More promisingly, an antisense strategy to down-regulate Bcl-2 and restore death sensitivity is currently under clinical evaluation for the treatment of melanoma [141].

While drugs that could suppress anti-apoptotic proteins have yielded some promising results, attempts to increase sensitivity by mimicking the structures of pro-apoptotic proteins has also led to the discovery of some novel BH3 mimetics (Fig. 3). One such well understood BH3 mimetic is ABT-737. ABT-737 mimics the BH3-only protein Bad and has been found to bind and inhibit Bcl-2, Bcl-w and Bcl-X_L. However, it displays a weak affinity to MCL-1 [142]. Thus cancer cells that overexpress MCL-1, are resistant to this small molecule compound. To that end, in a small cell lung cancer model,

ABT-737 was shown to sensitize cells to apoptosis induced by chemotherapeutic agents [143]. Of note, ABT-737 is highly effective against cancers with elevated expression of Bcl-2 and is an efficient apoptosis inducer in the presence of Bax and Bak [144].

Another group of BH3 peptides synthesized by Shangary et al. to mimic Bax and Bad BH3 domains demonstrated effective engagement of the intrinsic pathway with the release of cyt *c*, even in Bcl-2 and Bcl-X_L overexpressing cells [145]. BH3 peptides have also been shown to trigger oligomerization of Bax and Bak, resulting in MOMP and cyt *c* release [146]. Though the use of BH3 mimetics holds much promise, the use of BH3 peptides in cancer therapeutics has been limited due their poor cell permeability, bioavailability, solubility and metabolic stability *in vivo*. To address these issues, tagged proteins [147] or proteins with chemical modifications [148] have been employed.

6. Concluding remarks

Restoration of cell death pathways via the targeting of mitochondrial proteins is an attractive concept that emerged following the identification of the central orchestrators of this pathway. Indeed, this pathway is frequently impaired in cancer cells and contributes to the development of resistance to conventional chemotherapy. Several small molecules, targeted anti/pro-oxidants and antisense oligonucleotides have been designed to activate pro-apoptotic proteins as well as to block anti-apoptotic proteins and are currently under clinical evaluation. Results of clinical trials will determine whether the promise that these strategies hold will be realized for a significant improvement in the clinical management of cancers that are refractory to conventional interventions.

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