Too much death can kill you: inhibiting intrinsic apoptosis to treat disease

Kaiming Li[1,2], Mark F van Delft[1,2] and Grant Dewson[1,2]

[1] The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Melbourne, VIC 3052, Australia, Melbourne

[2] Department of Medical Biology, University of Melbourne, Royal Parade, Melbourne, VIC 3052, Australia, Melbourne.

Correspondence to:

Grant Dewson, dewson@wehi.edu.au, Tel:+61393452935

Mark van Delft, vandelft@wehi.edu.au, Tel:+61393452555

KEYWORDS

BAK, BAX, BCL-2, intrinsic apoptosis, mitochondria

Abstract

Apoptotic cell death is implicated in both physiological and pathological processes. Since many types of cancerous cells intrinsically evade apoptotic elimination, induction of apoptosis has become an attractive and often necessary cancer therapeutic approach. Conversely, some cells are extremely sensitive to apoptotic stimuli leading to neurodegenerative disease and immune pathologies. However, due to several challenges,

pharmacological inhibition of apoptosis is still only a recently emerging strategy to combat pathological cell loss. Here, we describe several key steps in the intrinsic (or mitochondrial) apoptosis pathway that represent potential targets for inhibitors in disease contexts. We also discuss mechanisms of action, advantages and limitations of small-molecule and peptide-based inhibitors that have been developed to date. These inhibitors serve as important research tools to dissect apoptotic signaling and may foster new treatment to reduce unwanted cell loss.

Introduction

Apoptosis is a highly regulated process of cell death that is important for multicellular organisms to maintain normal embryonic development and tissue homeostasis (Ke *et al*, 2013; Singh *et al*, 2019). Ligation of cell surface death receptors, such as members of the tumour necrosis factor (TNF) receptor superfamily, initiates *extrinsic apoptosis*, while stresses including growth factor withdrawal and DNA damage activate *intrinsic* (or *mitochondrial*) *apoptosis*, which is orchestrated by the B-cell lymphoma 2 (BCL-2) family of proteins (Czabotar *et al*, 2014). The BCL-2 family consists of three groups of members with opposing functions. The dynamic interplay between these proteins, including with other non-BCL-2 proteins, determines cell fate in response to apoptotic stress. The anti-apoptotic members include BCL-2 and its homologues (MCL-1, BCL-X_L, BCL-W and A1/BFL1). The pro-apoptotic members include a group of pro-apoptotic proteins termed BH3-only proteins (BAD, BIK, BID, HRK, BIM, NOXA, PUMA and BMF) that initiate the pathway by triggering activation of a second pro-apoptotic group, the executioner proteins BAX, BAK and BOK. BAX and BAK permeabilise the mitochondrial outer membrane (MOM) upon activation and either is critical for apoptosis in most cells (Lindsten *et al*, 2000; Wei *et al*, 2001), whilst the function of BOK is less well resolved (Echeverry *et al*, 2013; Hsu *et al*, 1997; Llambi *et al*, 2016). Mitochondrial outer membrane permeabilisation (MOMP) by BAX and/or BAK is considered the "point of no return" in the apoptosis pathway as it leads to irrevocable damage to mitochondria that disrupts the generation of ATP by oxidative phosphorylation and also causes the release of mitochondrial components into cytosol that trigger end-stage apoptotic events. In particular, cytochrome *c* when released from the mitochondrial intermembrane space ignites the activation of caspases, a conserved family of cysteine-dependent aspartate-specific proteases. Activated caspases proteolytically cleave multiple proteins to dismantle the cell and prepare it for clearance by resident phagocytic

cells (Slee *et al*, 2001). Additionally, caspases actively suppress the innate immune response to Danger-Associated Molecular Patterns (DAMPs) such as mitochondrial DNA (mtDNA) and reactive oxygen species (ROS), which are released from damaged mitochondria during apoptosis (Riley *et al*, 2018; White *et al*, 2014).

Aside from the role of apoptosis in normal physiology, both silencing and overactivation of apoptosis can lead to pathological conditions. Inactivation of this pathway is a hallmark of cancer (Hanahan & Weinberg, 2011). Hence, since the discovery that BCL-2 promotes tumourigenesis by impeding cell death (Vaux *et al*, 1988), significant effort has been expended in targeting the apoptotic machinery to treat cancer. An improved understanding of apoptosis regulation underpinned the advancement of small-molecule inhibitors, termed *BH3 mimetics*, which induce mitochondrial apoptosis by inhibiting specific anti-apoptotic BCL-2 members, as promising anti-cancer therapeutics (Adams & Cory, 2007; Czabotar *et al.*, 2014). The approval of the BCL-2-selective BH3 mimetic drug, Venclexta/venetoclax/ABT-199 to treat chronic lymphocytic leukemia and lymphoma has confirmed that targeting the cell death machinery for therapy is a tractable and promising strategy (Roberts *et al*, 2016).

Despite the successes in the developing agonists of intrinsic apoptosis and the considerable evidence that excessive or inappropriate apoptosis drives a variety of pathologies including acute and chronic degenerative diseases, the clinical development of targeted small molecule inhibitors of cell death has lagged (Butler *et al*, 2003; Springer *et al*, 1999; Vila *et al*, 2001). This is in part due to our incomplete understanding of the complex nature of apoptosis signaling, crosstalk between intrinsic and extrinsic apoptosis and also with other death pathways, and a scarcity of structural data on the protein interaction network, particularly as many of these events occur in a membrane environment. Moreover, early attempts to block apoptosis likely focused on targeting the wrong checkpoint in the pathway. With positive outcomes in preclinical disease models, pan-caspase inhibitors were considered a promising means to limit tissue injuries caused by unwanted cell death (Yaoita *et al*, 1998). However, inhibiting caspases has largely failed in clinical trials because of severe side effects and unsatisfactory efficacy (O'Brien, 2009). The reason for these failings is likely multi-factorial and may reflect emerging non-apoptotic roles of certain caspases and their key roles in mediating the immune response to pathogens. However, it is also now recognised that targeting caspases that act at a late stage in the intrinsic apoptosis cascade, is unlikely to be protective as these cells are already destined to die due to the irrevocable

damage to mitochondria (Figure 1). Hence, targeting molecular events upstream of caspase activation, may provide improved opportunity for the selective blockade of mitochondrial apoptosis. More recently, efforts have culminated in the discovery of selective small-molecule inhibitors of BAX and/or BAK that target the "point of no return" in mitochondrial apoptosis to provide more selective and lasting cytoprotective effects (Garner *et al*, 2019; van Delft *et al*, 2019).

There is significant interest in blocking death receptor signalling and extrinsic apoptosis including the development of several FDA-approved antibodies that neutralise TNF signaling that has been reviewed elsewhere (Croft *et al*, 2013). In this review, we will focus on the emerging concept of inhibiting *intrinsic apoptosis*. We will describe the molecular machineries in mitochondrial apoptosis and discuss the exciting therapeutic opportunities to intervene and block excessive mitochondrial apoptosis in disease. Then we will discuss selective inhibitors of mitochondrial apoptosis pathways in terms of their modes of action. Moreover, we will describe preclinical use of these inhibitors, with a focus on potential pitfalls and how they may be developed for future clinical translation.

Intrinsic apoptosis: A multitude of players

Intrinsic apoptosis can be divided into three key phases: 1) The balance of interactions between BCL-2 family proteins, 2) BAX/BAK-mediated MOMP, and 3) The activation of apoptotic caspases. Each of these phases represent potential targets for therapeutic intervention to impede apoptotic cell death, although long-term protection will only be afforded through targeting steps 1 or 2.

BCL-2 and non-BCL-2 proteins orchestrate the life-or-death decision

BCL-2 family proteins are characterised by one (BH3-only members) or more BCL-2 homology (BH) domains. Irrespective of their ability to prevent or induce apoptosis, those proteins with more than one BH domain adopt a similar overall fold that is characterised by a buried BH3 domain and a hydrophobic surface groove (Petros *et al*, 2004). Through these conserved sequence motifs and structural elements, the pro-apoptotic and anti-apoptotic BCL-2 proteins partake in dynamic and competitive interactions to strike a delicate balance that serves as the first checkpoint for mitochondrial apoptosis (Figure 1). This balance is upset, however, when a cell receives diverse apoptotic stimuli, as specific BH3-only proteins

are activated or upregulated to interact with anti-apoptotic and executioner members to tip the balance toward cell death.

BH3-only proteins insert their BH3 domain into the hydrophobic surface groove on binding BAK and BAX, or at an alternative "rear-pocket" involving residues in the BAX/BAK α1 and α6, thereby triggering a coordinated series of activating conformation changes in the executioners, including exposure of their own BH3 domain (Czabotar *et al*, 2013; Dewson *et al*, 2008; Moldoveanu *et al*, 2013; Wei *et al*, 2000) (Figure 2A). BH3-only proteins likewise bind to the hydrophobic groove of the anti-apoptotic BCL-2 proteins, thereby limiting the latter's ability to sequester newly activated BAK and BAX. As a result, activated BAK and BAX are free to oligomerise on the MOM to cause permeabilisation. Initially BAK and BAX form homodimers by inserting their everted BH3 domain into the hydrophobic groove of a neighbouring molecule (Brouwer *et al*, 2014; Czabotar *et al.*, 2013; Dewson *et al.*, 2008; Subburaj *et al*, 2015). Homodimers then form higher-order oligomers that permeabilise the MOM through a mechanism that is yet to be clearly defined, but likely involves interaction with MOM lipids in a toroidal lipidic pore (Cowan *et al*, 2020; Qian *et al*, 2008; Salvador-Gallego *et al*, 2016; Terrones *et al*, 2004). The similar protein interfaces employed in these varied interactions between pro-apoptotic and anti-apoptotic proteins poses conceptual challenges when exploiting these interfaces for targeted drug development.

While much focus has been given to BCL-2 family proteins, important roles for other non-BCL-2 proteins as gatekeepers of MOMP is emerging. For example, voltage-dependent anion channel 2 (VDAC2) is proposed to influence BAK and BAX apoptotic function. VDAC2 interacts with inactive BAK to impair its oligomerisation and thus limit MOMP (Cheng *et al*, 2003). VDAC2 is also required for BAK and BAX to target mitochondria effectively, and consequently is necessary for the apoptotic function of BAX (Lauterwasser *et al*, 2016; Ma *et al*, 2014; Naghdi *et al*, 2015). These seemingly opposing functions in controlling apoptosis are yet to be fully resolved. Additionally, VDAC1, the most abundant protein in the MOM, is proposed to oligomerise and initiate cytochrome *c* release independent of BAX/BAK in certain circumstances (Huang *et al*, 2015). Whilst cells that lack all three VDAC isoforms can still undergo apoptosis, suggesting that they are ultimately dispensable (Baines *et al*, 2007), the VDACs remain potentially important arbiters of mitochondrial integrity during apoptotic signalling and so are potential targets to manipulate apoptosis (van Delft *et al.*, 2019).

Mitochondrial membrane dynamics in apoptosis

As a consequence of MOMP, a number of components in the mitochondrial intermembrane space are released into the cytosol to trigger end-phase apoptotic signaling. Pro-apoptotic factors include cytochrome *c* (Liu *et al*, 1996), Second Mitochondria-derived Activator of Caspase (SMAC)/Direct IAP Binding protein with Low pI (DIABLO) (Du *et al*, 2000; Verhagen *et al*, 2000), and the serine protease OMI/high temperature requirement A2 (HTRA2) (Suzuki *et al*, 2001). However, recent findings have challenged the notion of MOMP as the "point of no return" in intrinsic apoptosis. Incomplete MOMP (termed "minority MOMP") leads to limited caspase activation, which is insufficient to trigger cell death. Instead, limited caspase activity triggered by minority MOMP can cause genomic instability that drives tumorigenesis (Ichim *et al*, 2015). Incomplete MOMP in epithelial cells may also contribute to immune defense against infection (Brokatzky *et al*, 2019). Since caspases attenuate innate immune responses to damaged mitochondria restricted caspase activation after minority MOMP can lead to activation of cGAS/STING-dependent production of inflammatory cytokines (Rongvaux *et al*, 2014; White *et al.*, 2014). Although limited mitochondrial permeabilisation can be tolerated in certain cells, the physiological relevance of minority MOMP is not fully resolved. However, it remains a potential mechanism that may contribute to inflammatory or chronic degenerative conditions.

In addition to the rupture of the MOM, changes in the mitochondrial inner membrane (MIM) may also influence the kinetics of apoptosis. Some apoptogenic components, including the majority of cytochrome *c,* reside in the mitochondrial cristae, structures formed by invaginations of the MIM (Scorrano *et al*, 2002). The constrictions of the cristae, the cristae junctions, are mainly governed by Optic Atrophy 1 (OPA1), a dynamin related GTPase that facilitates MIM fusion (Frezza *et al*, 2006). Low OPA1 expression (Olichon *et al*, 2003), dissociation of OPA1 oligomers (Griparic *et al*, 2004), or cleavage by the metalloprotease OMA1 (Jiang *et al*, 2014), promotes cristae opening and the full release of cristae components during apoptosis, including cytochrome *c*, to promote caspase activity and the end-phase of apoptosis (Frezza *et al.*, 2006; Yamaguchi *et al*, 2008) .

Whilst OPA1 located in the MIM links mitochondrial fusion to suppression of mitochondrial apoptosis, mitochondrial fission that occurs during early apoptosis is implicated to promote cell death (Karbowski *et al*, 2004). Dynamin-related protein 1 (DRP-1), a key mediator of mitochondrial fission, was demonstrated to trigger MOMP partially by interacting with BCL-2 proteins (Frank *et al*, 2001; Karbowski *et al*, 2002).

DRP-1 oligomerises and induces MOM constriction primarily at ER-mitochondria contact sites to enable the transmission of Ca^{2+} from ER to mitochondria, which triggers mitochondrial apoptosis (Prudent *et al*, 2015).

Whilst in most settings manipulation of mitochondrial dynamics and remodelling is unlikely to dictate the ultimate fate of a cell whose MOM is breached, it may influence the kinetics of caspase activation and cell death. So, in light of recent discoveries that cells may tolerate incomplete MOMP and limited caspase activation, these processes may indeed represent targets to determine cell fate.

Caspases and inhibitor of apoptosis proteins: the counterbalancing acts

Apoptotic caspases can be functionally subdivided into *initiators* (caspase-8, -9 and -10) and *effectors* (caspase-3, -6 and -7). Following MOMP, released cytochrome *c* promotes the oligomerisation of Apoptotic Protease Inhibiting Factor-1 (APAF-1) into a heptameric complex termed the apoptosome that serves as a platform for auto-activation of initiator caspase-9. Subsequently, caspase-9 proteolytically activates the effector caspases, which in turn cleave hundreds of substrates to manifest the characteristics of apoptotic cells including membrane blebbing, cell shrinkage, chromosomal DNA fragmentation and the presentation of signals that guide efferocytosis by resident phagocytes.

As part of the endogenous checkpoints negatively regulating apoptosis, a group of E3 ubiquitin ligases named Inhibitor of Apoptosis Proteins (IAPs) inhibit caspases, through three distinct mechanisms. IAPs can bind to the N-terminal IAP-binding motif (IBM) of caspase-9 to sequester and inhibit it (Srinivasula *et al*, 2001). A range of apoptogenic mitochondrial components including SMAC/DIABLO and OMI/HTRA2 contain similar N-terminal IBMs after maturation, and upon release into the cytosol, they compete with caspase-9 for binding to IAPs, enabling caspase-9 release and activation. IAPs can also competitively inhibit effector caspase-3 and caspase-7 by occupying their active site to hinder proteolysis of true substrates (Chai *et al*, 2001; Huang *et al*, 2001; Riedl *et al*, 2001). Lastly, the E3 ligase activities of IAPs promote the ubiquitination and proteasome-directed degradation of caspases (Hao *et al*, 2004).

Crosstalk between apoptosis and other regulated cell death modalities

In most circumstances, apoptosis is considered immunologically silent due to the suppression of DAMPs, the preservation of cell membrane integrity and efferocytotic clearance of the

apoptotic cell. In contrast, more recently defined death modalities including caspase-1-dependent pyroptosis and RIPK1/RIPK3/MLKL-mediated necroptosis are characterised by permeabilisation of the plasma membrane leading to the release of cytoplasmic contents that trigger inflammatory responses (Silke *et al*, 2015). Despite these stark differences in outcome, a picture is now emerging of a highly coordinated interplay between these different cell death modalities that determines how cells respond to specific stressors, exemplified recently by the highly coordinated and adaptable response to infection (Doerflinger *et al*, 2020). Whilst the molecular control of this interplay is still being resolved, the machineries involved have been found to play roles in multiple cell death modalities. For example, the ligation of death receptors can result in either extrinsic apoptosis or necroptosis, and can also trigger mitochondrial apoptosis, with caspase activity determining pathway execution. Understanding the complex interplay and potential redundancies between these pathways is key to exploiting them to limit pathogenic cell death.

Excessive mitochondrial apoptosis in disease

Excessive apoptosis participates in the pathology of both acute and chronic degenerative diseases. Significant gains have been made in understanding the role of mitochondrial apoptosis in pathological conditions based on genetic profiling of patients, gene expression analysis and genetically engineered model organisms (Table 1). Whilst potential roles for non-apoptotic cell death pathways have been implicated in various pathologies, we will focus here on the collective evidence indicating that excessive mitochondrial apoptosis is central to the loss of specific cell types and succedent tissue degeneration.

Mitochondrial apoptosis in acute degeneration

Ischaemic injury

Apoptosis plays a key role in the pathogenesis of ischaemic organ injuries such as in the heart and brain. Acute myocardial infarction is one of the common cardiac syndromes and a leading cause of death. Numerous studies have shown that increased apoptotic loss of cardiomyocytes and other cells in the heart is an important component in the pathogenesis of myocardial infarction and heart failure (Narula *et al*, 1996; Olivetti *et al*, 1997). Ischaemia-reperfusion (I/R) to resolve the infarct also induces apoptosis of cardiomyocytes *in vivo* (Gottlieb *et al*, 1994). Studies on transgenic mice showed that overexpression of BCL-2 protected against injury of cardiomyocytes after I/R as indicated by reduced markers of

apoptosis and significantly smaller infarct volumes (Brocheriou *et al*, 2000; Chen *et al*, 2001). Similarly, BAX deletion reduced apoptosis and improved myocardial function following I/R injury (Hochhauser *et al*, 2007; Hochhauser *et al*, 2003). Consistent with these findings, targeted deletion of the BH3-only protein PUMA significantly preserved cardiac function in a mouse model of I/R (Toth *et al*, 2006). Interestingly, cardiac-specific deletion of caspase-3 and caspase-7 did not significantly reduce cardiomyocyte death after ischaemic insult (Inserte *et al*, 2016), indicating that targeting executioner caspases is too late in the apoptotic pathway to halt myocardial I/R injury in the face of mitochondrial damage in the cardiomyocyte.

Apoptosis shapes embryonic development of the nervous system, with multiple mouse models of defective apoptosis exhibiting neural development defects (Ke *et al*, 2018; Li *et al*, 1997; Zou *et al*, 1997). Terminally differentiated neurons are necessarily highly resistant to cell death including apoptosis. Mitochondrial apoptosis elicited by certain pathological insults in post-mitotic neurons is likely mediated by BAX, given that mature neurons seemingly express only a truncated variant of BAK that cannot itself cause mitochondrial damage (Sun *et al*, 2003; Uo *et al*, 2005). Accumulating evidence shows that apoptosis of neurons occurs in the brain following ischemic stroke. Samples from patients post-ischaemia exhibited cytosolic cytochrome *c* indicative of mitochondrial permeabilisation and activated caspase-3 in neurons (Rami *et al*, 2003). Consistently, in a mouse model of cerebral ischaemia, caspase-3 activation, DNA fragmentation and apoptotic bodies were observed (Namura *et al*, 1998). *Bax* deletion also reduced neuronal apoptosis and infarct volume in a middle-cerebral artery occlusion model of ischaemic stroke (D'Orsi *et al*, 2015), consistent with BAX being the main executioner in neurons. Remarkably, apoptosis contributes to ischaemic neuronal death in young brains more so than in adult brains, substantiating the notion that sensitivity to apoptosis decreases after development (Zhu *et al*, 2005).

Traumatic brain injury

Although the immediate damage as a result of mechanical trauma to the brain is likely irreversible, the consequent biochemical alterations of regulated cell death pathways that lead to tissue degeneration in the penumbra is potentially amenable to intervention. Initially, tissue degeneration after brain trauma was linked solely to unregulated necrosis, while the involvement of apoptosis has emerged more recently. Brain tissue samples from traumatic brain injury patients compared with controls indicated altered expression of anti- and

pro-apoptotic proteins that suggested a tipping of the balance toward apoptosis (Clark *et al*, 1999). In addition, elevated expression of APAF-1 and cleaved caspase-3 were observed at injury sites in rat models induced by traumatic injuries of the spinal cord (Springer *et al.*, 1999) or brain (Yakovlev *et al*, 2001). Compared with wild-type mice, *Bax* knockout mice exhibited significantly reduced neuronal death following traumatic brain injury, although this protection did not manifest in improved cognition, attributed to potential developmental defects in *Bax* null mice (Tehranian *et al*, 2008). Collectively, these biochemical features of apoptosis after traumatic injury suggest that application of neuroprotective protocols targeting apoptosis could improve patient outcome.

Mitochondrial apoptosis in chronic degenerative diseases

Neurodegenerative diseases

Evidence suggests that mitochondrial apoptosis of various neuronal subtypes contributes to chronic neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and multiple sclerosis (MS) (Table 1). However, despite significant effort, attempts to stave off neuronal loss in these degenerative diseases to achieve neuroprotection have failed. There is unfortunately no cure for neurodegeneration and currently approved treatment strategies are limited to relieving symptoms. The major challenges in limiting the neurodegenerative process itself are a lack of understanding of the varied triggers for neuronal cell death in these complex diseases and the mode of cell death responsible for neuronal loss.

Parkinson's disease

The motor symptoms of PD are linked to the selective loss of dopaminergic neurons predominantly in the *substantia nigra pars compacta* region of the brain. The correlation between dopaminergic neuron apoptosis and PD is under debate, as initial studies showed no significant differences in the expression of BCL-2 proteins in PD patient samples (Banati *et al*, 1998; Jellinger, 2000; Wüllner *et al*, 1999). However, elevated BAX and also activated caspase-3 have been reported in post-mortem brain samples (Tatton, 2000). Moreover, deletion of BAX was sufficient to prevent the death of cultured neurons and PD-like motor deficits in mice treated with the mitochondrial toxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Gomez-Lazaro *et al*, 2008; Vila *et al.*, 2001). Intriguingly, although BAX-deletion inhibited dopaminergic neuron death in

6-OHDA-treated mice, it failed to significantly reduce motor deficits, suggesting that blocking death could not functionally rescue the neurons (Kim *et al*, 2011). Overexpression of the anti-apoptotic XIAP was also shown to be neuroprotective in an MPTP-induced mouse model of PD (Crocker *et al*, 2003; Eberhardt *et al*, 2000). Whether these acute toxin-induced models replicate the chronic cell death signalling in patients with familial or idiopathic PD, however, is questionable.

A mechanistic link between early onset PD and mitochondrial apoptosis has been established via the E3 ubiquitin ligase Parkin and its upstream regulator PTEN-induced kinase 1 (PINK1). The genes encoding Parkin (*PRKN/PARK2*) and PINK1 (*PINK1/PARK6*) are PD susceptibility genes, with mutations known to cause over 50% of juvenile onset autosomal recessive PD (Kitada *et al*, 1998). The mitochondrial serine/threonine kinase PINK1 protects neurons from intrinsic apoptosis, while PD-associated mutations and a kinase-inactive mutation abrogated this protective effect (Petit *et al*, 2005). Furthermore, when activated by PINK1, Parkin likewise promotes cell survival by ubiquitinating BAK and BAX to impair their activity (Bernardini *et al*, 2019; Johnson *et al*, 2012). However, following severe and irreparable mitochondrial damage induced by agents such as valinomycin, Parkin actually promotes apoptosis through the ubiquitination and degradation of anti-apoptotic MCL-1 (Carroll *et al*, 2014; Zhang *et al*, 2014), suggesting that Parkin controls the tipping point for neuronal survival in response to graded mitochondrial damage. Interestingly, in addition to ubiquitination, other post-translational modifications are also linked to neuronal apoptosis in PD. An S-nitrosylated form of X-linked IAP (XIAP), which has compromised ability to inhibit caspases, is enriched in PD patients and mouse models (Tsang *et al*, 2009). Likewise, elevated S-nitrosylation of Parkin is observed in both PD animal models and patient samples that impairs its ligase activity and neuroprotective function (Chung *et al*, 2004). OPA1 missense mutations have been found in patients with PD, further implicating the mitochondria (Carelli *et al*, 2015). However, whether dysregulated OPA1 predisposes dopaminergic neurons to apoptosis or impairs mitochondrial function is unclear.

Alzheimer's disease

AD is the most common neurodegenerative disease and is characterised by extracellular deposits of amyloid-β (Aβ) and phosphorylated tau that are thought to drive neuronal loss. Expression of various pro-apoptotic proteins including BAX and BAK, but also

anti-apoptotic BCL-2 proteins, are elevated in susceptible neurons in AD patients (Su *et al*, 1997). Additionally, *in vitro* treatment of primary neurons with aggregation prone $A\beta_{1-42}$ peptide induced expression of the BH3-only protein BIM. The consequent neuronal cell death was blocked by deletion of BAX, but not caspase-3, consistent with the need to block mitochondrial apoptosis upstream of perturbation of the MOM (Kudo *et al*, 2012; Selznick *et al*, 2000). This dependency on BAX-mediated apoptosis for Aβ-induced neuronal toxicity was subsequently confirmed in *ex vivo* hippocampal brain slices (Kudo *et al.*, 2012). However, whether ablation of apoptosis protects from the cognitive deficits in mouse models of AD is unclear.

Amyotrophic lateral sclerosis/motor neurone disease

ALS/MND pathogenesis is driven by the apoptotic death of motoneurons. The first gene associated with familial ALS was Cu/Zn-superoxide dismutase (SOD1) (Rosen *et al*, 1993). Mutant SOD1 localises predominantly to the mitochondrial intermembrane space (MIS), where it can trigger cytochrome *c* release to induce apoptotic death (Magrané *et al*, 2009; Takeuchi *et al*, 2002). Studies revealed that mutant SOD1 can initiate apoptotic pathways by either directly compromising mitochondrial integrity (Pedrini *et al*, 2010), or by sequestering anti-apoptotic BCL-2 (Pasinelli *et al*, 2004). *In vitro*, early apoptotic markers increased in a motoneuron-like cell line expressing mutant SOD1 (Joshi *et al*, 2018). In *in vivo* mouse models of ALS the pro-apoptotic proteins BIM and PUMA were upregulated (Hetz *et al*, 2007; Kieran *et al*, 2007). Whilst deletion of BAX was seemingly sufficient for neuroprotection and to maintain motor function and prolong survival in SOD1 mutant (G93A) mice, it failed to stop axonal denervation, questioning the utility of anti-apoptosis agents in ALS (Gould *et al*, 2006). However, that deletion of both BAX and BAK was functionally neuroprotective and prolonged survival in the $SOD1^{G93A}$ mouse model, suggests such a strategy could be ameliorative (Reyes *et al*, 2010).

Huntington's Disease

HD arises due to the expansion of a CAG trinucleotide repeat in the *huntingtin* (*HTT*) gene, which leads to an expanded polyglutamine (polyQ) stretch in the mutant protein. Mitochondrial apoptosis is activated in the striatum of severe HD patients and murine models of HD (Kiechle *et al*, 2002). *In vitro*, mutant HTT can trigger apoptosis with evidence of chromatin condensation, DNA laddering and caspase-3 activation, while wide-type HTT is

protective against apoptotic stimuli (Rigamonti *et al*, 2000; Saudou *et al*, 1998). The role of mutant and wide-type HTT in apoptosis was further supported by studies on proteins that interact with HTT. Normally, Huntingtin-Interacting Protein 1 (HIP-1) is sequestered by HTT (Kalchman *et al*, 1997), but increased polyQ length of mutant HTT disrupts this sequestration, enabling free HIP-1 to activate caspase-9 and -3 (Choi *et al*, 2006). It has been shown that mutant HTT can also translocate to the nucleus to activate p53, which in turn increases expression of NOXA and PUMA to trigger apoptosis (Steffan *et al*, 2000). Consistently, brains of HD patients and transgenic mouse models have elevated p53, while genetic deletion of p53 alleviated neurodegeneration and behavioral abnormalities (Bae *et al*, 2005).

Degenerative retinal diseases

A body of studies indicate that apoptosis of human retinal cells including retinal ganglion cells, photoreceptor cells and endothelial cells is at the root of degenerative retinal diseases such as age-related macular degeneration and diabetic retinopathy (Mizutani *et al*, 1996). Indeed, haplo-insufficiency of OPA1, the guardian of mitochondrial cristae formation, is responsible for autosomal dominant optic atrophy as a result of retinal ganglion cell degeneration (Alexander *et al*, 2000). Behr syndrome, which is characterised by early-onset optic atrophy, is also directly linked to heterozygous mutations in the *OPA1* gene (Schaaf *et al*, 2011). Besides OPA1, quantitative analysis determined that anti-apoptotic protein BCL-X_L is downregulated after optic injury (Levin *et al*, 1997). BCL-X_L deficiency also accelerates retinal cell loss in mouse models of retinal degeneration, whereas its overexpression has the opposite effect (Harder *et al*, 2012; Liu *et al*, 2001). Similarly, ectopic BCL-2 expression preserved retinal ganglion cells during both natural retinal development in mouse embryos and stress-induced optic damage in adult mice (Bonfanti *et al*, 1996; Cenni *et al*, 1996). Beside ganglion cells, photoreceptor cells were also preserved by BCL-2 overexpression in mouse models of retinal degeneration (Chen *et al*, 1996; Nir *et al*, 2000). BAX-deficient mice exhibited long-term preservation for retinal cells after damage (Semaan *et al*, 2010; Yang *et al*, 2004). Furthermore, retinal capillaries in patients with diabetic retinopathy are characterised by increased apoptosis, while blocking endothelial cell apoptosis facilitates tissue revascularisation (Grant *et al*, 2020). Overall, these pre-clinical studies support a role for intrinsic apoptosis in retinal degeneration.

Degenerative bone disease

Chondrocytes are crucial for adequate bone function. Apoptosis of chondrocytes is necessary during normal joint development, while accelerated apoptotic cell death may lead to chronic diseases such as osteoarthritis. This degenerative joint disease is characterised by degradation of articular cartilage and changes in the subchondral bone. However, whether chondrocyte apoptosis is a cause or a result of cartilage degeneration in osteoarthritis is debatable. Chondrocytes from patients with osteoarthritis had enhanced apoptosis in the cartilage sections compared with controls (Pérez *et al*, 2005) with the degree of apoptosis significantly correlating with clinical grade (Hashimoto *et al*, 1998). Animal models of experimental osteoarthritis also confirmed that during the early phases of osteoarthritis, chondrocyte apoptosis contributes to the pathogenesis of cartilage degradation (Almonte-Becerril *et al*, 2010).

Besides osteoarthritis, osteoporosis is another common disorder of bone degeneration resulting from increased survival of osteoclasts (OCs) and/or increased apoptosis of osteoblasts (OBs) and osteocytes, leading to a suppression of bone formation. Which cell type is predominantly altered to cause osteoporosis is currently unknown. Glucocorticoid treatment is a common cause of osteoporosis, and promotes apoptotic death of osteoblasts and osteocytes, suggesting that this glucocorticoid-induced bone disease is the result of aberrant apoptosis of bone cells (O'Brien *et al*, 2004). The induction of human osteoblast apoptosis was associated with an increased ratio of pro-apoptotic proteins to anti-apoptotic proteins (Jilka *et al*, 1998). Mechanistically, glucocorticoids can also activate caspase 3-mediated apoptosis in cultured cells (Liu *et al*, 2004). Another hormone, androgen, has a distinct function in skeletal homeostasis. It has been reported that androgen enhanced osteoblast apoptosis by decreasing BCL-2 expression in a post-transcriptional manner (Wiren *et al*, 2006).

Overexpression of BCL-X_L or BCL-2 has been shown to increase bone volume and retain bone strength during aging in transgenic mice (Moriishi *et al*, 2016; Pantschenko *et al*, 2005). Consistent with these findings, selective deletion of BAK and BAX in osteoblasts likewise increased bone formation (Jilka *et al*, 2014), suggesting that mitochondrial apoptosis is important in degenerative bone diseases.

Considering the significant and increasing medical and economic costs of degenerative diseases of aging, the need for novel therapeutic and preventative strategies is pressing.

Preservation of degenerating tissues has been proposed as one avenue for therapeutic intervention, including the inhibition of mitochondrial apoptosis. However, it should be noted that although apoptosis is implicated in many of these chronic degenerative conditions (Table 2), definitive evidence for mitochondrial apoptosis as a major driver of disease pathology is still lacking, with other cell death pathways such as necroptosis and pyroptosis also implicated to play a role. Even if apoptotic signalling drives the disease pathology, blocking it may trigger alternative, potentially highly inflammatory, cell death modalities that may be detrimental. Furthermore, even if the cell death program is arrested, the preserved "zombie" cells may still be functionally impaired due to altered gene expression patterns, disrupted cellular signalling or damaged intracellular structures. This raises the conceptual question whether protecting cells that are destined to die due to these underlying cellular defects would actually be beneficial. So, although resolving contextual issues is going to be key, an ability to efficiently and specifically suppress early apoptotic events in non-proliferative and indispensable cell types is likely to have significant potential for the treatment of certain degenerative diseases.

SELECTIVE INHIBITION OF MITOCHONDRIAL APOPTOSIS

Given the central role of apoptosis in the pathologies discussed above, targeted pharmacological inhibition of apoptosis may represent a promising therapeutic strategy. Many putative apoptosis inhibitors, identified through both target-based and unbiased phenotypic screens, are thought to act upon targets localised to mitochondria. Some are proposed to act directly upon the BCL-2 effector proteins BAX and/or BAK, while others are believed to impair apoptosis through other mechanisms. Whilst several inhibitors have been described, none have yet progressed beyond the early stages of pre-clinical development. We will summarise several of the most recently described inhibitors below and place them in the context of our current understanding of the apoptosis pathway.

Blocking caspase activity

For many years, caspases were the most actively pursued target for inhibiting apoptosis, owing largely to their key role in driving the widespread cleavage of cellular protein substrates during the culminating phase of the apoptotic cell death signaling cascade. Prior experience with drugging proteases facilitated the rapid identification of specific caspase inhibitors, some of which progressed to clinical trials aiming to provide cytoprotective

benefit in settings such as organ transplantation and metabolic diseases associated with liver damage (Table 2). Unfortunately, caspase inhibitors have largely underwhelmed in clinical trials (Table 2) (Garcia-Tsao *et al*, 2019; Ratziu *et al*, 2012), which may be due in part to their poor pharmacokinetic properties and the very high fractional inhibition of caspases required to effectively block their action (Methot *et al*, 2004). That caspases have many functions beyond apoptosis further complicates their use in patients (Carlile *et al*, 2004; Fujita *et al*, 2008; Guery *et al*, 2011; Jacquel *et al*, 2009; Sordet *et al*, 2002),

Blocking the apoptosome is another potential way to limit caspase activity and apoptosis, and both small molecule and peptoid inhibitors of APAF-1 have been described (Lademann *et al*, 2003; Wang *et al*, 2016). These have shown some *in vivo* efficacy in animal models of stem cell transplantation as well as cisplatin-induced ototoxicity and ischemic kidney injury (Orzáez *et al*, 2014; Shim *et al*, 2013). Intriguingly, as APAF-1 inhibitors block caspase activation downstream of MOMP, their mode of action has been shown to involve the recovery of early apoptotic cells in an autophagy-dependent fashion (Gortat *et al*, 2015). The same reliance on autophagy was noted for caspase inhibitors to preserve cell growth potential following MOMP (Colell *et al*, 2007).

Although it is possible for a cell to recover following minority MOMP, this tends to occur at low frequency. In the main, once a cell undergoes MOMP it is committed to die. Hence, although inhibiting caspase activation or apoptosome formation downstream of BAX/BAK-mediated MOMP may slow cell death, it is generally not an effective way to confer cytoprotection. Long-term cellular protection from insults that trigger intrinsic apoptosis can be achieved more efficiently by targeting regulators upstream of mitochondrial damage. Indirect approaches that quench stress signals, such as anti-oxidants or ROS scavengers represent one option. However, conceptually a more direct way to achieve this would be with molecules that preserve mitochondrial integrity through targeting BAX/BAK or proteins that modulate their activity.

Preserving mitochondrial integrity to prevent cell death

Mitochondria have a central role as the platform for intrinsic apoptosis, while also critical for cellular metabolism, iron cluster biogenesis and calcium signaling. Hence, a disrupted MOM is generally considered a death knell for a cell. As a potent source of pro-inflammatory DAMPs, mitochondrial damage can also have non-cell-autonomous effects on surrounding

cells and tissues. Thus, there is much interest in generating small molecules that protect mitochondrial integrity. A number of key players implicated in mitochondrial integrity have been identified, and some have been successfully targeted with small molecules. Continued development of these inhibitors will provide not only a greatly improved toolkit to decipher mitochondrial functions in cell survival and death, but also an expanded arsenal of small-molecule drug leads to inhibit pathological apoptosis.

Inhibitors of VDAC1 oligomerisation

Mammalian voltage-dependent anion channels (VDAC1, VDAC2, VDAC3) are responsible for transporting ions and metabolites across the MOM, but can also regulate MOMP. In the case of VDAC2, this occurs through modulating BAK and BAX (Cheng *et al.*, 2003; Ma *et al.*, 2014), whilst VDAC1 is argued to form oligomeric pores that enable the release of mitochondrial cytochrome *c* independently of BAX and BAK (Huang *et al.*, 2015). The pore-forming propensity of VDAC1 does not fit with the consensus view that BAX and BAK are required for cytochrome *c* release and that VDACs in general are dispensable (Baines *et al.*, 2007; Wei *et al.*, 2001). Nevertheless, VDAC1 has been targeted with small molecules to preserve mitochondrial integrity. 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), an anion conductance inhibitor, has been shown to interact non-specifically with VDAC1 and inhibit its oligomerisation (Keinan *et al*, 2010). Small molecules that selectively prevent VDAC1 oligomerisation were also identified from a high-throughput cell-based phenotypic screen relying on bioluminescence resonance energy transfer (BRET) to monitor VDAC1 oligomerisation (Ben-Hail *et al*, 2016). A series of analogues, including VBIT-4, were shown to interact with VDAC1, inhibit mitochondrial dysfunction, and block apoptosis of cultured cells (Ben-Hail *et al.*, 2016).

The role of VDAC1 in apoptosis may in fact be quite specialised with recent findings suggesting that oligomerisation of VDAC1 enables mtDNA release and an interferon (IFN) response without initiating typical cell death processes associated with cytochrome c release and caspase activation. This mtDNA release was blocked by VBIT-4 in mouse models of systemic lupus erythematosus (Kim *et al*, 2019) and more recently TDP-43-driven ALS (Yu *et al*, 2020). Therefore, rather than having broad utility in limiting apoptosis for cytoprotection, inhibitors of VDAC1 oligomerisation may be beneficial to limit inflammatory responses caused by the release of mitochondrial DAMPs.

Inhibitors of the mitochondrial fission protein DRP-1

In cells, Dynamin-Related Protein 1 (DRP-1)-induced mitochondrial fission plays an active role in apoptosis (Estaquier & Arnoult, 2007; Frank *et al.*, 2001; Lee *et al*, 2004). In response to mitochondrial fission induced by nitric oxide (NO) in neurons, BAX translocated to, and selectively aggregated at, fission sites, whilst blocking DRP-1 inhibited BAX foci formation (Yuan *et al*, 2007). These findings suggest that DRP-1 might be a useful target in numerous degenerative diseases.

One widely used compound that was originally described as a DRP-1 inhibitor is the quinazolinone derivative mitochondrial division inhibitor-1 (mdivi-1). mdivi-1 was identified from a phenotypic screen for small molecules that would preserve mitochondrial architecture and counteract the growth arrest in a temperature-sensitive mitofusin mutant yeast strain (Cassidy-Stone *et al*, 2008). It was proposed that mdivi-1 inhibited mitochondrial fission by impairing the GTPase activity of DNM-1, the yeast homologue of DRP-1. Although originally proposed to act similarly in mammalian cells, mdivi-1 has been shown to be a very weak inhibitor of human DRP-1 and instead to attenuate cell death by inhibiting mitochondrial respiration complex I and ROS production independently or DRP-1 (Bordt *et al*, 2017). Although its precise mode of action remains uncertain, mdivi-1 has demonstrated neuroprotection in animal models of ischaemic stroke (Grohm *et al*, 2012), traumatic brain injury (Wu *et al*, 2016), Parkinson's disease (Rappold *et al*, 2014), Alzheimer's disease (Reddy *et al*, 2018) and Huntington's disease (Zhao *et al*, 2018).

A cell-permeating peptide, P110, has also been rationally designed to selectively inhibit both the GTPase activity of DRP-1 as well as its interaction with mitochondrial fission 1 protein (FIS-1), an adaptor that recruits DRP-1 to the MOM (Qi *et al*, 2013). In cultured neurons, P110 preserved mitochondrial function and blocked excessive fission and apoptosis caused by a neurotoxin responsible for atypical forms of parkinsonism (Qi *et al.*, 2013). It has also demonstrated benefit in mouse models of retinal degeneration (Kim *et al*, 2015) and ALS (Joshi *et al.*, 2018), highlighting the potential of targeting DRP-1 to limit pathological apoptosis in these settings.

Whilst these *in vivo* studies are of interest and suggest that modulating DRP-1 may have therapeutic benefit, it remains unclear whether suppressing mitochondrial fission limits intrinsic apoptosis or rather it potentiates mitochondrial activity. Moreover, although inhibition of mitochondrial fission may slow events downstream of mitochondrial damage,

much like the inhibition of caspases, it is unlikely to confer long-term protection, hence the rationale for targeting mitochondrial fission to block cell death remains unclear.

Compounds targeting mitochondrial respiration

mdivi-1 is not the only apoptosis inhibitor proposed to target a component of the mitochondrial electron transfer chain. Another was found through a small molecule library screen for inhibitors of BAX/BAK-mediated apoptosis induced by BIM expression (Jiang *et al*, 2016). That molecule, compound A, was shown to covalently bind succinate dehydrogenase subunit B of respiratory complex II. Compound A afforded clonogenic protection to cells in culture and limited the loss of dopaminergic neurons in a 6-OHDA-induced mouse model of PD. Intriguingly, although pre-treatment with compound A reduced cytochrome *c* release it had only a modest impact on BAX oligomerisation. Compound A was therefore proposed to act downstream of BAK/BAX oligomerisation, challenging the notion that BAX/BAK oligomerisation and MOMP is the death knell for a cell. It remains under debate whether disruption of the electron transport chain and mitochondrial respiration are consequences of mitochondrial apoptosis or are events that guide it. Although these compounds are early in their development, their mode of action is unique and they have revealed interesting new biology.

Inhibitors of the pro-apoptotic BCL-2 effector proteins BAX and BAK

Given that a variety of apoptotic signals culminate in activating BAX and BAK to damage mitochondria as the pivotal step in intrinsic apoptosis, there has been intense interest in developing targeted small-molecule inhibitors of these critical effector proteins.

Molecules that influence lipid bilayers

There is an emerging appreciation that MOM lipids play an active role in MOMP, either to act as a bridge that links BAX/BAK oligomers in the proteolipidic pore or to promote membrane curvature, which appears key to MOM rupture (Basanez *et al*, 2002; Cowan *et al.*, 2020). Interestingly, several molecules known to alter the structural and elastic properties of lipid bilayers have been reported to inhibit BAX-mediated MOMP and cytochrome *c* release, including dibucaine, propranolol and cholesterol (Christenson *et al*, 2008; Polster *et al*, 2003). It is possible that these hydrophobic molecules, and some of the others described

below, might exert their anti-apoptotic function in part by altering the physical properties of the MOM.

Proteinaceous inhibitors

Protein and peptide inhibitors of BAX and BAK can help to uncover novel modes of regulation, with insights to be gained from the study of viral inhibitors. Viruses often develop strategies to block host cell apoptosis to promote their persistence and replication. In many cases these inhibitors are structural homologues of BCL-2, but several non-BCL2 BAX/BAK inhibitors have also been described. One example is the cytomegalovirus protein m38.5/vMIA (viral Mitochondria-Localised Inhibitor of Apoptosis), which binds to a novel regulatory site on BAX through a 20 amino acid helical domain that both stabilises cytosolic BAX and prevents MOM insertion and oligomerisation (Figure 2B) (Arnoult *et al*, 2004; Ma *et al*, 2012). Another cytomegalovirus protein m41.1/vIBO (viral inhibitor of BAK oligomerisation) is instead a selective inhibitor of BAK (Cam *et al*, 2010). The mode of action of this short 57-amino acid protein is less clear, but could equally shed new insight to facilitate the rational design of peptide-based or small molecule inhibitors.

The mitochondrial-derived peptide humanin is another naturally occurring peptide inhibitor of BAX (Guo *et al*, 2003), which has been shown to confer cytoprotection in a variety of disease models (Hazafa *et al*, 2021). Humanin is proposed to prevent BAX activation and translocation to mitochondria through a direct interaction, but the nature of this interaction remains unclear (Guo *et al.*, 2003), and other interacting partners including BID have also been reported (Hazafa *et al.*, 2021; Morris *et al*, 2020). *In vitro* evidence now suggests that humanin may impair apoptosis by aggregating BAX and BID into stable fibril structures (Morris *et al.*, 2020; Morris *et al*, 2019).

Peptide inhibitors of BAK have also been developed using structure-guided design. It was understood that BH3-only proteins trigger BAX/BAK activation through transient interaction with the hydrophobic surface groove of BAX/BAK (Figure 2A). Upon BH3-only protein dissociation, BAX/BAK change conformation to expose their BH3 domain to facilitate homodimerisation through the same hydrophobic surface groove. Thus, a BH3-peptide that stably interacts with the BAX or BAK groove may impede activation and oligomerisation. Guided by structures of the BIM BH3 domain bound to BAK, BH3 peptides were designed to incorporate a non-natural amino acid predicted to stabilise a charge interaction with BAK (Brouwer *et al*, 2017). As anticipated, the modified peptide interacted

more stably with the BAK hydrophobic groove, but did not induce significant BAK conformation change and prevented other BH3 domains from engaging with and activating BAK (Brouwer *et al.*, 2017). These studies represent the first rationally designed inhibitor of BAK or BAX and highlight the canonical hydrophobic groove as a key site to inhibit their apoptotic activity.

Anti-apoptotic proteins sequester the exposed BH3 domain of BH3-only proteins (termed *Mode 1*) or of activated BAX and BAK (termed *Mode 2*) in their own hydrophobic groove to block BAX/BAK activation and homo-oligomerisation (Llambi *et al*, 2011). However, regions in anti-apoptotic proteins other than the hydrophobic groove might also inhibit BAX or BAK, as a synthetic stapled peptide based on the BH4 domain in the BCL-2 N-terminus bound to BAX and inhibited early steps in BAX conformation change induced by a BIM BH3 peptide (Barclay *et al*, 2015). Photo-affinity cross-linking coupled with mass spectrometry identified multiple residues contacted by the peptide. A hotspot of labelling suggested that the BH4 peptide bound BAX at a site comprising residues in α2 and α3, whilst possibly also involving residues in α1 and α6 distal to the canonical hydrophobic groove (Figure 2B). Although the BH4 peptide competitively inhibited binding of an activator BH3 peptide to the groove, an allosteric mechanism may also contribute to its inhibitory effect.

Synthetic antibody fragments (Fab) that bind and inhibit BAX activation have also been described (Uchime *et al*, 2016). 3G11, a representative Fab, was shown to bind the rear activation site of BAX comprising residues in α1 and α6. It was proposed to block BH3-only proteins from binding to the same region to trigger conformational changes in BAX.

Carbazole compounds/Mitochondrial Channel Inhibitors (iMACs)

Brominated carbazole derivatives, termed Mitochondrial Channel Inhibitors (iMACs), were among the first small molecule inhibitors of BAX/BAK described. They were identified based on their ability to block the permeabilisation of artificial liposomes and mitochondria by recombinant BAX (Bombrun *et al*, 2003). These compounds blocked, and possibly reversed, oligomerisation of both BAX and BAK, inhibited cytochrome *c* release triggered by recombinant BID (tBID) and protected cells from treatment with staurosporine or Interleukin-3 withdrawal (Peixoto *et al*, 2009). Interestingly, these chemicals are structurally similar to Latrepirdine, an antihistamine drug that was shown to have protective effects on neurons (Bachurin *et al*, 2001).

Recently, the same brominated carbazole compounds were again identified as BAX inhibitors in a liposome permeabilisation screen. NMR studies indicated that these molecules, termed BAI1 (iMAC1) and BAI2 (iMAC2), bound directly to BAX with micromolar affinity, predominantly contacting residues in the BAX α3, α4 and α5, close to, but distinct from, the binding site of vMIA inhibitory peptide and BCL-2 BH4 peptide (Figure 2B and C) (Garner *et al.*, 2019). The molecules were shown to inhibit early events in BAX activation including membrane association and exposure of the BH3 domain (Garner *et al.*, 2019), but not the preceding N-terminal epitope exposure (Amgalan *et al*, 2020), suggesting that binding of the inhibitor to inactive BAX must prevent a discrete conformational transition in the stepwise activation of BAX (Figure 3). Allosteric stabilisation of the inactive conformer of BAX was proposed to explain their mode of action, although given the proximity of their binding to the hydrophobic groove (Figure 2C), direct competition with activating BH3-only proteins may also contribute. The compounds inhibited mitochondrial depolarisation, caspase-3/7 activation and cell death induced by TNF-α/cycloheximide in *Bak* knockout mouse embryonic fibroblasts (MEFs), but not *Bax* knockout MEFs, indicating that the inhibitors were selective for BAX (Garner *et al.*, 2019). Further, BAI1/iMAC1 limited BAX-mediated doxorubicin-induced cardiomyopathy in mouse models, but it did not compromise the efficacy of doxorubicin-induced cancer cell killing either *in vivo* and *in vitro* (Amgalan *et al.*, 2020), suggesting that BAK was unrestrained in the tumour cells. However, this specificity for BAX is difficult to reconcile with the ability of the inhibitors to block apoptosis of wild-type MEFs that express both BAX and BAK (Garner *et al.*, 2019) and a previous study that suggested activity against BAK (Peixoto *et al.*, 2009).

An *in vivo* screen for pro-neurogenic molecules uncovered another carbazole compound, P7C3, that when directly infused into the brain of mice could enhance neuron viability (Pieper *et al*, 2010). P7C3 was further developed to improve potency and reduce toxicity (MacMillan *et al*, 2011; Naidoo *et al*, 2014), and this chemical series exhibited neuroprotective effects in animal models of PD and ALS (De Jesús-Cortés *et al*, 2012; Tesla *et al*, 2012), retinal dystrophies (Asai-Coakwell *et al*, 2013), and traumatic brain injury (Blaya *et al*, 2014). The compound also demonstrated antidepressant effects, possibly by preserving immature neurons (Walker *et al*, 2015). Due to its structural relatedness to the brominated carbazoles (Bombrun *et al.*, 2003), P7C3 was initially proposed to limit neuronal degeneration by restraining BAX activity. However, indirect mechanisms involving

nicotinamide phosphoribosyltransferase (Wang *et al*, 2014; Yin *et al*, 2014) and glycogen synthase kinase 3β have also been proposed (Gu *et al*, 2017).

BAX channel inhibitors (BCIs)

BAX channel inhibitors (BCI1 and BCI2) are another class of small molecule BAX inhibitor identified through lipid bilayer permeabilisation assays (Hetz *et al*, 2005). These compounds inhibited apoptosis in cells induced by the broad kinase inhibitor staurosporine and also suppressed hippocampal damage in a rodent brain ischaemia model (Hetz *et al.*, 2005). Although not as potent in a following study, these inhibitors impaired conductance through channels formed by BAX oligomers downstream of BAX activation and membrane insertion, (Peixoto *et al.*, 2009). However, BAX oligomerisation was not directly assessed in these studies and these inhibitors have not progressed, hence their mechanism of action has not been confirmed.

Inhibitors of BAX/BAK activation

A small molecule series that blocks both BAX and BAK was also identified using a tBID/BAX-mediated liposome permeabilization screen. These inhibitors (MSN-50, MSN125, DAN004) reduced both BAX and BAK apoptotic activity on liposomes with low micromolar IC50 and provided long-term protection in cultured cell lines and primary cortical neurons (Niu *et al*, 2017). Mechanistically, the compounds inhibited the oligomerisation of BAX and BAK, whilst not impacting their ability to localise to the MOM. Cross-linking analysis revealed that the formation of BAX/BAK dimers and higher order oligomers were both reduced. Evidence for direct binding to BAX or BAK has not yet been observed, but the ability of these compounds to inhibit oligomerisation of both BAX and BAK suggests that they target a conserved interface, consistent with the ability of BAX and BAK to co-associate through BH3:groove interactions during apoptosis (Ma et al CDD 2014). Given that MSN-125 also prevented exposure of an N-terminal epitope characteristic of BAX activating conformation change suggests these inhibitors may also block interaction with BH3-only proteins, potentially implicating the hydrophobic groove or rear pocket comprising α1 and α6 as BAX activation trigger sites (Czabotar *et al.*, 2013; Gavathiotis *et al*, 2008). With recent studies indicating that higher order oligomerisation of BAX and BAK may be mediated by protein:lipid as opposed to protein:protein interactions (Cowan *et al.*, 2020; Li *et*

al, 2017; Uren *et al*, 2017), understanding how these inhibitors engage BAX and BAK may provide important new insight into the role MOM lipids play in BAX/BAK oligomerisation.

Recently, Eltrombopag, a thrombopoietin receptor agonist approved by the FDA for the treatment of thrombocytopenia, was identified through an *in silico* screen to have structural similarity to the BAX activator BAM7 (Gavathiotis *et al.*, 2008; Spitz *et al*, 2021). Yet despite a similar binding site at the α1/6 rear pocket of BAX, in contrast to BAM7, Eltrombopag inhibited BAX activation *in vitro* and in cellular assays, although its protective effect on 3T3 cells that express both BAX and BAK is difficult to reconcile with its lack of activity in BAX knockout MEFs (Spitz *et al.*, 2021). Whilst the contribution of this BAX inhibitory effect to the therapeutic activity of Eltrombopag is unknown, this compound adds to the library of tool compounds to further interrogate and potentially modulate BAX apoptotic activity.

WEHI-9625: a BAK-selective inhibitor

Liposome-based assays have been the richest source of identifying BAX/BAK inhibitors, but for technical reasons these assays are typically performed using BAX to drive permeabilisation. As such, the identified inhibitors have been more likely to target BAX than BAK. Using a different strategy centered on a cell-based phenotypic screen for small molecules that could protect cultured cells from cell death induced by a BH3-mimetic drug, a series of BAK-selective inhibitors was identified (van Delft *et al.*, 2019). The lead molecule developed, WEHI-9625, was shown to selectively inhibit BAK-driven apoptosis in cells with low nanomolar IC50. Using genetics and chemical biology its target was resolved as VDAC2, with WEHI-9625 preserving the interaction between BAK and VDAC2 when challenged with BH3-peptides and other apoptotic triggers. In doing so, WEHI-9625 blocked early steps in BAK activation including conformation change and homodimerisation, thereby preserving long-term clonogenic survival (Figure 3). Without structures of the BAK:VDAC2 complex the molecular detail of how WEHI-9625 stabilises their association remains unclear, but this study provides a path forward for the development of selective BAK inhibitors and advances our understanding of the mechanisms that control BAK apoptotic function. It also highlights the potential of BAX/BAK binding partners as putative targets to modulate apoptosis and the advantage to understanding the complexities of these interactions for therapeutic targeting.

Selective vs dual-specificity BAX/BAK inhibitors

BAX and BAK are constitutively expressed in most cell types and mediate apoptosis in a redundant manner. Hence, although targeting one executioner alone may prove partially protective, effective blockade of apoptosis may only come when both proteins are inhibited. In this case, dual-specificity inhibitors that exploit common features of BAX and BAK may be advantageous (Niu et al., 2017). However, inhibitors with dual-specificity conceptually carry greater risk of on-target toxicity that may limit their clinical utility, especially in chronic diseases for which long term dosing is required. In certain circumstances, where cell death specifically relies on either BAX or BAK, selective inhibition may be a feasible proposition. For instance, apoptosis in post-mitotic neurons is likely BAX-dependent as mature neurons have limited expression of full-length BAK that is competent to mediate MOMP (Deckwerth *et al*, 1996). Hence, deletion of BAX alone is often sufficient to confer protection to neurons in various animal models of both acute and chronic neurodegeneration (Table 1).

Conclusions

The last two decades have marked an era of understanding the role of apoptosis in disease, with remarkable recent research and clinical progress in the therapeutic modulation of apoptotic cell death. Given the intensifying impact of excessive apoptosis in disease pathogenesis, a variety of pre-clinical apoptosis inhibitors have been developed, providing invaluable means to investigate molecular mechanisms and explore therapeutic opportunities to address unmet clinical need. However, several barriers need to be overcome for these targeted apoptosis inhibitors to progress to the clinic including improved understanding of the mechanism of action, enhanced potency, selectivity, cell permeability and evidence of pathway modulation in cells. This would be facilitated by structural information on target engagement for structure-activity relationship studies. The capacity to permeate the blood-brain barrier is an additional hurdle that must be overcome for these compounds to be of use in the treatment of acute neuronal injury or neurodegenerative disease. Furthermore, given the essential role in apoptosis in limiting tumour development, there are understandable concerns that systemic, long-term administration of apoptosis inhibitors to treat chronic degenerative diseases may actually promote carcinogenesis. Acute administration of inhibitors, for example to block pathogenic cell death following traumatic injury or ischaemic stroke circumvents this issue, whilst phased or targeted drug delivery could limit this

potential risk in treating more chronic conditions. Moreover, given that neither *Bax*$^{-/-}$ nor *Bak*$^{-/-}$ mice are predisposed to tumour development (Knudson *et al*, 2001; Knudson *et al*, 1995; Lindsten *et al.*, 2000), drugs that target BAX or BAK specifically to limit cell death in specific cells or tissues would be invaluable in this endeavour. Although further insights are needed to inform the utility of these inhibitors in specific *in vivo* disease models, pharmacological inhibition of cell death has the emerging potential to transform the treatment of degenerative conditions, just as targeted agonists of apoptosis are transforming the treatment of various cancers.

Acknowledgements

The authors acknowledge Peter Czabotar and Guillaume Lessene for advice and discussions in the preparation of the manuscript. This work is supported by funding from the Bodhi Education Fund (GD) and grants from the National Health and Medical Research Council Australia to GD (GNT2001406, GNT2004446) and MvD (GNT1158137). This work was supported by operational infrastructure grants through the Australian Government Independent Research Institute Infrastructure Support Scheme (9000587) and the Victorian State Government Operational Infrastructure Support, Australia.

Conflict of interest

GD and MvD are employees of the Walter and Eliza Hall Institute which receives royalty payments relating to the use of Venclexta/venetoclax.

Glossary

AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALS/AST	Alanine transaminase /aspartate transaminase
APAF-1	Apoptotic Protease-Activating Factor 1
BAK	BCL-2 antagonist/killer
BAX	BCL-2-associated X protein
BAI	BAX Inhibitor
BCI	BAX Channel Inhibitor
BCL-2	B-cell lymphoma 2

BH	BCL-2 homology
DRP-1	Dynamin-Related Protein-1
HD	Huntington's disease
IAP	Inhibitor of Apoptosis Protein
I/R	Ischaemia/reperfusion
iMAC	Mitochondrial Channel Inhibitor
MND	Motor neurone disease
MIM	mitochondrial inner membrane
MIS	mitochondrial intermembrane space
MOM	mitochondrial outer membrane
MOMP	mitochondrial outer membrane permeabilisation
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
6-OHDA	6-hydroxydopamine
OPA1	Optic Atrophy 1
PD	Parkinson's disease
SNpc	*substantia nigra pars compacta*
SOD1	Superoxide dismutase 1
VDAC	Voltage-Dependent Anion Channel
vMIA	viral Mitochondria-Localised Inhibitor of Apoptosis

Table 1. Genetic evidence: apoptotic molecules mediating neuronal death

Diseases	Models with genes modulated	Outcome	Reference
Ischaemic stoke	Global ablation of caspase-3	Decreased cortical infarct volume	(Le *et al*, 2002)
	Constitutive deletion of *Bax*	Reduced infarct volume and brain water content	(D'Orsi *et al.*, 2015)
	Constitutive knockout of *Bid*	Reduced ischaemic damage	(Plesnila *et al*, 2001)
	Neuron-specific BCL-2 overexpression	Reduced brain infarct volume	(Martinou *et al*, 1994)
	Constitutive OPA1 overexpression	Alleviated ischaemic damage	(Varanita *et al*, 2015)
Traumatic brain injury	Constitutive *Bid* deletion	Decreased early post-traumatic damage with no long-term protection	(Bermpohl *et al*, 2006)
	Constitutive *Bax* deletion	Ameliorated neuronal death but did not improve cognitive behavior	(Tehranian *et al.*, 2008)
Alzheimer's disease	Constitutive *Bax* excision	Reduced neuronal cell loss induced by fibrillar Aβ peptide	(Kudo *et al.*, 2012)
Parkinson's disease	Viral delivery of XIAP into striatum	Prevented death of dopaminergic SNpc neurons	(Eberhardt *et al.*, 2000)
	Constitutive knockout of *Bax*	Preserved neurons in the SNpc before or after neurotoxic insults	(Vila *et al.*, 2001)

	Heterozygous knockout of *Mcl1* in Parkin-deficient mice	Sensitised *Park2*$^{-/-}$ mice to dopaminergic neurodegeneration and PD phenotype	(Ekholm-Reed *et al*, 2019)
	Viral delivery of *Opa1* into substantia nigra	Attenuated dopaminergic nigrostriatal denervation	(Ramonet *et al*, 2013)
	Bax deletion in 6-OHDA-treated mice.	Impaired the loss of dopaminergic neurons but did not rescue the motor deficits	(Kim *et al.*, 2011)
Amyotrophic lateral sclerosis	Constitutive deletion of *PUMA* in SOD1-mutant mice	Promoted motoneuron survival and delayed disease onset and motor dysfunction	(Kieran *et al.*, 2007)
	Constitutive overexpression of BCL-2 in SOD1-mutant mice	Delayed disease onset and prolonged survival, but did not alter disease duration	(Kostic *et al*, 1997)
	Neuronal *Bax/Bak* deletion in SOD1-mutant mice	Inhibited neuron loss and paralysis and extended survival.	(Reyes *et al.*, 2010)
Multiple sclerosis	Neuron-specific overexpression of BCL-2	Reduced axonal damage and clinical impairment	(Offen *et al*, 2000)
	Constitutive *Bax* excision	Reduced axonal damage, inflammatory infiltration and clinical impairment	(Lev *et al*, 2004)

Table 2. Clinical trials of caspases inhibitors Emricasan and Nivocasan as single agents

Agent	Trial identifier	Phase	Status	Indication(s)	Outcome

Emricasan (PF 03491390, IDN-6556)	NCT00080236	II	Completed	Liver transplantation	No results posted
	NCT00088140	II	Completed	Chronic hepatitis C virus infection	No results posted
	NCT01653899	I/II	Completed	Islet transplant to treat diabetes	No results posted
	NCT01912404	II	Terminated	Alcoholic hepatitis	N/A
	NCT01937130	II	Terminated	Acute liver failure	N/A
	NCT02039817	I	Completed	Renal impairment	
	NCT02077374	II	Completed	NASH, NAFLD	Decreased liver enzymes and biomarkers in NAFLD (Shiffman *et al*, 2019)
	NCT02121860	I	Completed	Liver impairment	
	NCT02138253	II	Completed	Liver damage caused by hepatitis C virus infection after liver transplantation	No significant difference in liver fibrosis
	NCT02230670	II	Completed	Liver cirrhosis	Improved outcome in liver function (Frenette *et al*, 2019)
	NCT02230683	II	Completed	Portal hypertension in subjects with liver cirrhosis	Reduced venous pressure and cleaved keratin as marker of caspase activity in patients with severe portal hypertension
	NCT02686762	II	Completed	NASH	Did not improve liver histology in patients with NASH fibrosis. May potentiate other cell death pathways

					(Harrison *et al*, 2020)
	NCT02960204	II	Completed	NASH and portal hypertension	No improvement in clinical outcomes (Garcia-Tsao *et al*, 2020)
	NCT03205345	II	Active, not recruiting	Decompensated NASH cirrhosis	No results posted
	NCT03479125	II	Terminated (development discontinued)	Post-treatment follow-up study for liver diseases	N/A
Nivocasan (GS-9450)	NCT00725803	II	Completed	Liver damage caused by hepatitis C virus infection	Reduced liver enzymes ALT/AST
	NCT00740610	II	Completed	NASH	Reduced liver enzymes ALT/AST and reduced cleaved keratin (Ratziu *et al.*, 2012)
	NCT00874796	II	Terminated (lab abnormalities and adverse events)	Chronic hepatitis C virus infection	N/A

References

Adams JM, Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26: 1324-1337

Alexander C, Votruba M, Pesch UE, Thiselton DL, Mayer S, Moore A, Rodriguez M, Kellner U, Leo-Kottler B, Auburger G *et al* (2000) OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nature genetics* 26: 211-215

Almonte-Becerril M, Navarro-Garcia F, Gonzalez-Robles A, Vega-Lopez MA, Lavalle C, Kouri JB (2010) Cell death of chondrocytes is a combination between apoptosis and autophagy during the pathogenesis of Osteoarthritis within an experimental model. *Apoptosis : an international journal on programmed cell death* 15: 631-638

Amgalan D, Garner TP, Pekson R, Jia XF, Yanamandala M, Paulino V, Liang FG, Corbalan JJ, Lee J, Chen Y *et al* (2020) A small-molecule allosteric inhibitor of BAX protects against doxorubicin-induced cardiomyopathy. *Nat Cancer* 1: 315-328

Arnoult D, Bartle LM, Skaletskaya A, Poncet D, Zamzami N, Park PU, Sharpe J, Youle RJ, Goldmacher VS (2004) Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. *Proc Natl Acad Sci U S A* 101: 7988-7993

Asai-Coakwell M, March L, Dai XH, Duval M, Lopez I, French CR, Famulski J, De Baere E, Francis PJ, Sundaresan P *et al* (2013) Contribution of growth differentiation factor 6-dependent cell survival to early-onset retinal dystrophies. *Hum Mol Genet* 22: 1432-1442

Bachurin S, Bukatina E, Lermontova N, Tkachenko S, Afanasiev A, Grigoriev V, Grigorieva I, Ivanov Y, Sablin S, Zefirov N (2001) Antihistamine agent Dimebon as a novel neuroprotector and a cognition enhancer. *Annals of the New York Academy of Sciences* 939: 425-435

Bae B-I, Xu H, Igarashi S, Fujimuro M, Agrawal N, Taya Y, Hayward SD, Moran TH, Montell C, Ross CA *et al* (2005) p53 mediates cellular dysfunction and behavioral abnormalities in Huntington's disease. *Neuron* 47: 29-41

Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 9: 550-555

Banati RB, Daniel SE, Blunt SB (1998) Glial pathology but absence of apoptotic nigral neurons in long-standing Parkinson's disease. *Movement disorders : official journal of the Movement Disorder Society* 13: 221-227

Barclay LA, Wales TE, Garner TP, Wachter F, Lee S, Guerra RM, Stewart ML, Braun CR, Bird GH, Gavathiotis E *et al* (2015) Inhibition of Pro-apoptotic BAX by a noncanonical interaction mechanism. *Mol Cell* 57: 873-886

Basanez G, Sharpe JC, Galanis J, Brandt TB, Hardwick JM, Zimmerberg J (2002) Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature. *J Biol Chem* 277: 49360-49365

Ben-Hail D, Begas-Shvartz R, Shalev M, Shteinfer-Kuzmine A, Gruzman A, Reina S, De Pinto V, Shoshan-Barmatz V (2016) Novel Compounds Targeting the Mitochondrial Protein VDAC1 Inhibit Apoptosis and Protect against Mitochondrial Dysfunction. *J Biol Chem* 291: 24986-25003

Bermpohl D, You Z, Korsmeyer SJ, Moskowitz MA, Whalen MJ (2006) Traumatic brain injury in mice deficient in Bid: effects on histopathology and functional outcome. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 26: 625-633

Bernardini JP, Brouwer JM, Tan IK, Sandow JJ, Huang S, Stafford CA, Bankovacki A, Riffkin CD, Wardak AZ, Czabotar PE *et al* (2019) Parkin inhibits BAK and BAX apoptotic function by distinct mechanisms during mitophagy. *EMBO J* 38: e99916

Blaya MO, Bramlett HM, Naidoo J, Pieper AA, Dietrich WD (2014) Neuroprotective efficacy of a proneurogenic compound after traumatic brain injury. *Journal of neurotrauma* 31: 476-486

Bombrun A, Gerber P, Casi G, Terradillos O, Antonsson B, Halazy S (2003) 3,6-dibromocarbazole piperazine derivatives of 2-propanol as first inhibitors of cytochrome c release via Bax channel modulation. *J Med Chem* 46: 4365-4368

Bonfanti L, Strettoi E, Chierzi S, Cenni MC, Liu XH, Martinou JC, Maffei L, Rabacchi SA (1996) Protection of retinal ganglion cells from natural and axotomy-induced cell death in neonatal transgenic mice overexpressing bcl-2. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 16: 4186-4194

Bordt EA, Clerc P, Roelofs BA, Saladino AJ, Tretter L, Adam-Vizi V, Cherok E, Khalil A, Yadava N, Ge SX *et al* (2017) The Putative Drp1 Inhibitor mdivi-1 Is a Reversible Mitochondrial Complex I Inhibitor that Modulates Reactive Oxygen Species. *Developmental cell* 40: 583-594.e586

Brocheriou V, Hagège AA, Oubenaïssa A, Lambert M, Mallet VO, Duriez M, Wassef M, Kahn A, Menasché P, Gilgenkrantz H (2000) Cardiac functional improvement by a human Bcl-2 transgene in a mouse model of ischemia/reperfusion injury. *J Gene Med* 2: 326-333

Brokatzky D, Dorflinger B, Haimovici A, Weber A, Kirschnek S, Vier J, Metz A, Henschel J, Steinfeldt T, Gentle IE *et al* (2019) A non-death function of the mitochondrial apoptosis apparatus in immunity. *EMBO J*

Brouwer JM, Lan P, Cowan AD, Bernardini JP, Birkinshaw RW, van Delft MF, Sleebs BE, Robin AY, Wardak A, Tan IK *et al* (2017) Conversion of Bim-BH3 from Activator to Inhibitor of Bak through Structure-Based Design. *Molecular Cell* 68: 659-+

Brouwer JM, Westphal D, Dewson G, Robin AY, Uren RT, Bartolo R, Thompson GV, Colman PM, Kluck RM, Czabotar PE (2014) Bak Core and Latch Domains Separate during Activation, and Freed Core Domains Form Symmetric Homodimers. *Molecular Cell* 55: 938-946

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52: 102-110

Cam M, Handke W, Picard-Maureau M, Brune W (2010) Cytomegaloviruses inhibit Bak- and Bax-mediated apoptosis with two separate viral proteins. *Cell Death Differ* 17: 655-665

Carelli V, Musumeci O, Caporali L, Zanna C, La Morgia C, Del Dotto V, Porcelli AM, Rugolo M, Valentino ML, Iommarini L *et al* (2015) Syndromic parkinsonism and dementia associated with OPA1 missense mutations. *Annals of neurology* 78: 21-38

Carlile GW, Smith DH, Wiedmann M (2004) Caspase-3 has a nonapoptotic function in erythroid maturation. *Blood* 103: 4310-4316

Carroll RG, Hollville E, Martin SJ (2014) Parkin sensitizes toward apoptosis induced by mitochondrial depolarization through promoting degradation of Mcl-1. *Cell Rep* 9: 1538-1553

Cassidy-Stone A, Chipuk JE, Ingerman E, Song C, Yoo C, Kuwana T, Kurth MJ, Shaw JT, Hinshaw JE, Green DR *et al* (2008) Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. *Dev Cell* 14: 193-204

Cenni MC, Bonfanti L, Martinou JC, Ratto GM, Strettoi E, Maffei L (1996) Long-term survival of retinal ganglion cells following optic nerve section in adult bcl-2 transgenic mice. *The European journal of neuroscience* 8: 1735-1745

Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y (2001) Structural basis of caspase-7 inhibition by XIAP. *Cell* 104: 769-780

Chen J, Flannery JG, LaVail MM, Steinberg RH, Xu J, Simon MI (1996) bcl-2 overexpression reduces apoptotic photoreceptor cell death in three different retinal degenerations. *Proc Natl Acad Sci U S A* 93: 7042-7047

Chen Z, Chua CC, Ho YS, Hamdy RC, Chua BH (2001) Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. *American Journal of Physiology Heart and Circulatory Physiology* 280: H2313-H2320

Cheng EH, Sheiko TV, Fisher JK, Craigen WJ, Korsmeyer SJ (2003) VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* 301: 513-517

Choi SA, Kim SJ, Chung KC (2006) Huntingtin-interacting protein 1-mediated neuronal cell death occurs through intrinsic apoptotic pathways and mitochondrial alterations. *FEBS letters* 580: 5275-5282

Christenson E, Merlin S, Saito M, Schlesinger P (2008) Cholesterol effects on BAX pore activation. *Journal of molecular biology* 381: 1168-1183

Chung KK, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, Dawson VL, Dawson TM (2004) S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304: 1328-1331

Clark RS, Kochanek PM, Chen M, Watkins SC, Marion DW, Chen J, Hamilton RL, Loeffert JE, Graham SH (1999) Increases in Bcl-2 and cleavage of caspase-1 and caspase-3 in human brain after head injury. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 13: 813-821

Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, Fitzgerald P, Guio-Carrion A, Waterhouse NJ, Li CW *et al* (2007) GAPDH and Autophagy Preserve Survival after Apoptotic Cytochrome c Release in the Absence of Caspase Activation. *Cell* 129: 983-997

Cowan AD, Smith NA, Sandow JJ, Kapp EA, Rustam YH, Murphy JM, Brouwer JM, Bernardini JP, Roy MJ, Wardak AZ *et al* (2020) BAK core dimers bind lipids and can be bridged by them. *Nat Struct Mol Biol*

Crocker SJ, Liston P, Anisman H, Lee CJ, Smith PD, Earl N, Thompson CS, Park DS, Korneluk RG, Robertson GS (2003) Attenuation of MPTP-induced neurotoxicity and behavioural impairment in NSE-XIAP transgenic mice. *Neurobiol Dis* 12: 150-161

Croft M, Benedict CA, Ware CF (2013) Clinical targeting of the TNF and TNFR superfamilies. *Nature reviews Drug discovery* 12: 147-168

Czabotar PE, Lessene G, Strasser A, Adams JM (2014) Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* 15: 49-63

Czabotar PE, Westphal D, Dewson G, Ma S, Hockings C, Fairlie WD, Lee EF, Yao S, Robin AY, Smith BJ *et al* (2013) Bax Crystal Structures Reveal How BH3 Domains Activate Bax and Nucleate Its Oligomerization to Induce Apoptosis. *Cell* 152: 519-531

D'Orsi B, Kilbride SM, Chen G, Perez Alvarez S, Bonner HP, Pfeiffer S, Plesnila N, Engel T, Henshall DC, Dussmann H *et al* (2015) Bax regulates neuronal Ca2+ homeostasis. *J Neurosci* 35: 1706-1722

De Jesús-Cortés H, Xu P, Drawbridge J, Estill SJ, Huntington P, Tran S, Britt J, Tesla R, Morlock L, Naidoo J *et al* (2012) Neuroprotective efficacy of aminopropyl carbazoles in a mouse model of Parkinson disease. *Proc Natl Acad Sci U S A* 109: 17010-17015

Deckwerth TL, Elliott JL, Knudson CM, Johnson EM, Jr., Snider WD, Korsmeyer SJ (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron* 17: 401-411

Dewson G, Kratina T, Sim HW, Puthalakath H, Adams JM, Colman PM, Kluck RM (2008) To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3:groove interactions. *Mol Cell* 30: 369-380

Doerflinger M, Deng Y, Whitney P, Salvamoser R, Engel S, Kueh AJ, Tai L, Bachem A, Gressier E, Geoghegan ND *et al* (2020) Flexible Usage and Interconnectivity of Diverse Cell Death Pathways Protect against Intracellular Infection. *Immunity* 53: 533-547 e537

Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102: 33-42

Eberhardt O, Coelln RV, Kugler S, Lindenau J, Rathke-Hartlieb S, Gerhardt E, Haid S, Isenmann S, Gravel C, Srinivasan A *et al* (2000) Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *J Neurosci* 20: 9126-9134

Echeverry N, Bachmann D, Ke F, Strasser A, Simon HU, Kaufmann T (2013) Intracellular localization of the BCL-2 family member BOK and functional implications. *Cell Death Differ* 20: 785-799

Ekholm-Reed S, Baker R, Campos AR, Stouffer D, Henze M, Wolf DA, Loring JF, Thomas EA, Reed SI (2019) Reducing Mcl-1 gene dosage induces dopaminergic neuronal loss and motor impairments in Park2 knockout mice. *Communications biology* 2: 125

Estaquier J, Arnoult D (2007) Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis. *Cell death and differentiation* 14: 1086-1094

Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Developmental cell* 1: 515-525

Frenette CT, Morelli G, Shiffman ML, Frederick RT, Rubin RA, Fallon MB, Cheng JT, Cave M, Khaderi SA, Massoud O *et al* (2019) Emricasan Improves Liver Function in Patients With Cirrhosis and High Model for End-Stage Liver Disease Scores Compared With Placebo. *Clin Gastroenterol Hepatol* 17: 774-783 e774

Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B *et al* (2006) OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* 126: 177-189

Fujita J, Crane AM, Souza MK, Dejosez M, Kyba M, Flavell RA, Thomson JA, Zwaka TP (2008) Caspase activity mediates the differentiation of embryonic stem cells. *Cell stem cell* 2: 595-601

Garcia-Tsao G, Bosch J, Kayali Z, Harrison SA, Abdelmalek MF, Lawitz E, Satapathy SK, Ghabril M, Shiffman ML, Younes ZH *et al* (2019) Randomized placebo-controlled trial of emricasan for non-alcoholic steatohepatitis-related cirrhosis with severe portal hypertension. *Journal of hepatology*

Garcia-Tsao G, Bosch J, Kayali Z, Harrison SA, Abdelmalek MF, Lawitz E, Satapathy SK, Ghabril M, Shiffman ML, Younes ZH *et al* (2020) Randomized placebo-controlled trial of emricasan for non-alcoholic steatohepatitis-related cirrhosis with severe portal hypertension. *J Hepatol* 72: 885-895

Garner TP, Amgalan D, Reyna DE, Li S, Kitsis RN, Gavathiotis E (2019) Small-molecule allosteric inhibitors of BAX. *Nat Chem Biol* 15: 322-330

Gavathiotis E, Suzuki M, Davis ML, Pitter K, Bird GH, Katz SG, Tu HC, Kim H, Cheng EH, Tjandra N *et al* (2008) BAX activation is initiated at a novel interaction site. *Nature* 455: 1076-1081

Gomez-Lazaro M, Galindo MF, Concannon CG, Segura MF, Fernandez-Gomez FJ, Llecha N, Comella JX, Prehn JH, Jordan J (2008) 6-Hydroxydopamine activates the mitochondrial apoptosis pathway through p38 MAPK-mediated, p53-independent activation of Bax and PUMA. *J Neurochem* 104: 1599-1612

Gortat A, Sancho M, Mondragón L, Messeguer À, Pérez-Payá E, Orzáez M (2015) Apaf1 inhibition promotes cell recovery from apoptosis. *Protein & cell* 6: 833-843

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 94: 1621-1628

Gould TW, Buss RR, Vinsant S, Prevette D, Sun W, Knudson CM, Milligan CE, Oppenheim RW (2006) Complete dissociation of motor neuron death from motor dysfunction by Bax deletion in a mouse model of ALS. *J Neurosci* 26: 8774-8786

Grant ZL, Whitehead L, Wong VH, He Z, Yan RY, Miles AR, Benest AV, Bates DO, Prahst C, Bentley K *et al* (2020) Blocking endothelial apoptosis revascularizes the retina in a model of ischemic retinopathy. *J Clin Invest*

Griparic L, van der Wel NN, Orozco IJ, Peters PJ, van der Bliek AM (2004) Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. *J Biol Chem* 279: 18792-18798

Grohm J, Kim SW, Mamrak U, Tobaben S, Cassidy-Stone A, Nunnari J, Plesnila N, Culmsee C (2012) Inhibition of Drp1 provides neuroprotection in vitro and in vivo. *Cell death and differentiation* 19: 1446-1458

Gu C, Zhang Y, Hu Q, Wu J, Ren H, Liu CF, Wang G (2017) P7C3 inhibits GSK3beta activation to protect dopaminergic neurons against neurotoxin-induced cell death in vitro and in vivo. *Cell death & disease* 8: e2858

Guery L, Benikhlef N, Gautier T, Paul C, Jego G, Dufour E, Jacquel A, Cally R, Manoury B, Vanden Berghe T *et al* (2011) Fine-tuning nucleophosmin in macrophage differentiation and activation. *Blood* 118: 4694-4704

Guo B, Zhai D, Cabezas E, Welsh K, Nouraini S, Satterthwait AC, Reed JC (2003) Humanin peptide suppresses apoptosis by interfering with Bax activation. *Nature* 423: 456-461

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144: 646-674

Hao Y, Sekine K, Kawabata A, Nakamura H, Ishioka T, Ohata H, Katayama R, Hashimoto C, Zhang X, Noda T *et al* (2004) Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. *Nature cell biology* 6: 849-860

Harder JM, Ding Q, Fernandes KA, Cherry JD, Gan L, Libby RT (2012) BCL2L1 (BCL-X) promotes survival of adult and developing retinal ganglion cells. *Molecular and cellular neurosciences* 51: 53-59

Harrison SA, Goodman Z, Jabbar A, Vemulapalli R, Younes ZH, Freilich B, Sheikh MY, Schattenberg JM, Kayali Z, Zivony A *et al* (2020) A randomized, placebo-controlled trial of emricasan in patients with NASH and F1-F3 fibrosis. *J Hepatol* 72: 816-827

Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M (1998) Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. *Arthritis and rheumatism* 41: 1266-1274

Hazafa A, Batool A, Ahmad S, Amjad M, Chaudhry SN, Asad J, Ghuman HF, Khan HM, Naeem M, Ghani U (2021) Humanin: A mitochondrial-derived peptide in the treatment of apoptosis-related diseases. *Life Sci* 264: 118679

Hetz C, Thielen P, Fisher J, Pasinelli P, Brown RH, Korsmeyer S, Glimcher L (2007) The proapoptotic BCL-2 family member BIM mediates motoneuron loss in a model of amyotrophic lateral sclerosis. *Cell death and differentiation* 14: 1386-1389

Hetz C, Vitte PA, Bombrun A, Rostovtseva TK, Montessuit S, Hiver A, Schwarz MK, Church DJ, Korsmeyer SJ, Martinou JC *et al* (2005) Bax channel inhibitors prevent mitochondrion-mediated apoptosis and protect neurons in a model of global brain ischemia. *J Biol Chem* 280: 42960-42970

Hochhauser E, Cheporko Y, Yasovich N, Pinchas L, Offen D, Barhum Y, Pannet H, Tobar A, Vidne BA, Birk E (2007) Bax deficiency reduces infarct size and improves long-term function after myocardial infarction. *Cell Biochem Biophys* 47: 11-20

Hochhauser E, Kivity S, Offen D, Maulik N, Otani H, Barhum Y, Pannet H, Shneyvays V, Shainberg A, Goldshtaub V *et al* (2003) Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice. *Am J Physiol Heart Circ Physiol* 284: H2351-H2359

Hsu SY, Kaipia A, McGee E, Lomeli M, Hsueh AJW (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proceedings of the National Academy of Sciences of the United States of America* 94: 12401-12406

Huang L, Han J, Ben-Hail D, He L, Li B, Chen Z, Wang Y, Yang Y, Liu L, Zhu Y *et al* (2015) A New Fungal Diterpene Induces VDAC1-dependent Apoptosis in Bax/Bak-deficient Cells. *J Biol Chem* 290: 23563-23578

Huang Y, Park YC, Rich RL, Segal D, Myszka DG, Wu H (2001) Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. *Cell* 104: 781-790

Ichim G, Lopez J, Ahmed SU, Muthalagu N, Giampazolias E, Delgado ME, Haller M, Riley JS, Mason SM, Athineos D *et al* (2015) Limited mitochondrial permeabilization causes DNA damage and genomic instability in the absence of cell death. *Mol Cell* 57: 860-872

Inserte J, Cardona M, Poncelas-Nozal M, Hernando V, Vilardosa Ú, Aluja D, Parra VM, Sanchis D, Garcia-Dorado D (2016) Studies on the role of apoptosis after transient myocardial ischemia: genetic deletion of the executioner caspases-3 and -7 does not limit infarct size and ventricular remodeling. *Basic Res Cardiol* 111: 18

Jacquel A, Benikhlef N, Paggetti J, Lalaoui N, Guery L, Dufour EK, Ciudad M, Racoeur C, Micheau O, Delva L *et al* (2009) Colony-stimulating factor-1-induced oscillations in phosphatidylinositol-3 kinase/AKT are required for caspase activation in monocytes undergoing differentiation into macrophages. *Blood* 114: 3633-3641

Jellinger KA (2000) Cell death mechanisms in Parkinson's disease. *Journal of neural transmission (Vienna, Austria : 1996)* 107: 1-29

Jiang X, Jiang H, Shen Z, Wang X (2014) Activation of mitochondrial protease OMA1 by Bax and Bak promotes cytochrome c release during apoptosis. *Proc Natl Acad Sci U S A* 111: 14782-14787

Jiang X, Li L, Ying Z, Pan C, Huang S, Li L, Dai M, Yan B, Li M, Jiang H *et al* (2016) A Small Molecule That Protects the Integrity of the Electron Transfer Chain Blocks the Mitochondrial Apoptotic Pathway. *Mol Cell* 63: 229-239

Jilka RL, O'Brien CA, Roberson PK, Bonewald LF, Weinstein RS, Manolagas SC (2014) Dysapoptosis of osteoblasts and osteocytes increases cancellous bone formation but exaggerates cortical porosity with age. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 29: 103-117

Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC (1998) Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 13: 793-802

Johnson BN, Berger AK, Cortese GP, Lavoie MJ (2012) The ubiquitin E3 ligase parkin regulates the proapoptotic function of Bax. *Proceedings of the National Academy of Sciences of the United States of America* 109: 6283-6288

Joshi AU, Saw NL, Vogel H, Cunnigham AD, Shamloo M, Mochly-Rosen D (2018) Inhibition of Drp1/Fis1 interaction slows progression of amyotrophic lateral sclerosis. *EMBO Mol Med* 10

Kalchman MA, Koide HB, McCutcheon K, Graham RK, Nichol K, Nishiyama K, Kazemi-Esfarjani P, Lynn FC, Wellington C, Metzler M *et al* (1997) HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. *Nature genetics* 16: 44-53

Karbowski M, Arnoult D, Chen H, Chan DC, Smith CL, Youle RJ (2004) Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis. *The Journal of cell biology* 164: 493-499

Karbowski M, Lee YJ, Gaume B, Jeong SY, Frank S, Nechushtan A, Santel A, Fuller M, Smith CL, Youle RJ (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *The Journal of cell biology* 159: 931-938

Ke F, Bouillet P, Kaufmann T, Strasser A, Kerr J, Voss AK (2013) Consequences of the combined loss of BOK and BAK or BOK and BAX. *Cell death & disease* 4: e650

Ke FFS, Vanyai HK, Cowan AD, Delbridge ARD, Whitehead L, Grabow S, Czabotar PE, Voss AK, Strasser A (2018) Embryogenesis and Adult Life in the Absence of Intrinsic Apoptosis Effectors BAX, BAK, and BOK. *Cell* 173: 1217-1230 e1217

Keinan N, Tyomkin D, Shoshan-Barmatz V (2010) Oligomerization of the mitochondrial protein voltage-dependent anion channel is coupled to the induction of apoptosis. *Mol Cell Biol* 30: 5698-5709

Kiechle T, Dedeoglu A, Kubilus J, Kowall NW, Beal MF, Friedlander RM, Hersch SM, Ferrante RJ (2002) Cytochrome C and caspase-9 expression in Huntington's disease. *Neuromolecular medicine* 1: 183-195

Kieran D, Woods I, Villunger A, Strasser A, Prehn JH (2007) Deletion of the BH3-only protein puma protects motoneurons from ER stress-induced apoptosis and delays motoneuron loss in ALS mice. *Proc Natl Acad Sci U S A* 104: 20606-20611

Kim J, Gupta R, Blanco LP, Yang S, Shteinfer-Kuzmine A, Wang K, Zhu J, Yoon HE, Wang X, Kerkhofs M *et al* (2019) VDAC oligomers form mitochondrial pores to release mtDNA fragments and promote lupus-like disease. *Science* 366: 1531-1536

Kim KY, Perkins GA, Shim MS, Bushong E, Alcasid N, Ju S, Ellisman MH, Weinreb RN, Ju WK (2015) DRP1 inhibition rescues retinal ganglion cells and their axons by preserving mitochondrial integrity in a mouse model of glaucoma. *Cell Death Dis* 6: e1839

Kim TW, Moon Y, Kim K, Lee JE, Koh HC, Rhyu IJ, Kim H, Sun W (2011) Dissociation of progressive dopaminergic neuronal death and behavioral impairments by Bax deletion in a mouse model of Parkinson's diseases. *PLoS One* 6: e25346

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605-608

Knudson CM, Johnson GM, Lin Y, Korsmeyer SJ (2001) *Bax* accelerates tumorigenesis in *p53*-deficient mice. *Cancer Research* 61: 659-665

Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ, Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 270: 96-99

Kostic V, Jackson-Lewis V, de Bilbao F, Dubois-Dauphin M, Przedborski S (1997) Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* 277: 559-562

Kudo W, Lee HP, Smith MA, Zhu X, Matsuyama S, Lee HG (2012) Inhibition of Bax protects neuronal cells from oligomeric Abeta neurotoxicity. *Cell death & disease* 3: e309

Lademann U, Cain K, Gyrd-Hansen M, Brown D, Peters D, Jäättelä M (2003) Diarylurea compounds inhibit caspase activation by preventing the formation of the active 700-kilodalton apoptosome complex. *Mol Cell Biol* 23: 7829-7837

Lauterwasser J, Todt F, Zerbes RM, Nguyen TN, Craigen W, Lazarou M, van der Laan M, Edlich F (2016) The porin VDAC2 is the mitochondrial platform for Bax retrotranslocation. *Sci Rep* 6: 32994

Le DA, Wu Y, Huang Z, Matsushita K, Plesnila N, Augustinack JC, Hyman BT, Yuan J, Kuida K, Flavell RA *et al* (2002) Caspase activation and neuroprotection in caspase-3- deficient mice after in vivo cerebral ischemia and in vitro oxygen glucose deprivation. *Proc Natl Acad Sci U S A* 99: 15188-15193

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ (2004) Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Molecular biology of the cell* 15: 5001-5011

Lev N, Barhum Y, Melamed E, Offen D (2004) Bax-ablation attenuates experimental autoimmune encephalomyelitis in mice. *Neuroscience letters* 359: 139-142

Levin LA, Schlamp CL, Spieldoch RL, Geszvain KM, Nickells RW (1997) Identification of the bcl-2 family of genes in the rat retina. *Investigative ophthalmology & visual science* 38: 2545-2553

Li MX, Tan IKL, Ma SB, Hockings C, Kratina T, Dengler MA, Alsop AE, Kluck RM, Dewson G (2017) BAK alpha 6 permits activation by BH3-only proteins and homooligomerization via the canonical hydrophobic groove. *Proceedings of the National Academy of Sciences of the United States of America* 114: 7629-7634

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91: 479-489

Lindsten T, Ross AJ, King A, Zong W, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K *et al* (2000) The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Mol Cell* 6: 1389-1399.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86: 147-157

Liu XH, Collier RJ, Youle RJ (2001) Inhibition of axotomy-induced neuronal apoptosis by extracellular delivery of a Bcl-XL fusion protein. *J Biol Chem* 276: 46326-46332

Liu Y, Porta A, Peng X, Gengaro K, Cunningham EB, Li H, Dominguez LA, Bellido T, Christakos S (2004) Prevention of glucocorticoid-induced apoptosis in osteocytes and osteoblasts by calbindin-D28k. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 19: 479-490

Llambi F, Moldoveanu T, Tait SWG, Bouchier-Hayes L, Temirov J, McCormick LL, Dillon CP, Green DR (2011) A Unified Model of Mammalian BCL-2 Protein Family Interactions at the Mitochondria. *Molecular cell* 44: 517-531

Llambi F, Wang YM, Victor B, Yang M, Schneider DM, Gingras S, Parsons MJ, Zheng JH, Brown SA, Pelletier S *et al* (2016) BOK Is a Non-canonical BCL-2 Family Effector of Apoptosis Regulated by ER-Associated Degradation. *Cell* 165: 421-433

Ma J, Edlich F, Bermejo GA, Norris KL, Youle RJ, Tjandra N (2012) Structural mechanism of Bax inhibition by cytomegalovirus protein vMIA. *Proc Natl Acad Sci U S A* 109: 20901-20906

Ma SB, Nguyen TN, Tan I, Ninnis R, Iyer S, Stroud DA, Menard M, Kluck RM, Ryan MT, Dewson G (2014) Bax targets mitochondria by distinct mechanisms before or during apoptotic cell death: a requirement for VDAC2 or Bak for efficient Bax apoptotic function. *Cell Death and Differentiation* 21: 1925-1935

MacMillan KS, Naidoo J, Liang J, Melito L, Williams NS, Morlock L, Huntington PJ, Estill SJ, Longgood J, Becker GL *et al* (2011) Development of proneurogenic, neuroprotective small molecules. *Journal of the American Chemical Society* 133: 1428-1437

Magrané J, Hervias I, Henning MS, Damiano M, Kawamata H, Manfredi G (2009) Mutant SOD1 in neuronal mitochondria causes toxicity and mitochondrial dynamics abnormalities. *Hum Mol Genet* 18: 4552-4564

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 13: 1017-1030

Methot N, Vaillancourt JP, Huang J, Colucci J, Han Y, Menard S, Zamboni R, Toulmond S, Nicholson DW, Roy S (2004) A caspase active site probe reveals high fractional inhibition needed to block DNA fragmentation. *J Biol Chem* 279: 27905-27914

Mizutani M, Kern TS, Lorenzi M (1996) Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 97: 2883-2890

Moldoveanu T, Grace CR, Llambi F, Nourse A, Fitzgerald P, Gehring K, Kriwacki RW, Green DR (2013) BID-induced structural changes in BAK promote apoptosis. *Nat Struct Mol Biol* 20: 589-597

Moldoveanu T, Liu Q, Tocilj A, Watson MH, Shore G, Gehring K (2006) The x-ray structure of a BAK homodimer reveals an inhibitory zinc binding site. *Molecular Cell* 24: 677-688

Moriishi T, Fukuyama R, Miyazaki T, Furuichi T, Ito M, Komori T (2016) Overexpression of BCLXL in Osteoblasts Inhibits Osteoblast Apoptosis and Increases Bone Volume and Strength. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 31: 1366-1380

Morris DL, Johnson S, Bleck CKE, Lee DY, Tjandra N (2020) Humanin selectively prevents the activation of pro-apoptotic protein BID by sequestering it into fibers. *J Biol Chem* 295: 18226-18238

Morris DL, Kastner DW, Johnson S, Strub MP, He Y, Bleck CKE, Lee DY, Tjandra N (2019) Humanin induces conformational changes in the apoptosis regulator BAX and sequesters it into fibers, preventing mitochondrial outer-membrane permeabilization. *J Biol Chem* 294: 19055-19065

Naghdi S, Varnai P, Hajnoczky G (2015) Motifs of VDAC2 required for mitochondrial Bak import and tBid-induced apoptosis. *Proc Natl Acad Sci U S A* 112: E5590-5599

Naidoo J, De Jesus-Cortes H, Huntington P, Estill S, Morlock LK, Starwalt R, Mangano TJ, Williams NS, Pieper AA, Ready JM (2014) Discovery of a neuroprotective chemical, (S)-N-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-fluoropropyl)-6-methoxypyridin-2-amine [(-)-P7C3-S243], with improved druglike properties. *Journal of medicinal chemistry* 57: 3746-3754

Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA (1998) Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18: 3659-3668

Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, Schmidt U, Semigran MJ, Dec GW, Khaw BA (1996) Apoptosis in myocytes in end-stage heart failure. *N Engl J Med* 335: 1182-1189

Nir I, Kedzierski W, Chen J, Travis GH (2000) Expression of Bcl-2 protects against photoreceptor degeneration in retinal degeneration slow (rds) mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20: 2150-2154

Niu X, Brahmbhatt H, Mergenthaler P, Zhang Z, Sang J, Daude M, Ehlert FGR, Diederich WE, Wong E, Zhu W *et al* (2017) A Small-Molecule Inhibitor of Bax and Bak Oligomerization Prevents Genotoxic Cell Death and Promotes Neuroprotection. *Cell Chem Biol* 24: 493-506 e495

O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS (2004) Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology* 145: 1835-1841

O'Brien T, and Dixit, V.M. (2009) Drug Discovery in Apoptosis. In: *eLS*, John Wiley & Sons, Ltd:

Offen D, Kaye JF, Bernard O, Merims D, Coire CI, Panet H, Melamed E, Ben-Nun A (2000) Mice overexpressing Bcl-2 in their neurons are resistant to myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE). *Journal of molecular neuroscience : MN* 15: 167-176

Olichon A, Baricault L, Gas N, Guillou E, Valette A, Belenguer P, Lenaers G (2003) Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. *J Biol Chem* 278: 7743-7746

Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, Di Loreto C, Beltrami CA, Krajewski S *et al* (1997) Apoptosis in the failing human heart. *N Engl J Med* 336: 1131-1141

Orzáez M, Sancho M, Marchán S, Mondragón L, Montava R, Valero JG, Landeta O, Basañez G, Carbajo RJ, Pineda-Lucena A *et al* (2014) Apaf-1 inhibitors protect from unwanted cell death in in vivo models of kidney ischemia and chemotherapy induced ototoxicity. *PloS one* 9: e110979

Pantschenko AG, Zhang W, Nahounou M, McCarthy MB, Stover ML, Lichtler AC, Clark SH, Gronowicz GA (2005) Effect of osteoblast-targeted expression of bcl-2 in bone: differential response in male and female mice. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 20: 1414-1429

Pasinelli P, Belford ME, Lennon N, Bacskai BJ, Hyman BT, Trotti D, Brown RH, Jr. (2004) Amyotrophic lateral sclerosis-associated SOD1 mutant proteins bind and aggregate with Bcl-2 in spinal cord mitochondria. *Neuron* 43: 19-30

Pedrini S, Sau D, Guareschi S, Bogush M, Brown RH, Jr., Naniche N, Kia A, Trotti D, Pasinelli P (2010) ALS-linked mutant SOD1 damages mitochondria by promoting conformational changes in Bcl-2. *Hum Mol Genet* 19: 2974-2986

Peixoto PM, Ryu SY, Bombrun A, Antonsson B, Kinnally KW (2009) MAC inhibitors suppress mitochondrial apoptosis. *Biochem J* 423: 381-387

Pérez HE, Luna MJ, Rojas ML, Kouri JB (2005) Chondroptosis: an immunohistochemical study of apoptosis and Golgi complex in chondrocytes from human osteoarthritic cartilage. *Apoptosis : an international journal on programmed cell death* 10: 1105-1110

Petit A, Kawarai T, Paitel E, Sanjo N, Maj M, Scheid M, Chen F, Gu Y, Hasegawa H, Salehi-Rad S *et al* (2005) Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. *The Journal of biological chemistry* 280: 34025-34032

Petros AM, Olejniczak ET, Fesik SW (2004) Structural biology of the Bcl-2 family of proteins. *Biochim Biophys Acta* 1644: 83-94

Pieper AA, Xie S, Capota E, Estill SJ, Zhong J, Long JM, Becker GL, Huntington P, Goldman SE, Shen CH *et al* (2010) Discovery of a proneurogenic, neuroprotective chemical. *Cell* 142: 39-51

Plesnila N, Zinkel S, Le DA, Amin-Hanjani S, Wu Y, Qiu J, Chiarugi A, Thomas SS, Kohane DS, Korsmeyer SJ *et al* (2001) BID mediates neuronal cell death after oxygen/ glucose deprivation and focal cerebral ischemia. *Proc Natl Acad Sci U S A* 98: 15318-15323

Polster BM, Basañez G, Young M, Suzuki M, Fiskum G (2003) Inhibition of Bax-induced cytochrome c release from neural cell and brain mitochondria by dibucaine and propranolol. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23: 2735-2743

Prudent J, Zunino R, Sugiura A, Mattie S, Shore GC, McBride HM (2015) MAPL SUMOylation of Drp1 Stabilizes an ER/Mitochondrial Platform Required for Cell Death. *Mol Cell* 59: 941-955

Qi X, Qvit N, Su YC, Mochly-Rosen D (2013) A novel Drp1 inhibitor diminishes aberrant mitochondrial fission and neurotoxicity. *J Cell Sci* 126: 789-802

Qian S, Wang W, Yang L, Huang HW (2008) Structure of transmembrane pore induced by Bax-derived peptide: evidence for lipidic pores. *Proc Natl Acad Sci U S A* 105: 17379-17383

Rami A, Sims J, Botez G, Winckler J (2003) Spatial resolution of phospholipid scramblase 1 (PLSCR1), caspase-3 activation and DNA-fragmentation in the human hippocampus after cerebral ischemia. *Neurochemistry international* 43: 79-87

Ramonet D, Perier C, Recasens A, Dehay B, Bové J, Costa V, Scorrano L, Vila M (2013) Optic atrophy 1 mediates mitochondria remodeling and dopaminergic neurodegeneration linked to complex I deficiency. *Cell death and differentiation* 20: 77-85

Rappold PM, Cui M, Grima JC, Fan RZ, de Mesy-Bentley KL, Chen L, Zhuang X, Bowers WJ, Tieu K (2014) Drp1 inhibition attenuates neurotoxicity and dopamine release deficits in vivo. *Nat Commun* 5: 5244

Ratziu V, Sheikh MY, Sanyal AJ, Lim JK, Conjeevaram H, Chalasani N, Abdelmalek M, Bakken A, Renou C, Palmer M *et al* (2012) A phase 2, randomized, double-blind, placebo-controlled study of GS-9450 in subjects with nonalcoholic steatohepatitis. *Hepatology* 55: 419-428

Reddy PH, Manczak M, Yin X, Reddy AP (2018) Synergistic Protective Effects of Mitochondrial Division Inhibitor 1 and Mitochondria-Targeted Small Peptide SS31 in Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* 62: 1549-1565

Reyes NA, Fisher JK, Austgen K, VandenBerg S, Huang EJ, Oakes SA (2010) Blocking the mitochondrial apoptotic pathway preserves motor neuron viability and function in a mouse model of amyotrophic lateral sclerosis. *J Clin Invest* 120: 3673-3679

Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS (2001) Structural basis for the inhibition of caspase-3 by XIAP. *Cell* 104: 791-800

Rigamonti D, Bauer JH, De-Fraja C, Conti L, Sipione S, Sciorati C, Clementi E, Hackam A, Hayden MR, Li Y *et al* (2000) Wild-type huntingtin protects from apoptosis upstream of caspase-3. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20: 3705-3713

Riley JS, Quarato G, Cloix C, Lopez J, O'Prey J, Pearson M, Chapman J, Sesaki H, Carlin LM, Passos JF *et al* (2018) Mitochondrial inner membrane permeabilisation enables mtDNA release during apoptosis. *EMBO J* 37

Roberts AW, Davids MS, Pagel JM, Kahl BS, Puvvada SD, Gerecitano JF, Kipps TJ, Anderson MA, Brown JR, Gressick L *et al* (2016) Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *N Engl J Med* 374: 311-322

Rongvaux A, Jackson R, Harman CC, Li T, West AP, de Zoete MR, Wu Y, Yordy B, Lakhani SA, Kuan CY *et al* (2014) Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. *Cell* 159: 1563-1577

Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX *et al* (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 362: 59-62

Salvador-Gallego R, Mund M, Cosentino K, Schneider J, Unsay J, Schraermeyer U, Engelhardt J, Ries J, Garcia-Saez AJ (2016) Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores. *EMBO J* 35: 389-401

Saudou F, Finkbeiner S, Devys D, Greenberg ME (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 95: 55-66

Schaaf CP, Blazo M, Lewis RA, Tonini RE, Takei H, Wang J, Wong LJ, Scaglia F (2011) Early-onset severe neuromuscular phenotype associated with compound heterozygosity for OPA1 mutations. *Molecular genetics and metabolism* 103: 383-387

Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell* 2: 55-67

Selznick LA, Zheng TS, Flavell RA, Rakic P, Roth KA (2000) Amyloid beta-induced neuronal death is bax-dependent but caspase-independent. *J Neuropathol Exp Neurol* 59: 271-279

Semaan SJ, Li Y, Nickells RW (2010) A single nucleotide polymorphism in the Bax gene promoter affects transcription and influences retinal ganglion cell death. *ASN neuro* 2: e00032

Shiffman M, Freilich B, Vuppalanchi R, Watt K, Chan JL, Spada A, Hagerty DT, Schiff E (2019) Randomised clinical trial: emricasan versus placebo significantly decreases ALT and caspase 3/7 activation in subjects with non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 49: 64-73

Shim G, Im S, Lee S, Park JY, Kim J, Jin H, Lee S, Im I, Kim DD, Kim SW *et al* (2013) Enhanced survival of transplanted human adipose-derived stem cells by co-delivery with liposomal apoptosome inhibitor in fibrin gel matrix. *Eur J Pharm Biopharm* 85: 673-681

Silke J, Rickard JA, Gerlic M (2015) The diverse role of RIP kinases in necroptosis and inflammation. *Nat Immunol* 16: 689-697

Singh R, Letai A, Sarosiek K (2019) Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nat Rev Mol Cell Biol* 20: 175-193

Slee EA, Adrain C, Martin SJ (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *Journal of Biological Chemistry* 276: 7320-7326

Sordet O, Rébé C, Plenchette S, Zermati Y, Hermine O, Vainchenker W, Garrido C, Solary E, Dubrez-Daloz L (2002) Specific involvement of caspases in the differentiation of monocytes into macrophages. *Blood* 100: 4446-4453

Spitz AZ, Zacharioudakis E, Reyna DE, Garner TP, Gavathiotis E (2021) Eltrombopag directly inhibits BAX and prevents cell death. *Nature communications* 12: 1134

Springer JE, Azbill RD, Knapp PE (1999) Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. *Nature medicine* 5: 943-946

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Fernandes-Alnemri T, Shi Y *et al* (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* 410: 112-116

Steffan JS, Kazantsev A, Spasic-Boskovic O, Greenwald M, Zhu YZ, Gohler H, Wanker EE, Bates GP, Housman DE, Thompson LM (2000) The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc Natl Acad Sci U S A* 97: 6763-6768

Su JH, Deng G, Cotman CW (1997) Bax protein expression is increased in Alzheimer's brain: correlations with DNA damage, Bcl-2 expression, and brain pathology. *J Neuropathol Exp Neurol* 56: 86-93

Subburaj Y, Cosentino K, Axmann M, Pedrueza-Villalmanzo E, Hermann E, Bleicken S, Spatz J, Garcia-Saez AJ (2015) Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species. *Nature communications* 6: 8042

Sun YF, Yu LY, Saarma M, Arumae U (2003) Mutational analysis of N-Bak reveals different structural requirements for antiapoptotic activity in neurons and proapoptotic activity in nonneuronal cells. *Mol Cell Neurosci* 23: 134-143

Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Molecular cell* 8: 613-621

Takeuchi H, Kobayashi Y, Ishigaki S, Doyu M, Sobue G (2002) Mitochondrial localization of mutant superoxide dismutase 1 triggers caspase-dependent cell death in a cellular model of familial amyotrophic lateral sclerosis. *The Journal of biological chemistry* 277: 50966-50972

Tatton NA (2000) Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. *Exp Neurol* 166: 29-43

Tehranian R, Rose ME, Vagni V, Pickrell AM, Griffith RP, Liu H, Clark RS, Dixon CE, Kochanek PM, Graham SH (2008) Disruption of Bax protein prevents neuronal cell death but produces cognitive impairment in mice following traumatic brain injury. *J Neurotrauma* 25: 755-767

Terrones O, Antonsson B, Yamaguchi H, Wang HG, Liu J, Lee RM, Herrmann A, Basanez G (2004) Lipidic pore formation by the concerted action of proapoptotic BAX and tBID. *J Biol Chem* 279: 30081-30091

Tesla R, Wolf HP, Xu P, Drawbridge J, Estill SJ, Huntington P, McDaniel L, Knobbe W, Burket A, Tran S *et al* (2012) Neuroprotective efficacy of aminopropyl carbazoles in a mouse model of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 109: 17016-17021

Toth A, Jeffers JR, Nickson P, Min J-Y, Morgan JP, Zambetti GP, Erhardt P (2006) Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* 291: H52-H60

Tsang AHK, Lee Y-I, Ko HS, Savitt JM, Pletnikova O, Troncoso JC, Dawson VL, Dawson TM, Chung KKK (2009) S-nitrosylation of XIAP compromises neuronal survival in Parkinson's disease. *Proceedings of the National Academy of Sciences* 106: 4900-4905

Uchime O, Dai Z, Biris N, Lee D, Sidhu SS, Li S, Lai JR, Gavathiotis E (2016) Synthetic Antibodies Inhibit Bcl-2-associated X Protein (BAX) through Blockade of the N-terminal Activation Site. *The Journal of biological chemistry* 291: 89-102

Uo T, Kinoshita Y, Morrison RS (2005) Neurons exclusively express N-Bak, a BH3 domain-only Bak isoform that promotes neuronal apoptosis. *J Biol Chem* 280: 9065-9073

Uren RT, O'Hely M, Iyer S, Bartolo R, Shi MX, Brouwer JM, Alsop AE, Dewson G, Kluck RM (2017) Disordered clusters of Bak dimers rupture mitochondria during apoptosis. *Elife* 6

van Delft MF, Chappaz S, Khakham Y, Bui CT, Debrincat MA, Lowes KN, Brouwer JM, Grohmann C, Sharp PP, Dagley LF *et al* (2019) A small molecule interacts with VDAC2 to block mouse BAK-driven apoptosis. *Nat Chem Biol* 15: 1057-1066

Varanita T, Soriano ME, Romanello V, Zaglia T, Quintana-Cabrera R, Semenzato M, Menabò R, Costa V, Civiletto G, Pesce P *et al* (2015) The OPA1-dependent mitochondrial cristae remodeling pathway controls atrophic, apoptotic, and ischemic tissue damage. *Cell Metab* 21: 834-844

Vaux DL, Cory S, Adams JM (1988) *Bcl*-2 gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. *Nature* 335: 440-442

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing inhibitor of apoptosis (IAP) proteins. *Cell* 102: 43-53

Vila M, Jackson-Lewis V, Vukosavic S, Djaldetti R, Liberatore G, Offen D, Korsmeyer SJ, Przedborski S (2001) Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Proc Natl Acad Sci U S A* 98: 2837-2842

Walker AK, Rivera PD, Wang Q, Chuang JC, Tran S, Osborne-Lawrence S, Estill SJ, Starwalt R, Huntington P, Morlock L *et al* (2015) The P7C3 class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis. *Molecular psychiatry* 20: 500-508

Wang G, Han T, Nijhawan D, Theodoropoulos P, Naidoo J, Yadavalli S, Mirzaei H, Pieper AA, Ready JM, McKnight SL (2014) P7C3 neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. *Cell* 158: 1324-1334

Wang Y, Cao Y, Zhu Q, Gu X, Zhu YZ (2016) The discovery of a novel inhibitor of apoptotic protease activating factor-1 (Apaf-1) for ischemic heart: synthesis, activity and target identification. *Sci Rep* 6: 29820

Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB, Korsmeyer SJ (2000) tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev* 14: 2060-2071

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292: 727-730

White MJ, McArthur K, Metcalf D, Lane RM, Cambier JC, Herold MJ, van Delft MF, Bedoui S, Lessene G, Ritchie ME *et al* (2014) Apoptotic caspases suppress mtDNA-induced STING-mediated type I IFN production. *Cell* 159: 1549-1562

Wiren KM, Toombs AR, Semirale AA, Zhang X (2006) Osteoblast and osteocyte apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. *Bone* 38: 637-651

Wu Q, Xia SX, Li QQ, Gao Y, Shen X, Ma L, Zhang MY, Wang T, Li YS, Wang ZF *et al* (2016) Mitochondrial division inhibitor 1 (Mdivi-1) offers neuroprotection through diminishing cell death and improving functional outcome in a mouse model of traumatic brain injury. *Brain research* 1630: 134-143

Wüllner U, Kornhuber J, Weller M, Schulz JB, Löschmann PA, Riederer P, Klockgether T (1999) Cell death and apoptosis regulating proteins in Parkinson's disease--a cautionary note. *Acta neuropathologica* 97: 408-412

Yakovlev AG, Ota K, Wang G, Movsesyan V, Bao WL, Yoshihara K, Faden AI (2001) Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21: 7439-7446

Yamaguchi R, Lartigue L, Perkins G, Scott RT, Dixit A, Kushnareva Y, Kuwana T, Ellisman MH, Newmeyer DD (2008) Opa1-mediated cristae opening is Bax/Bak and BH3 dependent, required for apoptosis, and independent of Bak oligomerization. *Mol Cell* 31: 557-569

Yang L, Bula D, Arroyo JG, Chen DF (2004) Preventing retinal detachment-associated photoreceptor cell loss in Bax-deficient mice. *Investigative ophthalmology & visual science* 45: 648-654

Yaoita H, Ogawa K, Maehara K, Maruyama Y (1998) Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation* 97: 276-281

Yin TC, Britt JK, De Jesús-Cortés H, Lu Y, Genova RM, Khan MZ, Voorhees JR, Shao J, Katzman AC, Huntington PJ *et al* (2014) P7C3 neuroprotective chemicals block axonal degeneration and preserve function after traumatic brain injury. *Cell Rep* 8: 1731-1740

Yu CH, Davidson S, Harapas CR, Hilton JB, Mlodzianoski MJ, Laohamonthonkul P, Louis C, Low RRJ, Moecking J, De Nardo D *et al* (2020) TDP-43 Triggers Mitochondrial DNA Release via mPTP to Activate cGAS/STING in ALS. *Cell* 183: 636-649 e618

Yuan H, Gerencser AA, Liot G, Lipton SA, Ellisman M, Perkins GA, Bossy-Wetzel E (2007) Mitochondrial fission is an upstream and required event for bax foci formation in response to nitric oxide in cortical neurons. *Cell death and differentiation* 14: 462-471

Zhang C, Lee S, Peng Y, Bunker E, Giaime E, Shen J, Zhou Z, Liu X (2014) PINK1 triggers autocatalytic activation of Parkin to specify cell fate decisions. *Curr Biol* 24: 1854-1865

Zhao Y, Sun X, Qi X (2018) Inhibition of Drp1 hyperactivation reduces neuropathology and behavioral deficits in zQ175 knock-in mouse model of Huntington's disease. *Biochemical and biophysical research communications* 507: 319-323

Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H, Blomgren K (2005) The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. *Cell death and differentiation* 12: 162-176

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of Caspase-3. *Cell* 90: 405-413

Figure Legends

Figure 1. Overview of the mitochondrial apoptosis pathway and opportunities for pharmacological inhibition.

Pro-apoptotic factors such as cytochrome *c*, SMAC/DIABLO and OMI/HTRA2 released from mitochondria trigger caspase-dependent apoptotic pathway. Mitochondrial DNA released from damaged mitochondria can trigger inflammation mediated by cGAS/STING or the NRLP3 inflammasome unless curtailed by caspases. Small molecule or peptide inhibitors have been developed that target distinct steps and players in mitochondrial apoptosis.

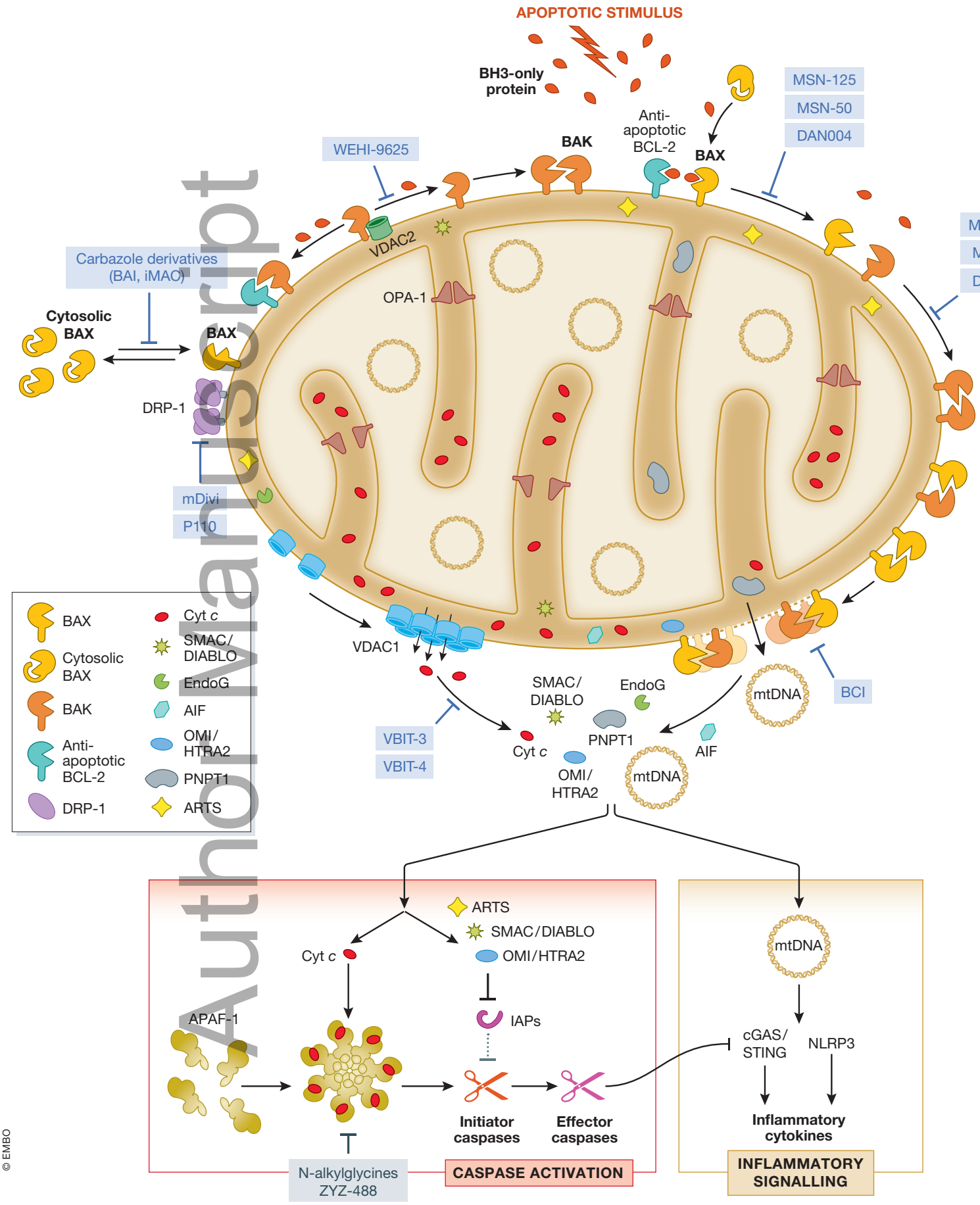
Figure 2. Modulation of BAX.

(A) BAX hydrophobic groove (α2-5) is a site for binding its C-terminal α9 transmembrane (TM) anchor and also activating BH3-only proteins. Soluble BAX (*surface representation, grey*) with its α9/TM shown (*magenta helix*) occupying the hydrophobic groove (PDB:2LR1). BIM BH3 peptide (*orange helix,* derived from PDB: 4ZIE) overlaid into the hydrophobic groove. **(B)** BAX (PDB: 2LR1) is inhibited by peptide from the cytomegalovirus protein vMIA (*blue*, (Ma *et al.*, 2012) and a stapled BCL-2 BH4 peptide. BAX residues implicated to interact with BCL-2 BH4 are indicated (*pink=low abundance labelling, purple=high abundance labelling*). **(C)** BAX inhibitory compound BAI1 (also known as iMAC1) binds proximally to the hydrophobic groove (*green*).

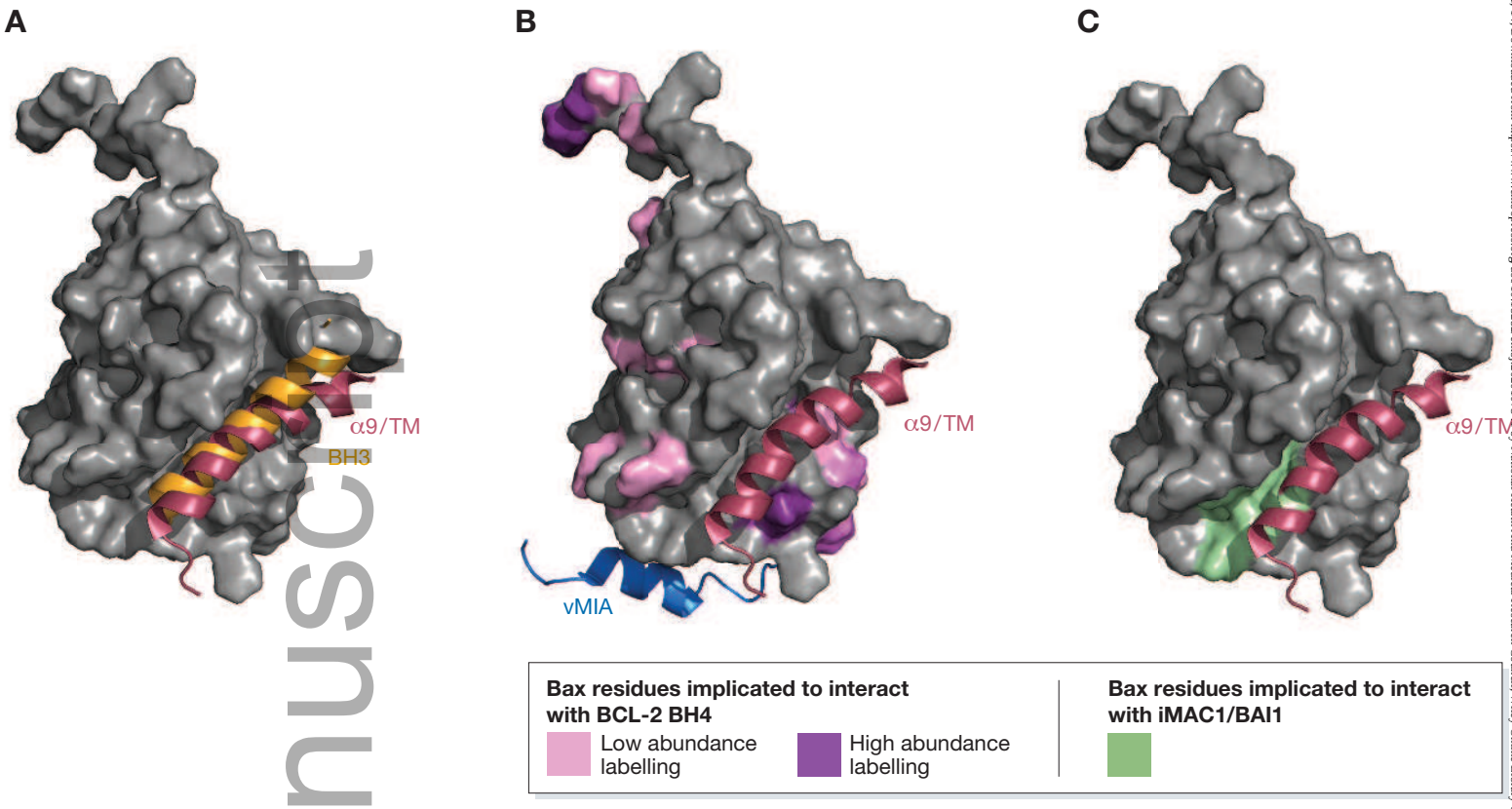
Figure 3. Inhibitory checkpoints in the stepwise activation of BAK and BAX

Stepwise activation of BAK and BAX with structures of transitional conformers. Inhibitors of BAK and/or BAX block at distinct steps in the activation pathway. **(1)** In healthy cells, inactive BAX is cytosolic with its transmembrane domain predominantly sequestered in its hydrophobic groove (see also Figure 2A), whilst BAK is sequestered in a protein complex containing VDAC2 with its C-terminal α9 transmembrane domain (*purple*) integrated into the MOM. The components of the complex and the molecular interactions between BAK and VDAC2 are currently unclear. *Structure*: Inactive conformer of monomeric BAK (PDB:2IMS(Moldoveanu *et al*, 2006)) with helix α1 (*wheat*), α2 (*marine*), α3 (*cyan*), α4 (*limon*), α5 (*red*), α6/7/8 (*grey*) indicated. The BH3 domain (α2) and hydrophobic binding groove

(α2/3/4/5) are the major sites for activation and oligomerisation. **(2)** Binding of a BH3 peptide (*orange*) promotes BAX mitochondrial localisation and dissociates BAK from VDAC2. The inhibitory compound WEHI-9625 behinds VDAC2 to stabilise its interaction with inactive BAK. *Structure*: BAK bound to BIM BH3 peptide (*orange*) (PDB:5VWY(Brouwer *et al.*, 2014)). **(3)** BH3-only protein binding instigates BAX/BAK conformation change involving eversion of the N-terminus (NT) and BH3 domain, eversion of the C-terminus (CT, BAX only), and separation dissociation of the core (α2-5) and latch (α6-8) domains. BCL-2 BH4 domain peptide with an engineered helix-stabilising hydrocarbon staple (*dash*) between two loop residues (*red*) and MAC/BAI compounds bind BAX to inhibit its conformation change. *Structure:* BAK bound with BIM BH3 peptide (*orange*) with its core (α1-5) and latch (α6-8) domains dissociated (PDB:5VWY(Brouwer *et al.*, 2014)). **(4)** Conformation change facilitates BAX/BAK homodimerisation via a BH3:groove interaction that precedes higher order oligomer formation and MOMP. The position of the BAK/BAX α1 (bounded by dashed line) in the activated conformer is unclear. Additionally, the structure of the BAK/BAX BH3:groove homodimers are currently limited to the α2-5 core domain. MSN compounds impair oligomerisation of BAX and BAK, MSN-125 also blocked BAX conformation change. *DAN004 inhibited BAX on liposomes, but was toxic to cells. BCI compounds limited channel activity/membrane permeabilisation mediated by BAX. *Structure*: BAK BH3-groove homodimer (structure limited to α2-5, PDB:4U2V(Brouwer *et al.*, 2014)). Helix α2 (BH3 domain) of one monomer binds the hydrophobic groove formed by helix α3, α4 and α5 of another monomer (shown in *pink*). **(5)** Binding of a mutant BIM BH3 peptide (*yellow*) to the BAK hydrophobic groove dissociates BAK from VDAC2, but prevents further conformation change and homo-oligomerisation thereby inhibiting BAK apoptotic activity. *Structure:* BAK bound with a mutant BIM BH3 peptide (*yellow*) (PDB:5VX0(Brouwer *et al.*, 2017)).



embj_2020107341_f1.eps



embj_2020107341_f2.eps