Review

Development of mitochondrial permeability transition inhibitory agents: a novel drug target

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ABSTRACT: Apoptosis is a genetically conserved mechanism that eliminates unnecessary or surplus cells and is also involved in the pathomechanism of a wide variety of diseases. The intrinsic pathway of apoptosis includes the mitochondria where numerous pro-apoptotic proteins are sequestered and their release marks the point-of-no-return, indicating the ultimate commitment to cell death. The *mitochondrial permeability transition* (mPT) is a mechanism enabling the release of Cytochrome-*c* (Cyt-*c*), AIF and other pro-apoptotic proteins, and is characterized by an alteration in the permeability of the organelle's membrane. This is due to reactive oxygen species or Ca^{2+} triggered dynamic assemble of a trans bi-membrane channel from various protein components including the voltage dependent anion channel, the adenine nucleotide translocase, the cyclophyllin D that enables solutes up to 1.5 kDa to pass through. The resultant influx of water into the mitochondrial matrix leads to mitochondrial swelling and the rupture of the membranes. Numerous agents can inhibit mPT including amiodarone, a widely used antiarrhythmic agent. Modification of this benzofuran derivate with nitroxides or their secondary amine derivates that exhibits antioxidant properties leads to the enhancement of mPT inhibitory effect of the original compound. Furthermore this hybrid compound is also capable of influencing the necrotic cell death pathway. This strategy may prove to be beneficial to increase the effectiveness of other mPT inhibitory agents. However, further studies are necessary to identify the components and structure of the permeability transition pore in order to design more effective mPT inhibitory compounds to fully exploit the therapeutic potential of this novel drug target.

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1. Introduction

Programmed cell death is a genetically conserved mechanism that has crucial implications in developmental processes and in the maintenance of tissue homeostasis. Although development largely relies on cell division and differentiation, carefully orchestrated cell death is also crucial to execute the morphological changes associated with a developing organism. In mature organisms the continuous proliferation of cells, at a variable rate depending on the specificity of the different tissues, is carefully balanced with the elimination of surplus cells in order to maintain the homeostasis of the tissues. As an example, roughly 500 billion lymphocytes are eliminated each day in an average human body to give room for the newly generated white blood cells (*1*). The theory of a cell death characterized by a sequence of pre-programmed event was formulated in the early 1970s (*2*). It is also called apoptosis, a word of Greek origin meaning the falling of leaves which suggests that this type of cell death is a natural phase of life.

Apoptosis is also associated with the development of various diseases: the disturbance of the balance between cell proliferation and removal affect the homeostasis of the tissues (*3*). If the rate of programmed cell death is increased it will lead to degenerative disorders. Diseases such as Parkinson's (*4*), Alzheimer's (*5*), and Huntington's disease (*6*), amyotrophic lateral sclerosis (*7*) are related to excessive loss of brain cells caused by increased level of apoptosis. Cardiovascular and cerebrovascular diseases such as acute myocardial infarction (*8*) or stroke (*9*) will initially result in necrotic cell death at the core area, while cells surrounding this pathological core will undergo apoptosis in due course significantly expanding the size of the damaged tissue. Degenerative joint diseases such as osteoarthritis (*10*) or rheumatoid arthritis (*11*) are also characterized by

excessive apoptosis.

Apoptosis is characterized by distinct morphological changes, such as the cell shrinkage and condensation due to the disruption of the cytoskeletal structure, condensation of the chromatin and the fragmentation of the nuclear membrane and of the DNA, the blebbing of the cell membrane leading to fragmentation and the formation of apoptotic bodies which are membrane bound cell fragments that are phagocytised by macrophages and neighbouring cells (*12*).

The components that regulate and execute apoptosis are genetically determined and are readily available in every cell. In order to prevent undesired or accidental activation of the apoptotic program, these components are inactivated and/or securely locked away in various cell compartments (*13*). Following an apoptotic stimuli, the dormant apoptotic machinery will spring into action to execute the predetermined program of cell death. Depending on whether the apoptotic stimuli arises from the external environment of the cell or from within the cell, two main pathways of the programmed cell death can be distinguished.

2. Apoptotic pathways and the effector machinery

When the programmed cell death is triggered *via* the *extrinsic apoptotic pathway*, the initiating signal derives from cells' external environment (*14*). In this case apoptosis is activated by the binding of a specific 'death ligand' (*e.g.* FasL, TRAIL, TNFα) to the death receptor (DR) located on the surface of the cell. These receptors belong to the TNF receptor family, and following the binding of the ligand, the internal part of the death receptor will recruit the protein FADD (Fas-associated death domain protein), adaptor proteins and initiator pro-caspases to form the death inducing signalling complex (DISC). This complex will activate the initiator caspases by stimulating their auto-cleavage which in turn will lead to a proteolytic cascade resulting in the activation of the effector caspases. The signal that initiates the extrinsic apoptotic pathway can also be the absence (*e.g.* withdrawal) of various growth or trophic factor (*e.g.* nerve growth factor; NGF) which are required for the normal growth and proliferation of the cells. These ligands stimulate cell survival *via* the PI3K/Akt (proteinkinase B) pathway that will phosphorylate and inactivate pro-apoptotic proteins to suppress the apoptotic process (*15*). The absence of such survival signal the apoptotic effector mechanism maintains its activity and executes the cell death program.

When the programmed cell death is triggered *via* the *intrinsic apoptotic pathway*, the initiating signal derives from cells' internal environment (*16*). In this case apoptosis is activated by a variety of stimuli such as DNA breaks, deprivation of oxygen or nutrients, accumulation of drugs or increased intracellular Ca^{2+} levels (*17*). This pathway is also termed as the mitochondrial apoptotic pathway since its activation is launched by the release of pro-apoptotic proteins from this organelle. Although the main cellular function of mitochondria is to provide chemical fuel for the cell in the form of ATP it also sequesters numerous proteins within its compartments engulfed by a dual-membrane structure that are release in response to stimuli. The most important of these intra-mitochondrial pro-apoptotic proteins include the following:

- **Cyt-*c*:** a protein that covalently binds its heme group and functions as an electron carrier between mitochondrial respiratory complexes III and IV in the inter-membrane space. When it is released to the cytosol, it facilitates the formation of apoptosome in concert with apoptosis activating factor-1 (Apaf-1), pro-caspase-9 and dATP to produce an active caspase-9 (*18*,*19*).
- **Smac/Diablo (second mitochondria-derived activator of caspases/ direct inhibitor of apoptosis-binding protein with low pI):** binds to and inactivates the cytosolic X-linked inhibitor of apoptosis protein (XIAP). XIAP directly interact with caspases in order to inhibit their activity (*20*). This inhibitory effect of XIAP is suspended by Smac/Diablo.
- **Omi/HtrA2:** is a serine protease with an apoptotic activity similar to Smac/Diablo; when released from mitochondria it also binds and inhibits the action of XIAP. However, unlike Smac/Diablo, Omi/HtrA2 also facilitates apoptosis by utilizing its protease activity in addition to the physical binding to XIAP (*21*).
- **AIF (apoptosis inducing factor):** is a mitochondrial flavoprotein that carries a NADH-oxidase domain which regulates mitochondrial respiratory complex I and is also indispensable for cell survival and for the integrity of the mitochondria (*22*). In response to apoptotic stimuli it translocates to the nucleus and induces chromatin condensation and large fragment DNA degradation.
- **Endo G (endonuclease-G):** is a nuclear DNA-encoded nuclease located in the inter-membrane space. Its apoptotic activity is similar to that of the AIF: it translocates to the nucleus and mediates oligonucleosomal DNA fragmentation (*23*).

Regardless of whether the signal initiating apoptosis arises from the external environment or from within the cell, apoptotic machinery is triggered into action in a predetermined manner, ultimately leading to the activation of effector caspases that execute the program.

The components of the *apoptotic machinery* belong to a family of evolutionary highly conserved proteins, exhibiting structural and functional similarities in various organisms from nematodes to humans. These are cystein proteases recognizing a specific sequence of

amino acids containing aspartate, hence they are termed as caspases (*24*). Present in the cells in an inactive form of pro-caspases, they are activated by proteolytic cleavage in an amplifying cascade. The pro-caspases at the initial stage of the cascade form the initiator caspases (caspases 2, 8, 9, 10) while the ones downstream form the effector caspases (caspases 3, 6, 7) that eventually cleave the cellular target proteins. All caspases have specific caspase recruitment domains (CARDs) allowing the binding of adaptor proteins and the formation of activation complexes in response to an apoptotic signal.

Although two different apoptotic pathways are described, in practice there is always cross-talk between them leading to both pathways being simultaneously involved in cell death albeit with the dominance of either one or the other. Intrinsic apoptotic pathway for example can be activated by the extrinsic route *via* the truncation and thus activation of the pro-apoptotic protein Bid (*25*). Mitochondria, however, play a crucial role in apoptosis: the translocation of the described (and possibly other yet unidentified) pro-apoptotic factors from the mitochondrial compartment to the cytosol marks the point of no return, where commitment to cell death becomes irreversible. Different models are used to describe the mechanism of mitochondrial pro-apoptotic protein release.

3. Mechanism of the mitochondrial pro-apoptotic protein release

The role of B-cell leukaemia (Bcl-2) proteins have been extensively studied (*26*). They are considered to play a crucial role in controlling the mitochondrial apoptotic pathway. Bcl-proteins are characterized by their BH (Bcl Homology) domains (Figure 1). Pro-apoptotic bcl-proteins have either 3 BH domains (BH1-3; *e.g.* Bax, Bak) or a single BH domain (BH3; *e.g.* bad, bid, bim, puma, noxa) while anti-apoptotic bcl-proteins have 4 BH domains (BH1-4; *e.g.* Bcl-2, Bcl-X_L). The anti-apoptotic family members form heterodimers with the pro-apoptotic multi-domain family members to prevent the activation of the cell death pathway. Apoptotic signals activate the single BH3 domain regulatory bcl-proteins by various stimuli such as proteolytic cleavage (*e.g.* Bid) or phosphorylation (*e.g.* Bad) causing them to promote the dissociation of the heterodimers. Subsequently, the pro-apoptotic proteins with BH1-3 domains oligomerize and form a pore in the outer membrane of the mitochondria (*27*), allowing the release of pro-apoptotic mitochondrial proteins from the inter-membrane space (Figure 2). Bcl-proteins can incorporate various signals in the apoptotic pathway. For example p53 can induce apoptosis by increasing the rate of transcription of numerous bcl-related genes, and also by activating BH multi-domain pro-apoptotic bax to bind to the mitochondrial outer membrane (*28*).

According to another explanation, which also describes the mitochondrial pro-apoptotic protein release, a trans-bi-membrane channel is formed (*29*). Mitochondria are surrounded by two membranes with the inner mitochondrial membrane having invaginations which form the mitochondrial cristae resulting in a larger overall surface than that of the outer mitochondrial membrane. Apoptotic stimuli leads to a channel formation that spans both mitochondrial inner and outer membranes. This non-selective channel (permeability transition pore; PTP) allows the passage of molecules with a cut-off at 1.5 kDa. Since mitochondria as the metabolic hubs of the cell, contain intermediates at a high concentration with high osmolar activity, it results

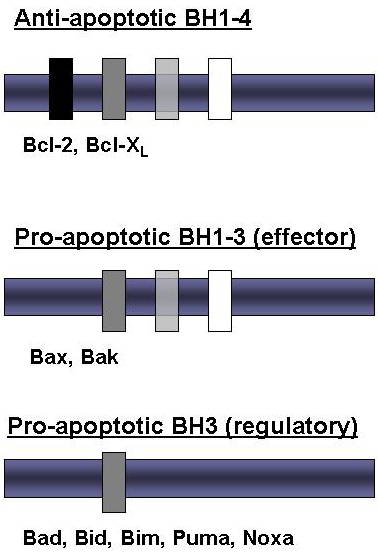


Figure 1. Bcl-proteins can be divided into three major groups depending on the number of bcl homology (BH) domains. Proteins with four BH domains have an anti-apoptotic function while those with one or three BH domains are pro-apoptotic.

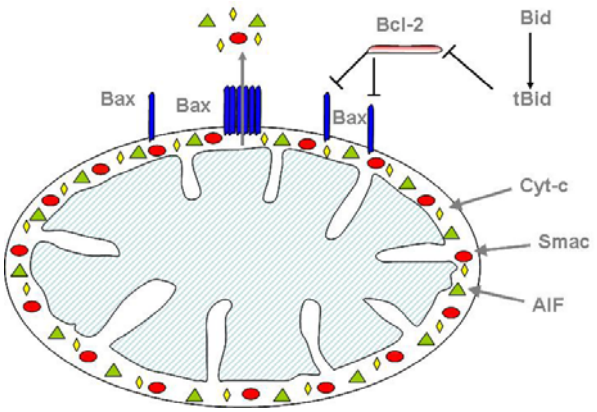


Figure 2. Pro-apoptotic BH1-3 bcl-proteins (Bax) are associated with anti-apoptotic BH1-4 bcl-proteins (Bcl-2). This heterodimer can be disrupted by BH3 pro-apoptotic proteins (tBid) facilitating the homo-oligomerization of the pro-apoptotic BH1-3 bcl-proteins (Bax). This leads to a formation of a pore that transverses the mitochondrial outer membrane, enabling the release of intra-mitochondrial pro-apoptotic proteins (*e.g.* Cyt-*c*, AIF, Smac/Diablo).

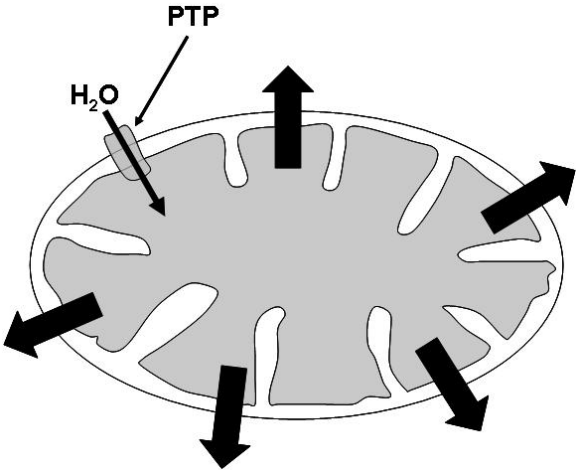


Figure 3. Assemble of a non-specific trans-bimembrane pore spanning both mitochondrial membrane results in H_2O influx due to osmosis. The subsequent mitochondrial swelling will eventually lead to the rupture of the mitochondrial outer membrane enabling the release of intra-mitochondrial pro-apoptotic proteins (*e.g.* Cyt-*c*, AIF, Smac/Diablo).

in the influx of water, the swelling of the mitochondria and the rupture of the outer membrane resulting in the release of pro-apoptotic proteins (Figure 3). This process is called the *mitochondrial permeability transition* (mPT). This route of apoptosis is typically characterized by excessive intracellular Ca^{2+} fluxes or the generation of substantial amount of reactive oxygen species (ROS). In a previous study we reported that a heme binding protein with BH3-like domain induced mPT that was sensitive to cyclosporine A (*30*). The composition of the PTP is not yet fully known, although a wide range of studies indicate that PTP is formed by the dynamic interaction of several molecular components (*31*). These include the voltage-dependent anion channel (VDAC) located in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane and cyclophilin D (CypD) found in the mitochondrial matrix forming the core components of the PTP (*32-34*). Additional components include the creatine kinase (CK) in the inter-membrane space, the peripheral-type benzodiazepine receptor (PBR) located in the outer membrane and two hexokinase isoforms (HKI and HKII) found in the cytosol (*35-37*). The mPT, including the assemble of the PTP and the release of Cyt-*c*, can be demonstrated experimentally in isolated mitochondria (*38*).

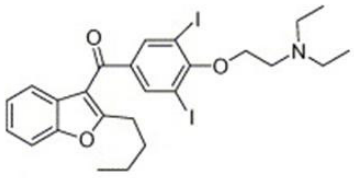
4. Mitochondrial permeability transition pore as novel drug target

Numerous pharmacological compounds can facilitate the mPT. Our previous studies for example have demonstrated the paclitaxel, an anticancer agent widely used in the clinical practice for the treatment of solid tumors or leukaemias induces mPT in addition to its previously described effect on the microtubular disassembly (*39*). Similar effect was observed in the case of cisplatin, another anticancer agents frequently used in practice. In general the activators of mPT may prove to be effective in the treatment of malignancies (*40*). The administration of mPT inhibitors in combination with conventional anticancer agents can potentiate the effectiveness of these drugs enabling their administration in a lower dose and thus reducing their associated side effects.

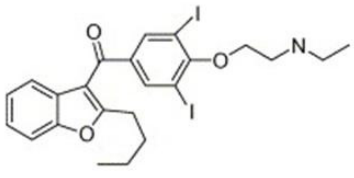
A wide range of pharmacological agent can exert an inhibitory effect on the mPT. One of the first agents proved to have an mPT inhibitory effect was cyclosporine A, an immunosuppressant compound (*41*). It binds to cyclophillin D in the mitochondrial matrix and inhibits its peptidyl-prolyl cis-trans isomerase activity which in turn prevents the formation of PTP (*42*). A study conducted to screen more than 1,000 previously established drugs for an effect on the mPT found 28 structurally related compounds including heterocyclic antidepressants and antipsychotic that possesses with inhibitory effect on the mPT (*43*). Another target for the inhibition of permeability transition is the peripheral-type benzodiazepine receptor (PBR) located in the outer mitochondrial membrane (*44*). This binds the specific PBR ligand 4'-chlorodiazepam, an agent that protects the myocardium during ischemia reperfusion injuries (*45*).

5. Amiodarone and structural analogues act as potent inhibitors of mPT

Previous studies have also identified an established pharmacological compound that inhibits mPT and protects the myocardium against oxidative damage sustained during ischemia-reperfusion injuries (*46*). The antiarrhythmic agent amiodarone (Figure 4) exerts a biphasic effect on the mPT: at lower concentrations it inhibits, while at higher concentrations it induces



Amiodarone



N-desethylamiodarone

Figure 4. Amiodarone, a benzfuran derivate (2-butyl-3-benzofuranyl-4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride) is a dominantly class III antiarrhythmic compound. Its main metabolite is *N*-Desethylamiodarone.

PTP formation as demonstrated in isolated rat liver mitochondria (*46*). It also had a beneficial effect on the myocardium during ischemia and reperfusion by facilitating the recovery of the level of high energy phosphate metabolites such as ATP and creatine phosphate respectively (*47*). It also prevented the translocation of apoptosis inducing factor from the mitochondria to the nucleus during ischemia and reperfusion in Langendorff-perfused rat hearts (*47*). Amiodarone is metabolized in the liver (*48*) and its major metabolite is the deseathyl-amiodarone (Figure 4). This metabolite however does not exert the beneficial effect demonstrated by amiodarone in equimolar concentration. It is particularly noteworthy that it did not have inhibitory effect on the mPT which is a characteristic of amiodarone. Since the difference in the effect on mPT between amiodarone and its major metabolite desethyl-amiodarone is solely due to structural alteration (*e.g.* the absence of the ethyl side chain from the amino group) we designed and developed paramagnetic and diamagnetic amiodarone analogues to evaluate their effect on mPT (*49*).

Amiodarone is among the most effective antiarrhythmic agents and frequently used in clinical practice to treat various ventricular and supraventricular arrhythmias (*50*). Its clinical application however is often limited by the severe side effects associated with its long term administration that include pancreas and liver fibrosis, thyroid dysfunction or the potentially life threatening thyreotoxicosis (*51*). Significant effort has been made in the past to enhance the pharmacokinetic properties of this compound. Amiodarone analogues produced by dealkylation, deiodination or deamination were evaluated to assess their toxic properties (*52*). Another research group introduced carboxymethoxy side chain to replace the tertiary amine to produce 2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (*53*), while others evaluated the substitution of the *n*-buthyl group with an isobuthyl ester. The discovery that amiodarone acts as an inhibitor of mPT (*46*) provides an explanation on the mechanism by which it reduces mortality after myocardial infarction (*54*). The ischemia-reperfusion type injury during myocardial infarction is characterized by ROS generation and the release of Ca^{2+} from the intracellular Ca^{2+}-stores and can be an etiological factor in inducing mPT and myocardial cell death during reperfusion. We demonstrated the beneficial effects of amiodarone on Langendorff-perfused rat hearts as well as in isolated mitochondria (*46,47*). In order to improve the effectiveness of amiodarone to inhibit mPT we produced analogues by varying the diethylaminoethyl side chain of phenolether and the 2-substituent on the benzofuran ring and evaluated the effect of these modifications in perfused hearts as well as in cultured cells or in isolated mitochondria (*49*). In the past decades numerous drugs that were modified with nitroxides or with their precursors exhibited additional beneficial features. This chemical group possesses antioxidant properties and is capable of scavenging ROS and RNS to protect cellular lipids and proteins from damaging oxidative insults (*55*). It prompted us to modify amiodarone and produce analogues that incorporate 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole and 1,2,5,6-tetrahydropyridine nitroxides or their amine precursors to supplement the antiarrhythmic agent with a superoxide dismutase (SOD) mimetic property (*49*). In order to improve the solubility and the ROS scavenging property of the amiodarone analogues, the nitroxide was reduced to secondary amine (*56*).

The majority of the amiodarone analogues did not exhibit biphasic effect on the mPT: while they inhibited the swelling in lower concentrations without inducing mPT when administered at higher doses. Those compounds that inhibit mPT with IC_{50} values comparable to that of the amiodarone, which was previously found to be 3.9 ± 0.8 μM (*46,47*), were selected for further evaluation. Amiodarone has an uncoupling effect on the mitochondria (*57*) and therefore dissipates the mitochondrial membrane potential ($\Delta\Psi$) with an ED_{50} of 4.2 ± 0.7 μM (*46,47*). The modified amiodarone analogues exerted uncoupling effect on the mitochondrial oxidative phosphorylation with a higher ED_{50} than that of the amiodarone (*49*). It should be noted, that a moderate uncoupling effect is believed to be beneficial since it decreases the level of noxious ROS production.

Those amiodarone analogues that exhibited low IC_{50} values in inhibiting mPT and a relatively high ED_{50} value for mitochondrial membrane potential dissipation in comparison to amiodarone were selected to evaluate their toxicological properties in cultured cells. Treating cardiomyocytes and liver cells with amiodarone and the selected analogues in the concentration up to 100 μM, which is significantly higher than the level in the tissues during prolonged drug administration (*47*), we were able to indentify that specific compound that had the lowest IC_{50} values for mPT inhibition, the highest ED_{50} value for dissipating the mitochondrial membrane potential, and showed the lowest toxicity in cultured cells (*49,58*). This agent is a 2-methyl-3-(3,5-diiodo-4-{2-[*N*-ethyl, *N*-(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)ethyl]}oxybenzoyl)benzofurane containing an additional 1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl component which possesses SOD-mimetic properties (Figure 5). The subcellular localization of this compound was assessed by quantitative HPLC-MS and this demonstrated an increased accumulation in the mitochondria in contrast to its level both in the cytosol and in the nucleus. More than 95% of this benzofuran derivate was taken up in the mitochondria of cultured cardiomyocytes which is similar to the level detected in the mitochondrial fraction of perfused hearts (93.2%). This agent

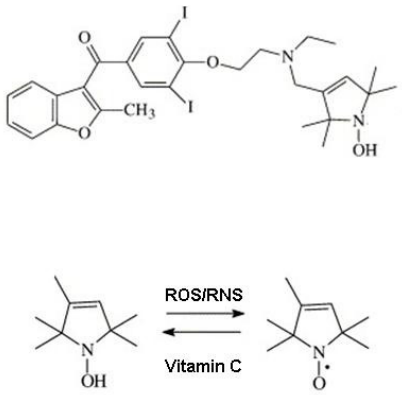


Figure 5. Modifying amiodarone with nitroxides provides antioxidant properties enabling the scavenging of ROS and RNS. An effective compound shown incorporates a 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole, 1,2,5,6-tetrahydropy ridine nitroxide. ROS induced oxidation of the *N*-hydroxyamine form is reduced by to nitroxide form by natural reducing agents (*e.g.* GSSG, thiols, ascorbate).

prevented the release of the pro-apoptotic proteins Cyt-*c*, AIF and Endo G from isolated mitochondria, and also decreased the caspase-3 activity in apoptotic cultured cells (*49*,*58*). The effect on the myocardial energy metabolism was also assessed in perfused heart by ^{31}P NMR spectroscopy and showed an enhanced recovery of high-energy phosphates in the myocardium following ischemia-reperfusion. Due to the antioxidant properties of the compound, lipid peroxidation as well as protein oxidation was decreased in this experimental model. Furthermore, the infarct size in the myocardium was also reduced (*58*).

The addition of a nitroxide compound to amiodarone to enhance the mPT inhibitory effect proved to be a successful approach. 2-methyl-3-(3,5-diiodo-4-{2-[*N*-ethyl, *N*-(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl) ethyl]}oxybenzoyl)benzofurane is the first representatives of a novel class of mPT inhibitors that exerts SOD-mimetic properties as well. These types of agents are capable of inhibiting not only the apoptotic but the necrotic cell death (*58*). This results in a significantly enhanced cyto-protection and cardio-protection which can be exploited further in the therapeutic practice. Modifying drug with antioxidant compounds may also prove to be a beneficial approach in the case of other classes of pharmacological agents as well (*59*).

6. Conclusion

Apoptosis is involved in the pathomechanism of numerous diseases. Since mitochondria play a crucial role marking the point of irreversibility by releasing the pro-apoptotic proteins it also presents an excellent opportunity for therapeutic intervention. Some pathological conditions, particularly those involving increased intracellular Ca^{2+} or ROS over-production, lead to the formation of the permeability transition pore providing a valid therapeutic target. It is also likely, however, that mPT plays a role in necrosis since the aggressive rupture of mitochondria membrane and thus subsequent inactivation of the oxidative phosphorylation will lead to prompt cell death (*60*). Therefore mPT inhibitors may also be able to influence necrosis. Based on this theory novel hybrid mPT inhibitors were developed, modified with nitroxide structures that have antioxidant properties. Further studies are necessary to identify the components and structure of the PTP in order to design more effective mPT inhibitory compounds. This is however challenging, since PTP formation is believed to be a dynamic and continuously altering interaction of a large number of proteins with each having other important functions in the mitochondrial physiology and it is very likely than some components are yet to be identified (*61*).

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