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Review Article

Mitochondrial permeability as a target for neurodegenerative disorders

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CONTENTS

Abstract

Mitochondria-dependent cell death is a primary mechanism of cell death in both acute and chronic neurodegenerative disorders, *e.g.*, stroke and Alzheimer's disease. Ischemia, glutamate excitotoxicity and reactive oxygen species (ROS) all target the mitochondria. In response to these lethal stimuli, the mitochondria release death factors, primarily cytochrome *c* (Cyt *c*), initiating the apoptotic cascade. Cyt *c* release occurs by at least two mechanisms, *i.e.*, mitochondrial permeability transition (mPT) and mitochondrial outer membrane permeabilization (MOMP) through a Bax channel. Blocking the release of Cyt *c* may have therapeutic potential in neurodegenerative disorders. Roche has described compounds that target the voltage-dependent anion channel component of the mPT. 2-Aminoethoxydiphenyl borate is able to inhibit Ca^{2+}-induced Ca^{2+} release, and tricyclic antidepressants can inhibit mPT. Substituted carbazoles from Serono are capable of blocking Bax channel formation and the release of Cyt *c* from isolated mitochondria. At MGI PHARMA, GPI-19410 was discovered to have a complex effect in blocking mitochondria-dependent cell death: it prevented mitochondrial depolarization in response to Ca^{2+}, blocked Ca^{2+}-induced mitochondrial swelling and Cyt *c* release, and blocked t-Bid-induced Cyt *c* release in a manner distinct from cyclosporin.

Introduction

The mitochondrion is an extraordinary organelle that not only produces life-giving energy to the healthy cell, but is also now known to mediate the release of death-inducing proteins that initiate death processes in the dysphoric cell (1, 2). Considerable attention is now being devoted to understanding the molecular mechanisms of mitochondria-dependent cell death in the hopes of identifying targeted pharmaceuticals that might turn on cell death for the treatment of cancer, or inhibit mitochondria-dependent cell death in degenerative disorders. This brief review is focused primarily on a discussion of targeted therapeutics for neurodegenerative disorders; discussions on mitochondria-targeted therapeutics for cancer are reviewed elsewhere (3-6).

Mitochondria-dependent cell death

The mechanisms causing a mammalian cell to die are not simple, whether it be cell death during development, disease or aging (7-9). Necrosis and apoptosis are the two broadly defined categories of cell death (10). Necrosis occurs from the "outside in" in response to overwhelming stress from oxygen-glucose deprivation (OGD), ischemia, glutamate excitotoxicity, reactive oxygen species (ROS) and environmental toxins. Apoptosis, or programmed cell death, can be initiated by the same stressors, but occurs in response to more subtle perturbations in OGD and ROS, or through signal transduction pathways (11-13). An example of this latter extrinsic pathway is apoptosis resulting from activation of "death receptors", *e.g.,* the tumor necrosis factor-α receptor (TNFR) or the p75 neurotrophin receptor; signal transduction can cause activation of caspase-8, initiating a cascade of activation of effector caspases, *e.g.,* caspase-3 and -7, that target the nucleus and kill the cell (14, 15). ROS can initiate apoptosis through an intrinsic pathway that acts directly on the mitochondria by involving proteins of the Bcl-2 family (12). The actual processes of cell death that occur are hypothesized to be determined by the energy status of a cell (16), and have been categorized in terms

of being mitochondria- and caspase-dependent or -independent (1, 7, 13, 17).

Mitochondria-dependent cell death is believed to underlie the death and degeneration of tissues in a variety of human disorders ranging from diseased liver (18, 19) and ischemic heart disease (20), to schizophrenia, stroke and Huntington's, Parkinson's and Alzheimer's diseases (12, 21-28). In the nervous system, cell death in both acute and chronic degenerative disorders, *e.g.,* stroke and Alzheimer's disease, is believed to result from three primary factors: 1) loss of oxidative substrates, *i.e*., OGD; 2) glutamate excitotoxicity with co-incident calcium overload; and 3) ROS, *e.g.,* superoxide radical, hydrogen peroxide and peroxynitrite. A target common to these stressors is the mitochondria. In response to lethal stimuli, the mitochondria will release mitochondrial death factors, including cytochrome *c* (Cyt *c*), second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO), apoptosis-inducing factor (AIF) and endonuclease G (1, 12, 25-32).

The molecular mechanisms that regulate the signaling and release of these mitochondria-derived death proteins are elusive, and the subject of large debate and definition (2, 7, 11, 33-35). What is clear, however, is that once these death proteins are released, particularly Cyt *c*, a complex cascade of events is initiated that results in cell death (1, 12). Cytoplasmic Cyt *c* triggers the oligomerization of apoptosis-activating factor-1 (Apaf-1), which recruits procaspase-9 and -3 into a complex known as the apoptosome. Active caspase-9 and -3 (and caspase-7) emerge to activate other proteases and nucleases, which kill the cell by degrading nuclear molecules critical to the survival of the cell (1, 2, 31, 36-38). Clearly, blocking the release of mitochondrial death factors, and particularly Cyt *c*, could have therapeutic potential for acute and chronic degenerative disorders.

Mechanisms of Cyt *c* release

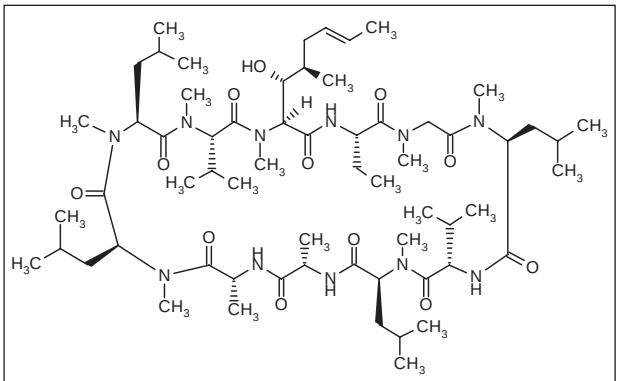
Cyt *c* is normally bound to the inner mitochondrial membrane (IMM) by an association with cardiolipin (39). In the nervous system, initiating signals of cell death due to excitotoxicity, ischemia and neurotrophic factor withdrawal are believed to create an environment of oxidative stress and Ca^{2+} overload (40-43). In response to such stress, Cyt *c* must dissociate from cardiolipin in the IMM and be released through the outer mitochondrial membrane (OMM). Cyt *c* release from mitochondria is thought to be mediated by at least two mechanisms: 1) mitochondrial permeability transition (mPT; Fig. 1A); and 2) mitochondrial outer membrane permeabilization (MOMP; Fig. 1B) (1, 44-46).

Mitochondrial permeability transition (mPT)

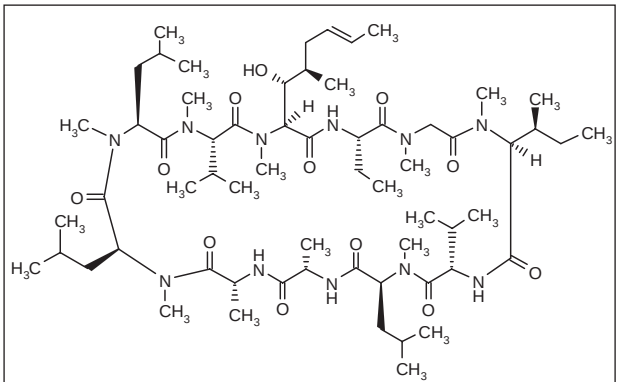
The classic mPT is the mitochondrial response to oxidative stress and/or Ca^{2+} overload. The IMM normally has a low permeability to ions and solutes. In response to stress, the IMM becomes permeable to 1,500-Da solutes, *i.e*., the permeability transition. There is a loss of mitochondrial membrane potential ($\Delta\psi$), and depletion of adenine nucleotides and respiratory substrates. The mitochondrial matrix osmotically accumulates water, resulting in mitotochondrial swelling that results in rupture of the OMM, release of Cyt *c* into the cytoplasm and activation of the apoptosome (2, 46-49).

The exact nature of the mPT remains obscure, despite detailed characterization of the molecular components. These components are believed to include the voltage-dependent anion channel (VDAC), an OMM protein, the IMM protein adenine nucleotide translocase (ANT) and cyclophilin D (Cyp-D) at outer and inner membrane contact sites (47, 50). Upon activation of the mPT, these proteins are thought to form an mPT pore now permeable to solutes with masses around 1,500 Da.

Cyclosporin (**1**) is an historically important drug used to pharmacologically characterize the mPT. Cyclosporin is a potent immunosuppressive drug that binds to the peptidylprolyl isomerase cyclophilin A (Cyp-A); the cyclosporin-cyclophilin complex is a potent inhibitor of the phosphatase calcineurin. In the mitochondria, cyclosporin targets Cyp-D (51). The classic mPT is defined by the ability of cyclosporin to block the mitochondrial swelling and depolarization response to Ca^{2+} overload (51, 52). Analogues of cyclosporin which lack the ability to inhibit calcineurin but retain the ability to bind



Cyclosporin (**1**)



NIM-811 (**2**)

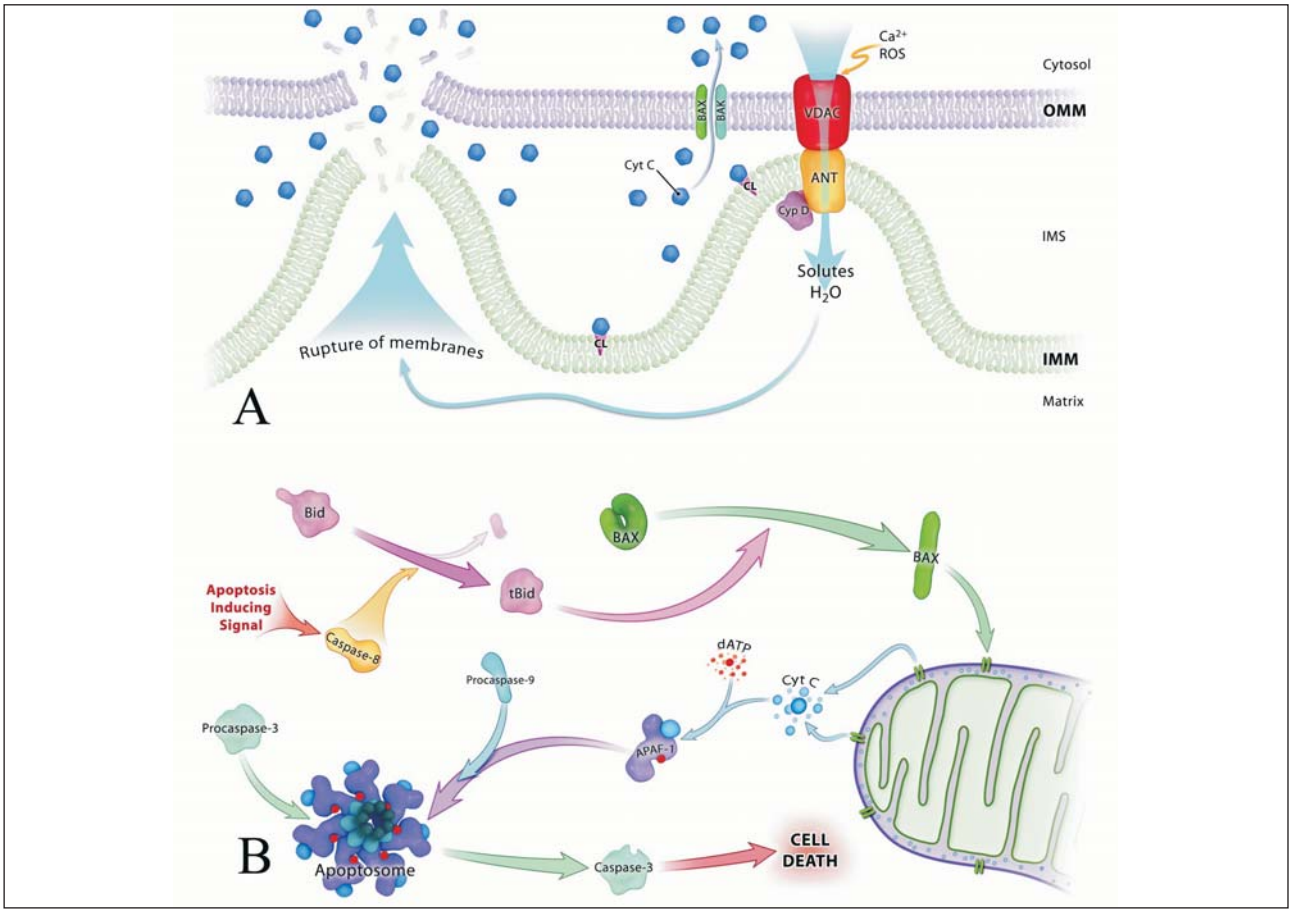


Fig. 1. Mechanisms of Cyt *c* release. **A.** Mitochondrial permeability transition (mPT)**.** In response to stress, the inner mitochondrial membrane (IMM) becomes permeable to 1,500-Da solutes. There is a loss of $\Delta\psi$, and depletion of adenine nucleotides and respiratory substrates. The mitochondrial matrix osmotically accumulates water, resulting in mitochondrial swelling, which results in rupture of the outer mitochondrial membrane (OMM) and release of Cyt *c* into the cytoplasm. IMS: intermembrane space; VDAC: voltage-dependent anion channel; ANT: adenine nucleotide translocase; Cyp-D: cyclophilin-D; CL: cardiolipin; Cyt *c*: cytochrome *c*; ROS: reactive oxygen species. Modified from Ref. 44. **B**. Mitochondrial outer membrane permeabilization. Permeability results from formation of a proteolipid pore controlled by members of the Bcl-2 family of proteins. Upon initiation of the extrinsic apoptotic cascade, initiator caspase-8 cleaves Bid to t-Bid. t-Bid activates the translocation and/or oligomerization of Bax (with Bak) to the OMM. This protein complex forms a channel or pore permeable to Cyt, resulting in a massive release of Cyt *c* into the cytosol and activating the formation of the apoptosome. Binding of Cyt *c* with dATP to APAF-1 triggers recruitment of procaspase-9 and procaspase-3 into the formation of the apoptosome oligomer. Activated caspase-3 is released, activating the subsequent processes of cell death (44).

to Cyp-A and Cyp-D also block mPT. Thus, *N*-methyl-4-isoleucine-cyclosporin A (NIM-811; **2**) blocks calcium-induced mPT (53). Such nonimmunosuppressive cyclosporin analogues have been useful for distinguishing between mPT- and calcineurin-mediated effects. Much of the pathophysiological involvement of mPT is inferred by the protective efficacy of cyclosporin (47).

Further understanding of the molecular nature of the mPT has come from studies using transgenic mice. Transgenic gene depletion experiments have examined the effect of deleting ANT, VDAC and Cyp-D on the mPT. Deletion or knockout (KO) of ANT or VDAC1 has little or no effect on Ca^{2+}-induced, cyclosporin-inhibitable mPT, *i.e.,* the response of the KO mice was similar to wild-type animals, indicating that although they may have a regulatory role, ANT and VDAC1 are not required for a functioning mPT pore (54, 55). Four laboratories have reported a significant effect upon deleting Cyp-D (56-59). Mitochondria from these KO animals are significantly less sensitive to Ca^{2+}, and although much higher concentrations of Ca^{2+} can still induce an mPT, the pore opening is insensitive to cyclosporin, indicating that Cyp-D is also a regulator, but not a key structural component, of the mPT pore (46-48). Although cells from the Cyp-D KO animals were still sensitive to apoptosis induced by staurosporine and etoposide, similar to wild-type animals, they were resistant to H_2O_2. Furthermore, *in vivo*, the Cyp-D KO mice were resistant to tissue injury induced by brain or cardiac ischemia. This implicates Cyp-D in necrotic cell death pathways induced by oxidative stress. However, in several apoptotic paradigms, cyclosporin can inhibit apoptotic cell death (46, 48). Similarly, fluoxetine, a volt-

age sensitizer of VDAC, is reported to decrease VDAC conductance, inhibit opening of the mPT pore and release of Cyt *c*, and protect against staurosporine-induced apoptotic cell death (60). Thus, several investigators caution that it is still premature to absolutely conclude that Cyp-D and mPT are involved in only necrotic death pathways and not apoptotic pathways (46-48).

Mitochondrial outer membrane permeabilization (MOMP)

MOMP is the result of the formation of a proteolipid pore controlled by members of the Bcl-2 family of proteins (11). Upon initiation of the extrinsic apoptotic cascade (13), initiator caspase-8 activates a cytoplasmic protein called Bid to a truncated form, *i.e*., t-Bid. t-Bid activates the translocation and/or oligomerization of Bax (with Bak) to the OMM. This protein complex forms a channel or pore permeable to Cyt *c* (and possibly Smac/DIABLO and AIF), resulting in a massive release of Cyt *c* into the cytosol and activation of the apoptosome.

Again, the molecular structures underlying the MOMP remain obscure. In the absence of Bax and Bak, MOMP does not occur (1, 35, 61-63). A Cyt *c*-permeable pore can be constructed in liposomes by oligomers of recombinant Bax (64). Translocation of cytosolic Bax to the mitochondria and/or oligomerization of Bax associated with mitochondria is induced by interaction with t-Bid (62, 65, 66). Dejean *et al.* (35) define the pore as a mitochondrial apoptosis-induced channel (MAC), a heterogeneous, high-conductance channel detected by patch clamping of mitochondria during t-Bid-induced release of Cyt *c* (35).

Although mPT and MOMP are largely considered to be independent, there is evidence of overlap between these molecular events. Bax is reported to affect VDAC or ANT function, influencing Cyt *c* release (67-70). When Bax and Bak are depleted by genetic knockout, the mitochondria from HCT 116 cells are reported to exhibit a Ca^{2+}-induced mPT, indicating that Bax has no major role in the mPT (71). However, in primary cerebellar granule neurons, when mitochondria-dependent apoptosis is initiated by trophic factor withdrawal, mPT, as indicated by mitochondrial swelling and loss of $\Delta\psi$, is an early event, followed by translocation of Bax to mitochondria and induction of MOMP; the phenomena, including Bax translocation, are blocked by cyclosporin (72).

The mPT pore, MOMP and/or MAC are hypothesized to be mechanisms for the release of other mitochondrial death factors, *e.g*., Smac/DIABLO and AIF (12). The temporal coincidence of the release of these factors with Cyt *c* is under active investigation, and appears to depend on the cell type and stressor used to initiate release (27, 30, 34, 73). For example, MEKK1 kinase overexpression in HEK293 cells induces release of Smac/DIABLO from mitochondria independent of Cyt *c* (74). However, in osteoblastoma cells where staurosporine induces Bax translocation and coincident release of both Cyt *c* and Smac/DIABLO in wild-type cells, genetic depletion of Cyt *c* disables Smac/DIABLO release following staurosporine induction, indicating a complexity in the regulation of release of these two proteins.

Mitochondria-dependent cell death in acute disorders

Much evidence supports a role for mitochondria/Cyt *c*/caspase-dependent cell death in models of focal ischemic stroke (28, 30, 75). Cerebral ischemia causes mitochondrial dysfunction immediately due to OGD. Both necrotic and apoptotic processes are activated (76). In the infarct core, the tissue dies due to lack of energy and collapse of cell membranes and ionic gradients. Although mitochondrial function can be restored in the partially perfused penumbra or reperfused lesion with re-supply of substrates, there is clear evidence of a secondary, delayed mitochondrial dysfunction, called secondary energy failure, which is believed to underlie a delayed apoptotic cell death following cerebral ischemia. Mitochondrial swelling is observed, Cyt *c* is released into the cytoplasm, caspases are activated and brain tissue dies (28, 43, 77-82).

The neuroprotection afforded by the antioxidants ebselen and NXY-059 correlates with a reduction in Cyt *c* release in the area at risk (83-85). Drugs that are known to block the mPT have been shown to reduce infarct volume and improve neurological outcome in rodent models of stroke. Wieloch and Siesjo have reported the protective effects of cyclosporin (86-89). However, the clinical application of cyclosporin is limited by its very poor brain penetration after systemic administration (90). In a definitive study, a nonimmunosuppressive analogue of cyclosporin was shown to reduce infarct size (91). Uchino *et al.* have recently reported the efficacy of a new Cyp-D inhibitor, FR-901495, which blocks mPT and tissue injury due to forebrain ischemia (88). Andrabi *et al.* reported that the antiapoptotic effects of melatonin *in vivo* were mediated by direct inhibition of the mPT (92). Another group reported a very complete analysis of heterocyclic drugs, exemplified by promethazine, in their potency to block the mPT, protect against OGD in culture and reduce infarct volume in a rodent model of stroke (24).

Mitochondria-dependent cell death in chronic disorders

Alzheimer's disease (AD) is a serious age-associated dementia, the diagnosis of which is still confirmed only by *post mortem* histological observation of extraordinary brain accumulation of amyloid plaques, the defining pathology of the dementia (93-95). Despite the genetic linkage to mutations in the amyloid precursor protein (APP) in a minority of cases (96), the cause(s) of nonfamilial, sporadic, senile dementia of the Alzheimer's type (SDAT) is unknown (97). Extensive study on the effects of the Aβ peptide precursor of AD plaque indicates that a primary mechanism of Aβ toxicity is the induction of ROS (98-102). In fact, chronic oxidative stress and glutamate excitotoxicity are hypothesized to underlie the apoptotic

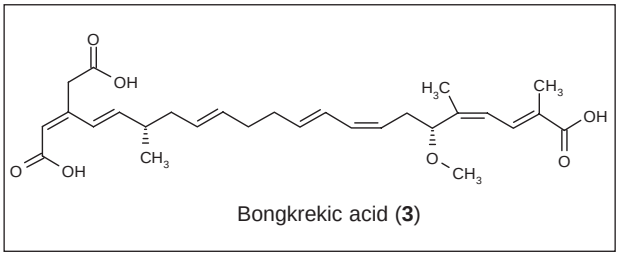
neuronal death associated with SDAT, very similar to the acute stress in ischemic stroke (25, 103, 104). The apoptotic neuronal death that occurs in AD (99, 105, 106) is hypothesized to involve mitochondrial dysfunction in the respiratory chain (107), and to be mediated by the release of mitochondrial death proteins such as Cyt *c* (12, 26, 103). Several laboratories are now focused on a mitochondrial hypothesis of AD (105, 108-110). Novel pharmaceutical agents that prevent mitochondrial dysfunction and/or the release of mitochondrial death proteins are speculated to slow or prevent the progression of disease (23, 97, 107).

Discovery and development of molecules that target mitochondrial permeability

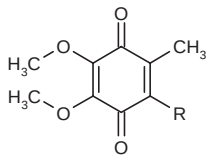
Much of the basic understanding of the mPT and its role in pathophysiology evolved from work with cyclosporin. The classic mPT and its involvement in ischemic and degenerative disorders is defined by the ability of cyclosporin to block the mPT and inhibit mitochondria-dependent cell death. Other mPT blockers with inhibitory effects similar to cyclosporin include bongkrekic acid (**3**), adenosine diphosphate (ADP), various ubiquinone analogues (**4**), and a number of amphipathic compounds such as spermine and trifluoroperazine. However, in most cases, these compounds have poor specificity, and except for bongkrekic acid and ADP, which are ligands for the ANT, the molecular targets are not well characterized. Nonetheless, the protective efficacy of cyclosporin in clinically important paradigms has spurred recent interest in the discovery of new classes of molecules which target the mitochondria and block membrane permeability and the release of cell death-inducing proteins.

VDAC-targeted agents

A group at Roche recently described compounds that target the VDAC component of the mPT (55, 111)**.** With an interest in identifying new inhibitors of the mPT, the Roche investigators screened a chemical compound library for the ability to inhibit Ca^{2+}-induced swelling in energized rat liver mitochondria. Compounds that inhibited swelling but did not interfere with mitochondrial respiration at the concentrations that blocked the mitochondrial permeability transition pore (mPTP) were characterized further. Several compounds with a common core structure, exemplified by Ro-04-2843 (**5**), inhibited the mPTP in the low micromolar range. The enone derivative Ro-68-3406 (**6**) was prepared and evaluated, and it too was found to be a potent mPTP inhibitor. A number of analogues were subsequently prepared, of which Ro-68-3400 (**7**) was the most potent, with an EC_{50} of 190 nM. In the same version of the swelling assay, cyclosporin had an EC_{50} of 300 nM, indicating that Ro-68-3400 is one of the most potent mPTP inhibitors described. The high potency of Ro-68-3400, coupled with the fact that it contains a reactive moiety (the α,β-unsaturated ketone), made it an attractive candidate for affinity labeling studies. Accordingly, the Roche group prepared the tritiated analogue and used it as an affinity probe. They identified the predominantly labeled 32-kDa protein to be isoform 1 of VDAC.



Bongkrekic acid (**3**)



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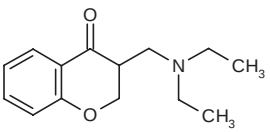
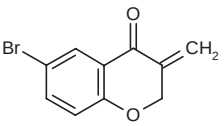
R = H (ubiquinone)
R = -$(CH_2)_9CH_3$ (decylubiquinone)
R = CH_3 ($TM\text{-}Ub_{10}$)



Ro-04-2843 (**5**)



Ro-68-3406 (**6**)

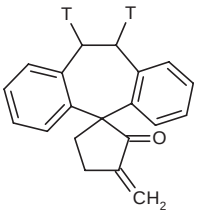
A recent report suggests that fluoxetine (Prozac), a 5-HT reuptake inhibitor, interacts with VDAC and decreases its conductance. The drug was shown to inhibit the mPT and release of Cyt *c*, and to protect against staurosporine-induced apoptotic cell death (60).

2-Aminoethoxydiphenyl borate

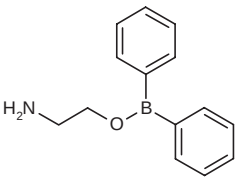
Gary Fiskum and co-workers at the University of Maryland School of Medicine have extensively studied

the mPT characteristics of brain mitochondria (12, 112, 113). Mitochondria from brain are particularly resistant to Ca^{2+}-induced swelling and are less responsive than mitochondria from other tissues to mPT blockade by cyclosporin. Fiskum's group has reported that the compound 2-aminoethoxydiphenyl borate (2-APB; **8**) is able to inhibit Ca^{2+}-induced Ca^{2+} release in rat brain mitochondria in the presence of physiological concentrations of ATP and Mg^{2+} (114). Cyclosporin did not inhibit mPT under these conditions. The inhibition of Ca^{2+}-induced Ca^{2+} release by 2-APB was concentration-dependent, and treatment with 2-APB also protected against Ca^{2+}-induced loss of $\Delta\psi$. Ca^{2+}-induced release of Cyt *c*, as well as release of matrix pyridine nucleotides, was likewise inhibited by 2-APB but not by cyclosporin.

Several classes of compounds structurally related to 2-APB have recently been shown to block mitochondrial channels. These compounds are discussed in the next two sections.



Ro-68-3400 (**7**)



2-APB (**8**)

Tricyclic antidepressants and related structures which block the mPTP and are effective in a stroke model

Kristal and co-workers screened a collection of 1,040 FDA-approved drugs for their ability to inhibit Ca^{2+}-induced mPT in energized rat liver mitochondria (24). The initial screen identified roughly two dozen compounds which afforded moderate protection against Ca^{2+}-induced swelling. These compounds were either tricyclic antidepressants or phenothiazine antipsychotics. Representative compounds identified are shown in Figure 2.

Most of the compounds provided statistically significant protection at concentrations < 30 μM, and about half were protective at 3 μM. Representative compounds from the chemical classes were shown not to alter normal mitochondrial physiology at relevant concentrations, suggesting that the protective effects were not associated with mitochondrial toxicity.

Promethazine was selected for further study as it was one of the more potent mPT inhibitors identified, and it is well tolerated at high doses in humans. Promethazine was neuroprotective against OGD *in vitro* and against middle cerebral artery occlusion (MCAO) *in vivo* (24). The protective effect of promethazine and other tricyclic compounds did not correlate with their ability to inhibit either calcineurin or phospholipase A_2. These results support the therapeutic utility of mPTP blockade for ischemia-related damage.

Compounds that block MOMP

Scientists at Serono reported that substituted carbazoles were capable of blocking Bax channel formation and the release of Cyt *c* from isolated mitochondria (115). Compounds (Table I; concentrations are 10 μM unless

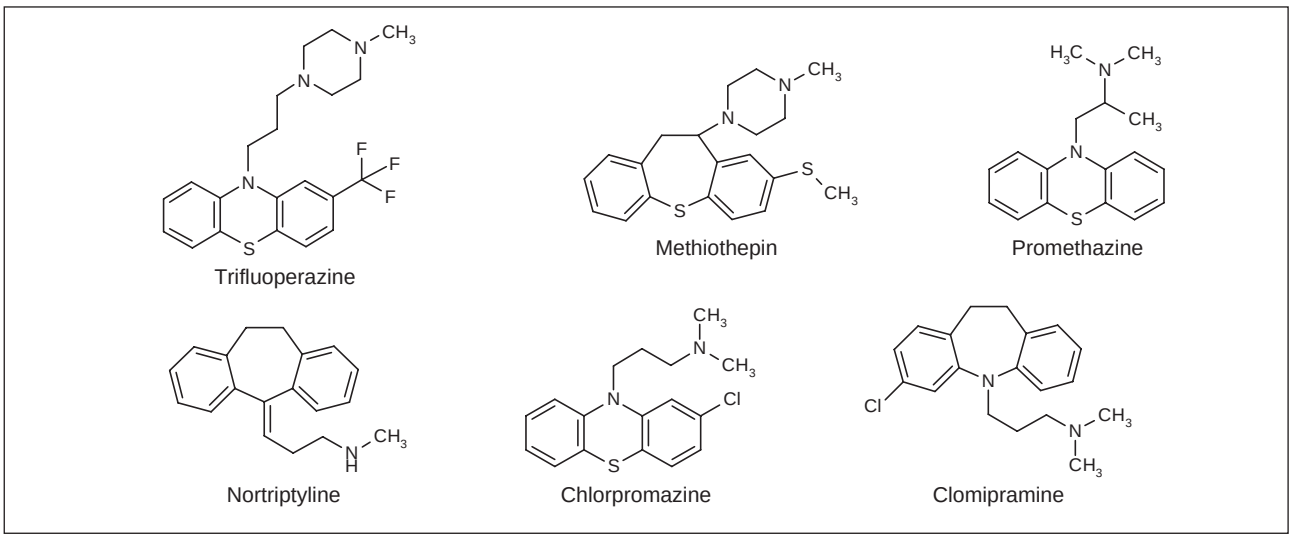
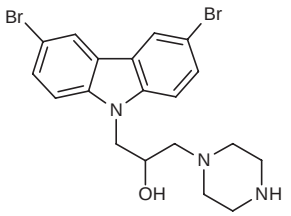
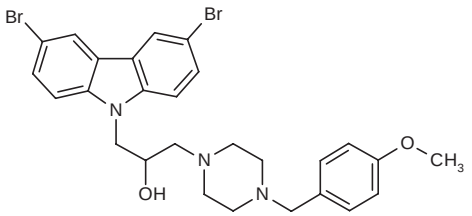
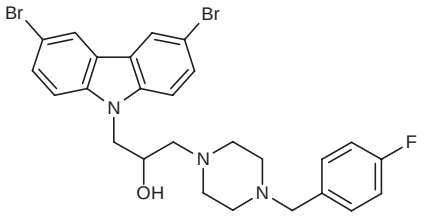
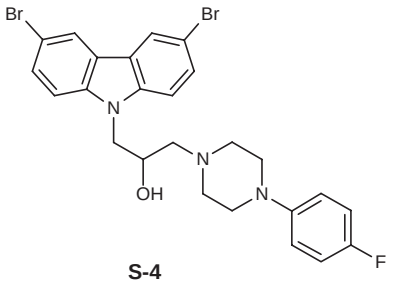
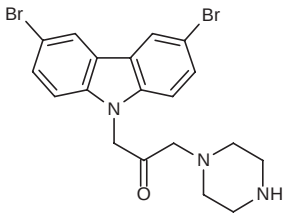
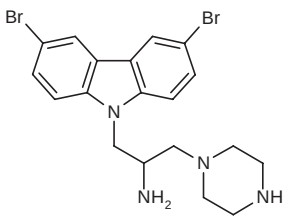


Fig. 2. Representative tricyclic antidepressants and phenothiazine antipsychotics that inhibit Ca^{2+}-induced mPT.

Table I: Inhibitory potencies of carbazoles in Bax channel liposome assays and in blocking Cyt c release from isolated mitochondria.

Compound	IC_{50} (μM) in Bax assay	% Inhibition of Cyt c release (concentration)
 **S-1**	0.52 ± 0.21	99 ± 2 (20 μM) 99 ± 4 (10 μM) 77 ± 7 (5 μM)
 **S-2**	0.37 ± 0.12	99 ± 2 (20 μM) 82 ± 4 (10 μM) 61 ± 7 (5 μM)
 **S-3**	0.48 ± 0.14	65 ± 6
 **S-4**	2.38 ± 0.48	15 ± 10
 **S-5**	0.29 ± 0.04	71 ± 5
 **S-6**	0.96 ± 0.10	71 ± 10

otherwise noted) were evaluated for their ability to inhibit the release of Cyt *c* from isolated mitochondria in which Bax proteins were activated by treatment with t-Bid. Compound S-1 was the most potent in this assay, producing nearly total inhibition of Cyt *c* release at 10 μM. There was some sensitivity to substitution on the piperazine ring with respect to activity; activity decreased when the 4-amino group was substituted with *para*-methoxybenzyl, *para*-fluorobenzyl and *para*-fluorophenyl. Oxidation of the hydroxyl of S-1 (to give S-5) or replacement by amino (S-6) caused modest decreases in activity.

The carbazoles were also tested for their ability to block formation of Bax channels in a fluorescence-based liposome assay. Compounds which potently inhibited Cyt *c* release from isolated mitochondria (*e.g.*, S-1) were found to be submicromolar Bax channel formation blockers, while compounds which were weak inhibitors of Cyt *c* release were less effective in the liposome assay (S-4). In order to gain insight into the mode of action of the inhibitors, derivatives of S-1 which incorporated a fluorescent moiety attached to the piperazine ring were prepared. HeLa cells were incubated with one of two different fluorescent-tagged inhibitors and treated with the mitochondrial marker Mitotraker. The inhibitors co-localized with the marker, suggesting interaction with mitochondrial components as a basis for their action.

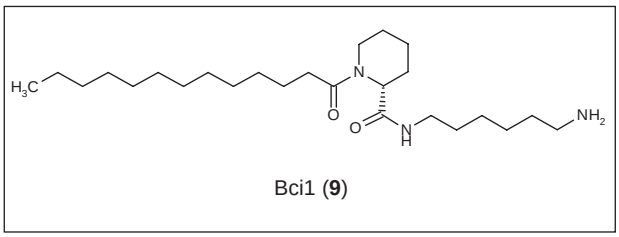
Subsequently, the Serono group identified structurally distinct Bax channel blockers from screening a large chemical library (63). These compounds, Bci1 (**9**) and Bci2 (**10**), were also shown to inhibit Bax channel formation in liposomes and prevented Cyt *c* release from isolated mitochondria. They did not affect VDAC channel conductance or gating properties. Both compounds protected HeLa cells against Bax-mediated apoptosis induced by staurosporine treatment, and were protective *in vivo* in a gerbil model of global ischemia.

Bis-urea and bis-amide modulators of mitochondrial function: molecules with multiple modes of action

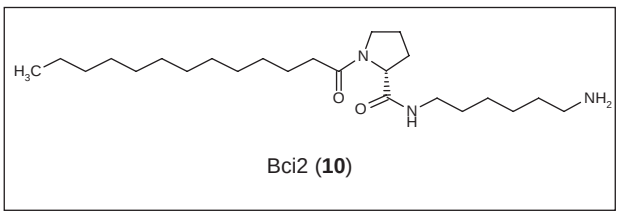
We have previously published data on the activity of simple aryl- and bisarylureas and -thioureas as inhibitors of the peptidylprolyl isomerase (PPIase; rotamase) activity of Cyp-A (116). Because of the known role of Cyp-D as a component of the mPT and the documented mPT-inhibitory activity of cyclosporin, a plate-reader absorbance assay which measures the ability of test compounds to inhibit toxin-induced swelling of mitochondria from rat liver was established as part of Guilford Pharmaceuticals' cyclophilin inhibitor program. After evaluating a number of cyclophilin rotamase inhibitors as inhibitors of mitochondrial swelling, it became apparent that the two activities were not well correlated.

Compounds were found to separate into three classes based on their activities as inhibitors of Cyp-D rotamase activity and their ability to inhibit mitochondrial swelling: 1) compounds that were potent inhibitors of cyclophilin enzymatic activity but were ineffective as swelling inhibitors; 2) compounds that were equipotent for both activities, including cyclosporin and several of its analogues; and 3) compounds that were weak or inactive as rotamase inhibitors but were highly effective at blocking the mPTP, such as GPI-15483 (**11**) and GPI-18297 (**12**).

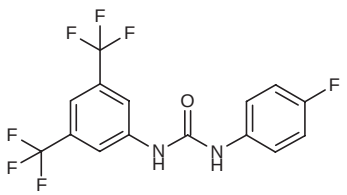
The initial screening for mitochondrial channel blockers used a deliberately de-energized preparation of rat liver mitochondria. Swelling was induced by diamide acting synergistically with calcium. This preparation was used to eliminate, theoretically, false-positive drug effects on mitochondrial respiration and membrane potential. A number of compounds were identified that achieved low nanomolar potency (Table II). Whereas the original leads (GPI-15483 and GPI-18297) were relatively insoluble (kinetic aqueous solubilities < 5 μg/ml), incorporation of the cyanoguanidine moiety into the simple ureas provided compounds which retained activity as swelling inhibitors and showed greatly enhanced aqueous solubility (*e.g.,* GPI-19410, or compound **28** in Table II, with a



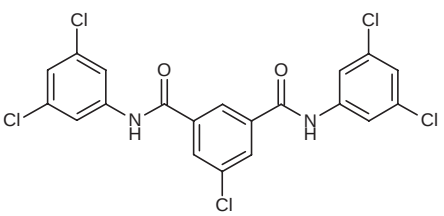
Bci1 (**9**)



Bci2 (**10**)

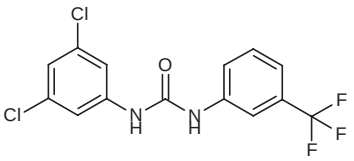
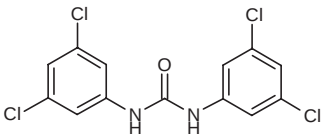
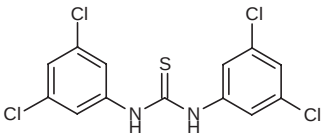
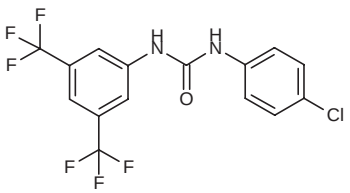
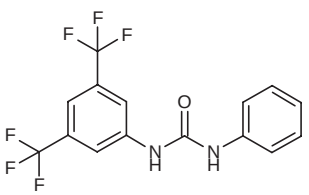
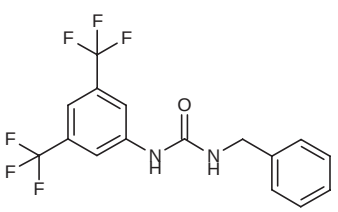


GPI-15483 (**11**)
EC_{50} = 600 nM



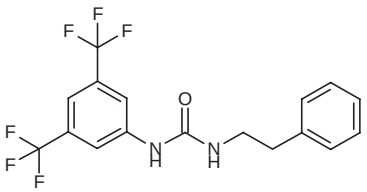
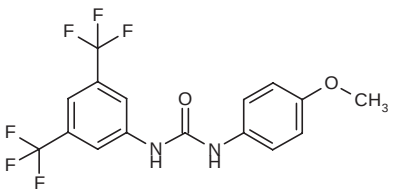
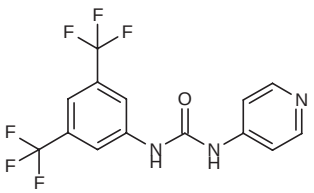
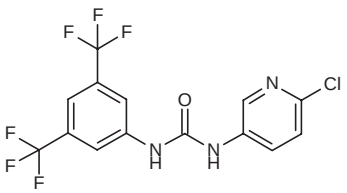
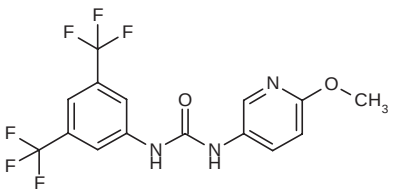
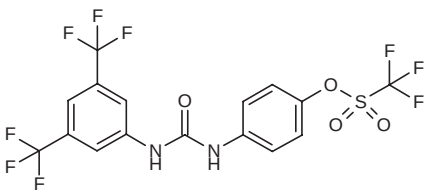
GPI-18297 (**12**)
EC_{50} = 10 nM

Table II: Potencies of test compounds for blocking mitochondrial permeability transition (mPT).

Compound	IC_{50} (μM)
 **13**	0.130
 **14**	0.819
 **15**	0.987
 **16**	0.797
 **17**	3.24
 **18**	7.29

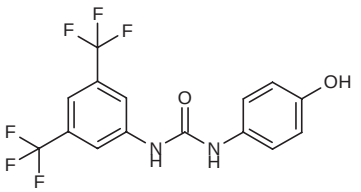
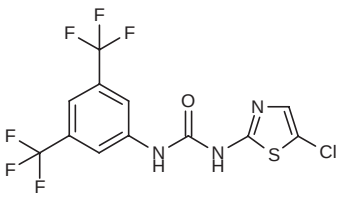
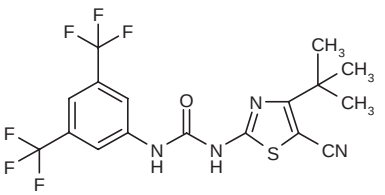
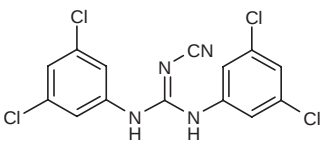
Continuation

Table II (Cont.): Potencies of test compounds for blocking mitochondrial permeability transition (mPT).

Compound	IC_{50} (μM)
 **19**	36% inhibition @ 3 μM
 **20**	2.87
 **21**	1.32
 **22**	0.160
 **23**	1.07
 **24**	0.166

Continuation

Table II (Cont.): Potencies of test compounds for blocking mitochondrial permeability transition (mPT).

Compound	IC_{50} (μM)
 **25**	6.22
 **26**	0.194
 **27**	0.030
 **28** GPI-19410	0.150

solubility of 40 μg/ml and an IC_{50} in the swelling assay of 150 nM). The incorporation of a pyridine ring into the urea structure was also tolerated, providing compounds such as **22**, from which water-soluble salts may be prepared.

In general, substitution of the aryl rings of the ureas with electron-withdrawing groups was favorable, while electron-donating groups were deleterious to activity. Replacement of a halo group with methoxy, for example, resulted in 5-10-fold loss of activity (compare, for example, compounds **16** and **20**, and **22** and **23**). Converting the phenolic hydroxyl of **25** (IC_{50} = 6.22 μM) to the strongly electron-withdrawing triflate group increased potency for inhibition of mPTP by 40-fold (**24**; IC_{50} = 166 nM). Moving the aryl rings away from the urea moiety decreased activity (**17-19**). Five-membered heterocycles were tolerated (**26**, **27**).

The prototype compound is GPI-19410 (**28**). The protective properties of this molecule were characterized in several paradigms. Work by Dubinsky *et al*. (117) found that in nonsynaptosomal brain mitochondria, GPI-19410 had no effect on mitochondrial respiration (state 4, state 3 or uncoupled) with either succinate-glutamate or pyruvate-malate as substrates. However, GPI-19410 prevented mitochondrial depolarization in response to Ca^{2+} (250 nmoles/mg mitochondrial protein), and blocked Ca^{2+}-induced mitochondrial swelling (Fig. 3).

In mouse liver mitochondria, GPI-19410 blocked Ca^{2+}-induced swelling and Cyt *c* release with an IC_{50} of

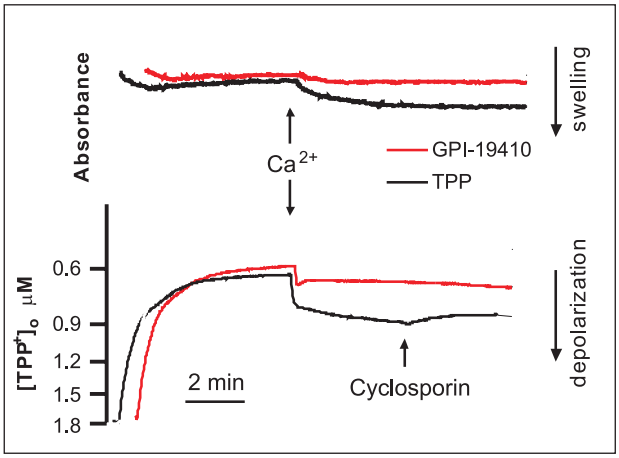


Fig. 3. GPI-19410 blockade of Ca^{2+}-induced $\Delta\psi$. Mitochondrial membrane potential ($\Delta\psi$) and light scattering were measured simultaneously in isolated CNS mitochondria at 37 ºC in a continuously stirred chamber, according to Dubinsky *et al.* (118). $\Delta\psi$ was followed by monitoring the distribution of tetraphenylphosphonium cation (TPP^+) between the external medium and the mitochondrial matrix with a TPP^+-sensitive electrode. Mitochondrial swelling was measured as the change of light scattering with a photodiode light detector (117).

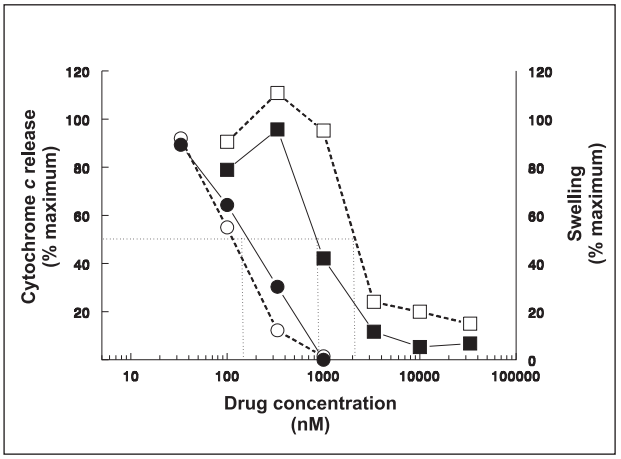


Fig. 4. GPI-19410 blockade of Ca^{2+}-induced Cyt *c* release. Mouse liver mitochondria were used in the primary drug screens. The Ca^{2+}-induced swelling was assayed in 96-well conical bottom plates in a final volume of 200 µl using a modification of conditions described by Antonsson (119), without Mg^{2+}, which antagonizes swelling. At the end of the swelling incubation, the 96-well plates were centrifuged and an aliquot of the supernatant from each well was assayed by HPLC for Cyt *c* content (120). Cyclosporin and GPI-19410 blocked swelling and Cyt *c* release with IC_{50} values of 100 mM and 1 µM, respectively.

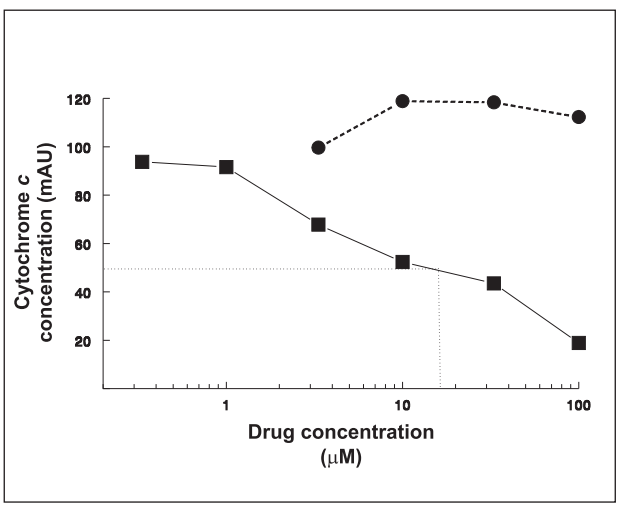


Fig. 5. GPI-19410 blockade of t-Bid-induced Cyt *c* release. The assay was modified from Antonsson (119) using 96-well conical bottom plates in a final volume of 200 µl. Mouse liver mitochondria co-purify with sufficient Bax to channel the release of Cyt *c* upon addition of t-Bid (12). Cyt *c* release was induced by addition of 6 nM human recombinant t-Bid. After 30 min at 30 °C, the plates were centrifuged and an aliquot of the supernatant from each well was assayed by HPLC for Cyt *c* content (120). GPI-19410 blocked t-Bid-induced Cyt *c* release with an IC_{50} of 20 µM; cyclosporin had no protective effect in this assay.

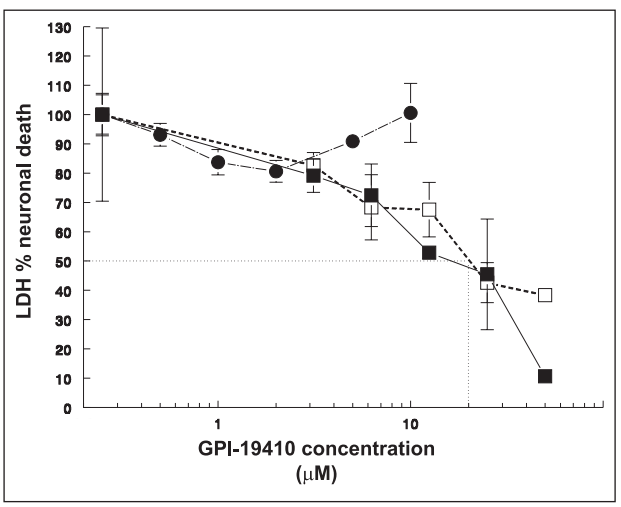


Fig. 6. GPI-19410 protects against NMDA toxicity in hippocampal cultures. Primary cultures of hippocampal neurons were prepared according to published methods (121). After 5-7 days *in vitro*, the media were removed and replaced with 200 µM NMDA in Hank's balanced salt solution. Cells were incubated with NMDA at 37 °C under normal atmospheric conditions for 20 min. The HBSS was removed and cells were returned to normal culture conditions. Cell death was analyzed by LDH assay 18-24 h after NMDA exposure. GPI-19410 blocked NMDA toxicity with an IC_{50} of 20 µM; cyclosporin had no protective effect in this assay.

1 µM; cyclosporin had an IC_{50} of 200 nM (Fig. 4). GPI-19410 also blocked t-Bid-induced Cyt *c* release in mouse liver mitochondria with an IC_{50} of 20 µM; cyclosporin had no effect on t-Bid-induced Cyt *c* release (Fig. 5). In tissue cultures of rat embryonic hippocampal neurons, GPI-19410 protected against 200 mM NMDA-induced toxicity; cyclosporin had no protective effect in this assay (Fig. 6). GPI-19410 may act via multiple mechanisms to block Cyt *c* release in stressed mitochondria in a manner distinct from cyclosporin.

References

1. Green, D.R., Kroemer, G. *The pathophysiology of mitochondrial cell death*. Science 2004, 305: 626.

2. Bernardi, P., Krauskopf, A., Basso, E. et al. *The mitochondrial permeability transition from in vitro artifact to disease target*. FEBS J 2006, 273: 2077-99.

3. Wang, Y., Perchellet, E.M., Ward, M.M. et al. *Antitumor triptycene analogs induce a rapid collapse of mitochondrial transmembrane potential in HL-60 cells and isolated mitochondria*. Int J Oncol 2006, 28: 161-72.

4. Armstrong, J.S. *Mitochondria: A target for cancer therapy*. Br J Pharmacol 2006, 147: 239-48.

5. Schmitt, E., Maingret, L., Puig, P.E. et al. *Heat shock protein 70 neutralization exerts potent antitumor effects in animal models of colon cancer and melanoma*. Cancer Res 2006, 66: 4191-7.

6. Debatin, K.M. *Apoptosis pathways in cancer and cancer therapy*. Cancer Immunol Immunother 2004, 53: 153.

7. Adams, J.M. *Ways of dying: Multiple pathways to apoptosis*. Genes Develop 2003, 17: 2481.

8. Yuan, J., Lipinski, M., Degterev, A. *Diversity in the mechanisms of neuronal cell death*. Neuron 2003, 40: 401.

9. Nicotera, P., Leist, M., Manzo, L. *Neuronal cell death: A demise with different shapes*. Trends Pharmacol Sci 1999, 20: 46.

10. Van Cruchten, S., Van den Broeck, W. *Morphological and biochemical aspects of apoptosis, oncosis and necrosis*. Anat Histol Embryol-J Vet Med Ser C 2002, 31: 214.

11. Green, D.R. *At the gates of death*. Cancer Cell 2006, 9: 328-30.

12. Polster, B.M., Fiskum, G. *Mitochondrial mechanisms of neural cell apoptosis*. J Neurochem 2004, 90: 1281.

13. Green, D.R. *Apoptotic pathways: Paper wraps stone blunts scissors*. Cell 2000, 102: 1.

14. Leong, K.G., Karsan, A. *Signaling pathways mediated by tumor necrosis factor alpha*. Histol Histopathol 2000, 15: 1303.

15. Sharma, K., Wang, R.X., Zhang, L.Y. et al. *Death the Fas way: Regulation and pathophysiology of CD95 and its ligand*. Pharmacol Ther 2000, 88: 333.

16. Nicotera, P., Leist, M., Fava, E., Berliocchi, L., Volbracht, C. *Energy requirement for caspase activation and neuronal cell death*. Brain Pathol 2000, 10: 276.

17. Cregan, S.P., Dawson, V.L., Slack, R.S. *Role of AIF in caspase-dependent and caspase-independent cell death*. Oncogene 2004, 23: 2785-96.

18. Zhao, K., Zhao, G.M., Wu, D. et al. *Cell-permeable peptide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury*. J Biol Chem 2004, 279: 34682.

19. Morin, D., Pires, F., Plin, C., Tillement, J.P. *Role of the permeability transition pore in cytochrome C release from mitochondria during ischemia-reperfusion in rat liver*. Biochem Pharmacol 2004, 68: 2065-73.

20. Halestrap, A.P. *The mitochondrial permeability transition pore in reperfusion injury and cardioprotection*. Cardiovasc J S Afr 2004, 15: S5.

21. Prabakaran, S., Swatton, J.E., Ryan, M.M. et al. *Mitochondrial dysfunction in schizophrenia: Evidence for compromised brain metabolism and oxidative stress*. Mol Psychiatr 2004, 9: 684.

22. Fiskum, G., Starkov, A., Polster, B.M., Chinopoulos, C. *Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease*. Ann NY Acad Sci 2003, 991: 111-9.

23. Kristal, B.S., Stavrovskaya, I.G., Narayanan, M.V. et al. *The mitochondrial permeability transition as a target for neuroprotection*. J Bioenerg Biomembr 2004, 36: 309.

24. Stavrovskaya, I.G., Narayanan, M.V., Zhang, W.H. et al. *Clinically approved heterocyclics act on a mitochondrial target and reduce stroke-induced pathology*. J Exp Med 2004, 200: 211.

25. Hynd, M.R., Scott, H.L., Dodd, P.R. *Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease*. Neurochem Int 2004, 45: 583.

26. Lindholm, D., Eriksson, O., Korhonen, L. *Mitochondrial proteins in neuronal degeneration*. Biochem Biophys Res Commun 2004, 321: 753.

27. Penninger, J.M., Kroemer, G. *Mitochondria, AIF and caspases - Rivaling for cell death execution*. Nat Cell Biol 2003, 5: 97.

28. Plesnila, N. *Role of mitochondrial proteins for neuronal cell death after focal cerebral ischemia*. Acta Neurochir Suppl 2004, 89: 15.

29. Daugas, E., Susin, S.A., Zamzami, N. et al. *Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis*. FASEB J 2000, 14: 729.

30. Dawson, V.L., Dawson, T.M. *Deadly conversations: Nuclear-mitochondrial cross-talk*. J Bioenerg Biomembr 2004, 36: 287-94.

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

32. Verhagen, AM., Ekert, P.G., Pakusch, M. et al. *Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins*. Cell 2000, 102(1): 43-53.

33. Chipuk, J.E., Bouchier-Hayes, L., Green, D.R. *Mitochondrial outer membrane permeabilization during apoptosis: The innocent bystander scenario*. Cell Death Differ 2006, 13(8): 1396-402.

34. Jemmerson, R., Dubinsky, J.M., Brustovetsky, N. *Cytochrome c release from CNS mitochondria and potential for clinical intervention in apoptosis-mediated CNS diseases*. Antioxid Redox Signal 2005, 7: 1158-72.

35. Dejean, L.M., Martinez-Caballero, S., Kinnally, K.W. *Is MAC the knife that cuts cytochrome c from mitochondria during apoptosis?* Cell Death Differ 2006, 13(8): 1387-95.

36. Halestrap, A.P., Doran, E., Gillespie, J.P., O'Toole, A. *Mitochondria and cell death*. Biochem Soc Trans 2000, 28: 170.

37. Jiang, X.J., Wang, X.D. *Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1*. J Biol Chem 2000, 275: 31199.

38. Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X. *Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome c*. Cell 1996, 86: 147.

39. Lutter, M., Fang, M., Luo, X., Nishijima, M., Xie, X., Wang, X. *Cardiolipin provides specificity for targeting of tBid to mitochondria*. Nat Cell Biol 2000, 2: 754.

40. Vesce, S., Kirk, L., Nicholls, D.G. *Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate*. J Neurochem 2004, 90: 683.

41. Schinder, A.F., Olson, E.C., Spitzer, N.C., Montal, M. *Mitochondrial dysfunction is a primary event in glutamate neurotoxicity*. J Neurosci 1996, 16: 6125.

42. Chang, L.K., Putcha, G.V., Deshmukh, M., Johnson, E.M. Jr. *Mitochondrial involvement in the point of no return in neuronal apoptosis*. Biochimie 2002, 84: 223.

43. Pivovarova, N.B., Nguyen, H.V., Winters, C.A., Brantner, C.A., Smith, C.L., Andrews, S.B. *Excitotoxic calcium overload in a subpopulation of mitochondria triggers delayed death in hippocampal neurons*. J Neurosci 2004, 24: 5611.

44. Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C., Kroemer, G. *Mechanisms of cytochrome c release from mitochondria*. Cell Death Differ 2006, 13: 1423-33.

45. Martinez-Caballero, S., Dejean, L.M., Jonas, E.A., Kinnally, K.W. *The role of the mitochondrial apoptosis induced channel MAC in cytochrome c release*. J Bioenerg Biomembr 2005, 37: 155-64.

46. Halestrap, A.P. *Calcium, mitochondria and reperfusion injury: A pore way to die*. Biochem Soc Trans 2006, 34: 232-7.

47. Forte, M., Bernardi, P. *Genetic dissection of the permeability transition pore*. J Bioenerg Biomembr 2005, 37: 121-8.

48. Zamzami, N., Larochette, N., Kroemer, G. *Mitochondrial permeability transition in apoptosis and necrosis*. Cell Death Differ 2005, 12(Suppl. 2): 1478-80.

49. Christophe, M., Nicolas, S. *Mitochondria: A target for neuroprotective interventions in cerebral ischemia-reperfusion*. Curr Pharm Des 2006, 12: 739-57.

50. Halestrap, A.P., Brennerb, C. *The adenine nucleotide translocase: A central component of the mitochondrial permeability transition pore and key player in cell death*. Curr Med Chem 2003, 10: 1507.

51. Waldmeier, P.C., Zimmermann, K., Qian, T., Tintelnot-Blomley, M., Lemasters, J.J. *Cyclophilin D as a drug target*. Curr Med Chem 2003, 10: 1485-506.

52. Sullivan, P.G., Rabchevsky, A.G., Waldmeier, P.C., Springer, J.E. *Mitochondrial permeability transition in CNS trauma: Cause or effect of neuronal cell death?* J Neurosci Res 2005, 79: 231-9.

53. Waldmeier, P.C., Feldtrauer, J.J., Qian, T., Lemasters, J.J. *Inhibition of the mitochondrial permeability transition by the non-immunosuppressive cyclosporin derivative NIM811*. Mol Pharmacol 2002, 62: 22-9.

54. Kokoszka, J.E., Waymire, K.G., Levy, S.E. et al. *The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore*. Nature 2004, 427: 461-5.

55. Krauskopf, A., Eriksson, O., Craigen, W.J., Forte, M.A., Bernardi, P. *Properties of the permeability transition in VDAC1(-/-) mitochondria*. Biochim Biophys Acta 2006, 1757(5-6): 590-5.

56. Baines, C.P., Kaiser, R.A., Purcell, N.H. et al. *Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death*. Nature 2005, 434: 658-62.

57. Nakagawa, T., Shimizu, S., Watanabe, T. et al. *Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death*. Nature 2005, 434: 652-8.

58. Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M.A., Bernardi, P. *Properties of the permeability transition pore in mitochondria devoid of cyclophilin D*. J Biol Chem 2005, 280: 18558-61.

59. Schinzel, A.C., Takeuchi, O., Huang, Z. et al. *Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia*. Proc Natl Acad Sci USA 2005, 102: 12005-10.

60. Nahon, E., Israelson, A., Abu-Hamad, S., Varda, S.B. *Fluoxetine (Prozac) interaction with the mitochondrial voltage-dependent anion channel and protection against apoptotic cell death*. FEBS Lett 2005, 579: 5105-10.

61. Desagher, S., Osen-Sand, A., Nichols, A. et al. *Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis*. J Cell Biol 1999, 144: 891.

62. Brustovetsky, N., Dubinsky, J.M., Antonsson, B., Jemmerson, R. *Two pathways for tBID-induced cytochrome c release from rat brain mitochondria: BAK- versus BAX-dependence*. J Neurochem 2003, 84: 196.

63. Hetz, C., Vitte, P.A., Bombrun, A. et al. *Bax channel inhibitors prevent mitochondrion-mediated apoptosis and protect neurons in a model of global brain ischemia*. J Biol Chem 2005, 280: 42960-70.

64. Eskes, R., Antonsson, B., Osen-Sand, A. et al. *Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions*. J Cell Biol 1998, 143: 217.

65. Roucou, X., Montessuit, S., Antonsson, B., Martinou, J.C. *Bax oligomerization in mitochondrial membranes requires tBid (caspase-8-cleaved Bid) and a mitochondrial protein*. Biochem J 2002, 368: 915.

66. Wei, M.C., Zong, W.X., Cheng, E.H. et al. *Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death*. Science 2001, 292: 727.

67. Banerjee, J., Ghosh, S. *Bax increases the pore size of rat brain mitochondrial voltage-dependent anion channel in the presence of tBid*. Biochem Biophys Res Commun 2004, 323: 310.

68. Csordas, G., Madesh, M., Antonsson, B., Hajnoczky, G. *tcBid promotes Ca^{2+} signal propagation to the mitochondria: Control of Ca^{2+} permeation through the outer mitochondrial membrane*. EMBO J 2002, 21: 2198.

69. Rostovtseva, T.K., Tan, W., Colombini, M. *On the role of VDAC in apoptosis: Fact and fiction*. J Bioenerg Biomembr 2005, 37: 129-42.

70. Adachi, M., Higuchi, H., Miura, S. et al. *Bax interacts with the voltage-dependent anion channel and mediates ethanol-induced apoptosis in rat hepatocytes*. Am J Physiol-Gastrointest Liver Physiol 2004, 287: G695.

71. De Marchi, U., Campello, S., Szabo, I., Tombola, F., Martinou, J.C., Zoratti, M. *Bax does not directly participate in the Ca^{2+}-induced permeability transition of isolated mitochondria*. J Biol Chem 2004, 279: 37415-22.

72. Precht, T.A., Phelps, R.A., Linseman, D.A. et al. *The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis*. Cell Death Differ 2005, 12: 255-65.

73. Arnoult, D., Parone, P., Martinou, J.C., Antonsson, B., Estaquier, J., Ameisen, J.C. *Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli*. J Cell Biol 2002, 159: 923.

74. Mendoza, F.J., Henson, E.S. and Gibson, S.B., *MEKK1-induced apoptosis is mediated by Smac/Diablo release from the mitochondria*. Biochem Biophys Res Commun 2005, 331: 1089-1198.

75. Saito, A., Hayashi, T., Okuno, S., Nishi, T., Chan, P.H. *Oxidative stress is associated with XIAP and Smac/DIABLO signaling pathways in mouse brains after transient focal cerebral ischemia*. Stroke 2004, 35: 1443.

76. Unal-Cevik, I., Kilinc, M., Can, A., Gursoy-Ozdemir, Y., Dalkara, T. *Apoptotic and necrotic death mechanisms are concomitantly activated in the same cell after cerebral ischemia*. Stroke 2004, 35: 2189.

77. Siesjo, B.K., Elmer, E., Janelidze, S. et al. *Role and mechanisms of secondary mitochondrial failure*. Acta Neurochir Suppl (Wien) 1999, 73: 7.

78. Ferrer, I., Friguls, B., Dalfo, E., Justicia, C., Planas, A.M. *Caspase-dependent and caspase-independent signalling of apoptosis in the penumbra following middle cerebral artery occlusion in the adult rat*. Neuropathol Appl Neurobiol 2003, 29: 472.

79. Ferrer, I., Planas, A.M. *Signaling of cell death and cell survival following focal cerebral ischemia: Life and death struggle in the penumbra*. J Neuropathol Exp Neurol 2003, 62: 329.

80. Graham, S.H., Chen, J. *Programmed cell death in cerebral ischemia*. J Cereb Blood Flow Metab 2001, 21: 99.

81. Benchoua, A., Guegan, C., Couriaud, C. et al. *Specific caspase pathways are activated in the two stages of cerebral infarction*. J Neurosci 2001, 21: 7127.

82. Kristian, T. *Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage*. Cell Calcium 2004, 36: 221-33.

83. Han, M., He, Q.P., Yong, G., Siesjo, B.K., Li, P.A. *NXY-059, a nitrone with free radical trapping properties inhibits release of cytochrome c after focal cerebral ischemia*. Cell Mol Biol (Noisy-le-Grand) 2003, 49: 1249.

84. Namura, S., Nagata, I., Takami, S., Masayasu, H., Kikuchi, H. *Ebselen reduces cytochrome c release from mitochondria and subsequent DNA fragmentation after transient focal cerebral ischemia in mice*. Stroke 2001, 32: 1906.

85. Andrabi, S.A., Sayeed, I., Siemen, D., Wolf, G., Horn, T.F. *Direct inhibition of the mitochondrial permeability transition pore: A possible mechanism responsible for anti-apoptotic effects of melatonin*. FASEB J 2004, 18: 869.

86. Ferrand-Drake, M., Zhu, C., Gido, G. et al. *Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c and caspase-3 activation in neurons exposed to transient hypoglycemia*. J Neurochem 2003, 85: 1431.

87. Li, P.A., Kristian, T., He, Q.P., Siesjo, B.K. *Cyclosporin A enhances survival, ameliorates brain damage, and prevents secondary mitochondrial dysfunction after a 30-minute period of transient cerebral ischemia*. Exp Neurol 2000, 165: 153.

88. Uchino, H., Morota, S., Takahashi, T. et al. *A novel neuroprotective compound FR901459 with dual inhibition of calcineurin and cyclophilins*. Acta Neurochir Suppl 2006, 96: 157-62.

89. Uchino, H., Elmer, E., Uchino, K. et al. *Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat*. Brain Res 1998, 812: 216.

90. Matsumoto, S., Isshiki, A., Watanabe, Y., Wieloch, T. *Restricted clinical efficacy of cyclosporin A on rat transient middle cerebral artery occlusion*. Life Sci 2002, 72: 591.

91. Khaspekov, L., Friberg, H., Halestrap, A., Viktorov, I., Wieloch, T. *Cyclosporin A and its nonimmunosuppressive analogue N-Me-Val-4-cyclosporin A mitigate glucose/oxygen deprivation-induced damage to rat cultured hippocampal neurons*. Eur J Neurosci 1999, 11: 3194.

92. Andrabi, S.A., Spina, M.G., Lorenz, P., Ebmeyer, U., Wolf, G., Horn, T.F. *Oxyresveratrol (trans-2,3',4,5'-tetrahydroxystilbene) is neuroprotective and inhibits the apoptotic cell death in transient cerebral ischemia*. Brain Res 2004, 1017: 98.

93. Katzman, R. *Alzheimer's disease*. New Engl J Med 1986, 314: 964.

94. Newell, K.L., Hyman, B.T., Growdon, J.H., Hedley-Whyte, E.T. *Application of the National Institute on Aging (NIA)-Reagan Institute criteria for the neuropathological diagnosis of Alzheimer disease*. J Neuropath Exp Neurol 1999, 58: 1147.

95. Atiya, M., Hyman, B.T., Albert, M.S., Killiany, R. *Structural magnetic resonance imaging in established and prodromal Alzheimer disease: A review*. Alz Dis Assoc Dis 2003, 17: 177.

96. Bertoli-Avella, A.M., Oostra, B.A., Heutink, P. *Chasing genes in Alzheimer's and Parkinson's disease*. Hum Genet 2004, 114: 413.

97. Mattson, M.P. *Pathways towards and away from Alzheimer's disease*. Nature 2004, 430: 631.

98. Butterfield, D.A. *Amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity: Implications for neurodegeneration in Alzheimer's disease brain. A review*. Free Radic Res 2002, 36: 1307.

99. Pereira, C., Ferreiro, E., Cardoso, S.M., de Oliveira, C.R. *Cell degeneration induced by amyloid-beta peptides: Implications for Alzheimer's disease*. J Mol Neurosci 2004, 23: 97.

100. Canevari, L., Abramov, A.Y., Duchen, M.R. *Toxicity of amyloid beta peptide: Tales of calcium, mitochondria, and oxidative stress*. Neurochem Res 2004, 29: 637.

101. Zhu, X., Raina, A.K., Lee, H.G., Casadesus, G., Smith, M.A., Perry, G. *Oxidative stress signalling in Alzheimer's disease*. Brain Res 2004, 1000: 32.

102. Keil, U., Bonert, A., Marques, C.A. et al. *Amyloid-beta induced changes in nitric oxide production and mitochondrial activity lead to apoptosis*. J Biol Chem 2004, 279(48): 50310-20.

103. Rego, A.C., Oliveira, C.R. *Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: Implications for the pathogenesis of neurodegenerative diseases*. Neurochem Res 2003, 28: 1563.

104. Williams, L.R. *Oxidative stress, age-related neurodegeneration, and the potential for neurotrophic treatment*. Cerebrovasc Brain Metab Rev 1995, 7: 55.

105. Eckert, A., Keil, U., Marques, C.A. et al. *Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease*. Biochem Pharmacol 2003, 66: 1627.

106. Ekshyyan, O., Aw, T.Y. *Apoptosis in acute and chronic neurological disorders*. Front Biosci 2004, 9: 1567-76.

107. Beal, M.F. *Mitochondrial dysfunction and oxidative damage in Alzheimer's and Parkinson's diseases and coenzyme q(10) as a potential treatment*. J Bioenerg Biomembr 2004, 36: 381.

108. Cash, A.D., Perry, G., Ogawa, O., Raina, A.K., Zhu, X., Smith, M.A. *Is Alzheimer's disease a mitochondrial disorder?* Neuroscientist 2002, 8: 489.

109. Cardoso, S.M., Santana, I., Swerdlow, R.H., Oliveira, C.R. *Mitochondria dysfunction of Alzheimer's disease cybrids enhances Aβ toxicity*. J Neurochem 2004, 89: 1417.

110. Jordan, J., Cena, V., Prehn, J.H. *Mitochondrial control of neuron death and its role in neurodegenerative disorders*. J Physiol Biochem 2003, 59: 129.

111. Cesura, A.M., Pinard, E., Schubenel, R. et al. *The voltage-dependent anion channel is the target for a new class of inhibitors of the mitochondrial permeability transition pore*. J Biol Chem 2003, 278: 49812-8.

112. Kristian, T., Weatherby, T.M., Bates, T.E., Fiskum, G. *Heterogeneity of the calcium-induced permeability transition in isolated non-synaptic brain mitochondria*. J Neurochem 2002, 83: 1297.

113. Fiskum, G., Murphy, A.N., Beal, M.F. *Mitochondria in neurodegeneration: Acute ischemia and chronic neurodegenerative diseases*. J Cereb Blood Flow Metab 1999, 19: 351.

114. Chinopoulos, C., Starkov, A.A., Fiskum, G. *Cyclosporin A-insensitive permeability transition in brain mitochondria: Inhibition by 2-aminoethoxydiphenyl borate*. J Biol Chem 2003, 278: 27382.

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

116. Wu, Y.Q., Belyakov, S., Choi, C. et al. *Synthesis and biological evaluation of non-peptidic cyclophilin ligands*. J Med Chem 2003, 46: 1112-5.

117. Brustovetsky, N., Brustovetsky, T., Jemmerson, R., Dubinsky, J.M. *Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane*. J Neurochem 2002, 80: 207.

118. Brustovetsky, N., Dubinsky, J.M. *Dual responses of CNS mitochondria to elevated calcium*. J Neurosci 2000, 20: 103.

119. Antonsson, B., Montessuit, S., Sanchez, B., Martinou, J.C. *Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells*. J Biol Chem 2001, 276: 11615.

120. Crouser, E.D., Gadd, M.E., Julian, M.W. et al. *Quantitation of cytochrome c release from rat liver mitochondria*. Anal Biochem 2003, 317: 67.

121. Nunez, J.L., Alt, J.J., McCarthy, M.M. *A new model for prenatal brain damage. I. GABAA receptor activation induces cell death in developing rat hippocampus*. Exp Neurol 2003, 181: 258.