Targeting mitochondrial permeability as a pharmacological cardioprotective strategy.

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Abstract

Ischemia-reperfusion injury is a leading cause of death in Western countries. Currently, the only treatment to reduce infarct size and to improve the clinical outcome after myocardial ischemia is the rapid restoration of blood flow and the development of reperfusion techniques has strongly reduced the morbidity and mortality in patients. However, the efficacy of this clinical approach is limited because cardiac reperfusion per se gives birth to cellular injury. During the last decades, multiple studies demonstrated that the pathological signals induced by ischemia-reperfusion converge towards mitochondria and that most of cell death in the heart is induced by the permeabilization of mitochondrial membranes in the early reperfusion. The search for drugs able to block or to inhibit mitochondrial membrane permeabilization has been the subject of growing interest. It gave birth to several pharmacological approaches to protect from myocardial ischemia-reperfusion injury in experimental models and clinical settings. This review describes these mitochondrial-targeting strategies with a focus on new pharmacological approaches which constitute real hope for the future.

Keywords: mitochondria, cardioprotection, mitochondrial permeability transition, ischemia-reperfusion

Introduction

Myocardial ischemia is a leading cause of death in developed countries and progresses continuously in emerging countries. Today, the only available treatment to reduce infarct size and to improve the clinical outcome after myocardial ischemia remains the restoration as rapidly as possible of the blood flow (reperfusion) in the occluded coronary arteries (by angioplasty, thrombolysis or surgery). However, the efficacy of this clinical strategy is limited because the restoration of blood flow in the ischemic myocardium paradoxically generates cellular lesions which are called "reperfusion injury". These lesions are mainly related to the massive cellular influx of Ca²⁺ and the production of reactive oxygen species (ROS). In the past decades, major efforts
have converged to find new "cardioprotective" pharmacological approaches that can be combined with reperfusion methods in order to reduce the lesions of reperfusion and thus the morbidity and the mortality following acute myocardial infarction (AMI).¹

This has been made possible by the enormous progresses that have been performed to decipher the cellular signaling pathways leading to cardioprotection after myocardial infarction and these advances provided several ways to develop pharmacological protective agents.¹

It is now well-established that the major cause of cell injuries during cardiac ischemia-reperfusion is the increase in the mitochondrial membrane permeability generated both by the formation of channels across the outer membrane and the opening of the mitochondrial transition pore (mPTP).²,³ This is a process whereby the inner mitochondrial membrane becomes permeable to solutes (For review see ⁴). Its opening is detrimental for the cell as it causes mitochondrial swelling, loss of membrane potential, inhibition of oxidative phosphorylation and may result in rupture of outer mitochondrial membrane leading to cell death.⁵,⁶

The limitation of mitochondrial membrane permeability during the reperfusion of the ischemic myocardium represents, therefore, a major objective to attenuate lethal reperfusion injury and to get the most benefit from reperfusion strategies. In the present review, we will discuss pharmacological strategies (1) acting on signaling pathways and/or endogenous factors which promote mitochondrial membrane permeabilization or (2) targeting mitochondrial channels involved in the permeabilization. This review does not provide a coverage of the cellular and mitochondrial events occurring during cardiac reperfusion after a prolonged period of ischemia since this has been well-reviewed in previous articles.⁷-¹⁰

1. Pharmacological strategies targeting signaling pathways involved in mitochondrial membrane permeabilization

The discovery of the cardioprotective effect of ischemic preconditioning, i.e., non lethal brief episodes of ischemia-reperfusion preceding a prolonged ischemic period, was a major step to decipher the mechanisms leading to cell death during reperfusion injury and the signaling pathways responsible for cytoprotection.¹ Ischemic preconditioning which was originally described by Murry et al. is one of the most powerful strategies to protect the heart against lethal reperfusion injury.¹¹ Studies demonstrated that ischemic preconditioning causes the release of four G protein-coupled receptors agonists, adenosine, bradykinin, opioid and sphingosine that activates a cascade of cardioprotective kinases named the “Reperfusion Injury Salvage Kinase” or RISK pathway.¹² Although there is still some debate, this cascade includes PI3kinase, Akt, ERK1/2 and downstream proteins such as glycogen synthase kinase-3β (GSK-3β), protein kinase G (PKG), Bad or endothelial nitric oxide synthase (eNOS). Other studies showed that protein kinase C (PKC) and mitochondrial ATP-sensitive potassium channels also belong to the ischemic preconditioning signaling pathway.¹³,¹⁴ Subsequently, another signaling pathway named the Survivor Activating Factor Enhancement (SAFE) pathway that is activated independently of the RISK pathway was identified. It is initiated by the Tumor necrosis factor alpha and involves the activation of the signal transducer and activator of transcription 3 (STAT-3)¹⁵ but appears to play a more important role in larger animals than in rodents.¹⁶
Although the overall signaling pathway is not completely understood, it is now accepted that these actors converge to mitochondria to prevent the formation of the mPTP which is considered to induce much of cell death in the heart in the first minutes of reperfusion. The mechanism by which the RISK pathway inhibits mPTP is unclear, but several candidates have been proposed which may act in concert to mediate mPTP inhibition.\textsuperscript{17,18}

Similarly to ischemic preconditioning, postconditioning (reperfusion-reocclusion cycles at the onset of reperfusion) and remote preconditioning (brief periods of ischemia applied in a distant tissue) promote cardioprotection mediated by a kinase response and a limitation of mPTP opening.\textsuperscript{19-22} However, recent studies suggest that the cardioprotection induced by remote and postconditioning could be independent from the RISK pathway in large animals.\textsuperscript{16-22}

Nevertheless, activation of RISK and SAFE pathways provides a possible therapeutic intervention to inhibit mPTP opening and to protect the heart at reperfusion and several pharmacological strategies have been used.

1.1. Targeting of the RISK pathway

Adenosine was the first G protein-coupled receptor ligand shown to mimic the cardioprotective effect of ischemic preconditioning when administrated prior to coronary occlusion in the animal.\textsuperscript{23} This experimental investigation was followed by two small clinical trials showing myocardial protection when adenosine was administrated at high doses during AMI as an adjunct to angioplasty and thrombolysis.\textsuperscript{24,25} Two larger randomized controlled studies also observed a reduction in infarct size\textsuperscript{26,27} but this pharmacological strategy failed to improve clinical outcomes.\textsuperscript{27} However, a recent meta-analysis evaluating all randomized trials, comparing intracoronary adenosine administration versus placebo in ST-segment myocardial infarction (STEMI) patients undergoing primary percutaneous coronary intervention (PCI), demonstrated a clinical benefit for adenosine in hard endpoints, such as adverse cardiovascular events.\textsuperscript{28}

Opioid receptors also activate the RISK pathway and morphine promotes potent cardioprotection in experimental studies when administrated upon reperfusion.\textsuperscript{29} This cardioprotective effect involves the inhibition of the mPTP.\textsuperscript{30} In a clinical study, morphine improved the protective effect of remote preconditioning during PCI.\textsuperscript{31} In a more recent study, Zhang et al. showed that morphine may provide enhanced cardioprotection against ischemia-reperfusion injury in children undergoing corrections of Tetralogy of Fallot but this study concerned only a small number of children.\textsuperscript{32} However a recent trial failed to reduce infarct size in STEMI patients.\textsuperscript{33}

Other G protein-receptor ligands such as the growth factor urocortin and the glucagon-like peptide-1 confirmed the link between the activation of the RISK pathway and the induction of a cardioprotective effect.\textsuperscript{34,35} This link exists also for a variety of other pharmacological agents activating membrane receptors. Experimental studies demonstrated that TGF\( \beta \)-1, insulin, insulin growth factor and erythropoietin elicited cardioprotection through the activation of the RISK pathway when administrated at reperfusion (for review see 36). Among these drugs, erythropoietin gave birth to several preclinical studies (for example 37-40) but also to some clinical trials. In patient with STEMI, the administration of high doses of erythropoietin did not show cardioprotective effect\textsuperscript{41-44} whereas low doses of the drug appeared to be beneficial.\textsuperscript{45} A large clinical study has been conducted but the results have not yet been published.\textsuperscript{46}
Besides to the drugs acting through membrane receptor mediated mechanisms, a number of agents confer cardioprotection by activating components of the RISK pathway downstream to these receptors. This is the case of statines which, in addition to their lipid lowering properties, were shown to stimulate different kinases belonging to the RISK pathway\textsuperscript{47-49} but also to activate the mitochondrial K\textsubscript{ATP} channel through nitric oxide production.\textsuperscript{50} Statins were investigated in clinical trials leading to mixed results.\textsuperscript{43,51,52}

Another pharmacological strategy is to directly target PKC which plays a crucial role in drug-induced cardioprotection. Epsilon-PKC is a primary cardioprotective PKC isoform, whereas delta-PKC promotes injury. In a recent clinical study, a selective inhibitor of delta-PKC, which reduced infarct size during ischemia-reperfusion in animal models, diminished myocardial necrosis and improved reperfusion in a pilot study during primary PCI\textsuperscript{53} but did not reduce biomarkers of myocardial injury in a larger trial.\textsuperscript{54}

1.2. Targeting of the SAFE pathway

The discovery of the SAFE pathway is more recent but its components are also the target of drugs that mimic conditioning and offer the opportunity to promote cardioprotection at reperfusion. In this context, high density lipoproteins, glyceryltrinitrate, cariporide, resveratrol and melatonin were shown to protect the heart against ischemia-reperfusion damage by activating the SAFE pathway and thereby inhibiting mPTP opening.\textsuperscript{55-58} It is important to emphasize that crosstalk between the RISK and the SAFE pathways have been described\textsuperscript{59} and some drugs first considered as selective activators of the RISK pathway also stimulate the SAFE pathway. For example, recent data indicate that the cardioprotective effect of morphine is also mediated by STAT3-activation\textsuperscript{60} and Brulhart-Meynet et al. demonstrated that Akt, STAT3 and ERK1/2 were similarly activated by high density lipoproteins.\textsuperscript{61} In the same way, erythropoietin-induced cardioprotection in a rodent model of prolonged hypothermic global ischemia-reperfusion injury seems to be mediated via activation of the SAFE cytoprotective signaling pathway.\textsuperscript{62} Similarly, the cardioprotective effect of atorvastatin is related to the inhibition of mPTP opening secondary to the activation of TNF-\alpha and the JAK/STAT pathway in early reoxygenation of the human myocardium, \textit{in vitro}.\textsuperscript{63}

2. Pharmacological modulation of endogenous factors inducing mPTP

It is well-established that mitochondrial Ca\textsuperscript{2+} overload is essential to activate mPTP opening at reperfusion and that other factors such as adenine nucleotide depletion, high phosphate concentrations and more particularly ROS are involved in the formation and/or in the regulation of the pore. These factors enhance the sensitivity of the mPTP for Ca\textsuperscript{2+} that possesses binding sites in the mitochondrial inner membrane facing the matrix.\textsuperscript{5} Therefore, inhibiting mitochondrial Ca\textsuperscript{2+} overload and limiting ROS, the primary activators of mPTP opening during cardiac ischemia-reperfusion, are relevant objectives to protect the heart.

2.1. Calcium

The majority of the studies indicate that cellular Ca\textsuperscript{2+} rises during ischemia and precedes irreversible injury of the cell.\textsuperscript{64} This is in agreement with the observation that hearts issued from mice lacking the Na\textsuperscript{+}/Ca\textsuperscript{2+} or the Na\textsuperscript{+}/H\textsuperscript{+} exchanger which both contribute to the rise in cellular Ca\textsuperscript{2+} during ischemia have decreased injury after ischemia-reperfusion.\textsuperscript{65-66} Inhibitors of these exchangers have been shown to limit ischemia-reperfusion injury\textsuperscript{67-69} and this constitutes an interesting strategy to attenuate mitochondrial Ca\textsuperscript{2+} overload and mPTP opening\textsuperscript{70,71}. Besides limiting Ca\textsuperscript{2+} accumulation, inhibition of Na\textsuperscript{+}/H\textsuperscript{+}
exchanger decreases intracellular pH which is also known to inhibit mPTP opening during early reperfusion.\(^9\)

Another way to limit Ca\(^{2+}\) overload is to act on the sarcoplasmic reticulum. The sarcoplasmatic reticulum is an important cellular Ca\(^{2+}\) sink which regulates cytosolic Ca\(^{2+}\) and improving sarcoplasmic reticulum Ca\(^{2+}\) handling has been shown to protect mitochondria against mPTP opening and to reduce ischemic injury.\(^72,73\) In this context, recent results revealed that the interaction between sarcoplasmic reticulum and mitochondria\(^74\) could play a role in mitochondrial Ca\(^{2+}\) overload at reperfusion and could constitute a target to protect cardiomyocytes against reperfusion injury.\(^75\)\(^-\)\(^77\)

Since Ca\(^{2+}\) is a key inducer of mPTP opening, inhibition of mitochondrial Ca\(^{2+}\) uniporter, the protein responsible for mitochondrial Ca\(^{2+}\) uptake\(^78\), was considered as a possible target. According to this hypothesis, Miyamae et al. reported that ruthenium red, a selective inhibitor of the Ca\(^{2+}\) uniporter, improved cardiac function in isolated rat hearts subjected to ischemia-reperfusion.\(^79\) This was confirmed with the use of a selective and potent mitochondrial Ca\(^{2+}\) uniporter blocker.\(^80,81\) This is in line with the results of Kwong et al. demonstrating that hearts from mice lacking the mitochondrial Ca\(^{2+}\) uniporter produced mitochondria resistant to mPTP opening upon Ca\(^{2+}\) overload and these hearts were protected from acute ischemia-reperfusion injury.\(^82\) Taken together, these data suggest that acute inhibition of the Ca\(^{2+}\) uniporter could be an interesting therapeutic approach to protect against cell death. However, opposite results indicate that it is necessary to better understand the mechanisms governing cell survival and cellular responses due to the loss of Ca\(^{2+}\) uniporter before developing therapies designed to prevent mitochondrial Ca\(^{2+}\) overload.\(^83\)

### 2.2. Reactive oxygen species (ROS)

Mitochondria are one of the key initiators of cellular ROS production. Electron leakage from the electron transport chain during respiration is generally considered as the main source of mitochondrial ROS but several other mitochondrial enzymatic systems have been found to produce ROS.\(^84\) In physiological conditions, ROS formed within mitochondria are eliminated by powerful antioxidant systems.\(^84\) During post-ischemic reperfusion, the sudden influx of oxygen leads to a burst of ROS\(^85,86\) which can overwhelm endogenous antioxidant systems. ROS produced during early reperfusion have been shown to be primary activators of mPTP and cardiomyocyte death.\(^87\) The sensitization of mPTP along with an increase in mitochondrial protein carbonylation during myocardial ischemia emphasizes the role of the oxidative stress.\(^88\)

The question is how an increase in ROS production can affect mPTP opening? Former studies performed in isolated mitochondria have mentioned the role of oxidation-reduction of critical protein residues which could influence mPTP opening.\(^89\) More particularly, the oxidation of thiol functions and cysteine residues, which is considered as an important mechanism to regulate protein structure, was described on proteins supposed to be involved in the formation of the pore or in the regulation of its opening. This concerns the adenine nucleotide translocase\(^90,91\), cyclophilin D (CypD)\(^92\), ATP synthase\(^93\) or complex I of the respiratory chain.\(^94\) The oxidation of cardiolipin can also contribute to the effect of ROS on mPTP opening. Indeed, cardiolipin is specific of mitochondria and is the main lipid of the inner membrane. Cardiolipine is located close to the sources of ROS production and contains high level of unsaturated fatty acids susceptible of lipid peroxidation. Oxidized cardiolipine was shown to sensitize heart mitochondria to mPTP opening.\(^95\)
Therefore, the use of antioxidant agents has emerged as a relevant approach to limit mPTP opening and thus ischemia-reperfusion injury. In addition, it was recently suggested that the contribution of ROS might be more necessary than Ca$^{2+}$ overload to maintain sustained mPTP opening and cell death at reperfusion\(^\text{96}\), reinforcing the suitability of this approach.

It should be noted that the reduction of oxidative stress was suggested to contribute to the cardioprotective effect of several therapeutic drugs such as the substrate of oxidative-phosphorylation pyruvate\(^\text{97-99}\), the non-selective β-adrenoceptor antagonist carvedilol\(^\text{100,101}\) or the anaesthetic drug propofol widely used in cardiac surgery.\(^\text{102,103}\)

Several strategies aimed at scavenging ROS or increasing their degradation using a wide range of natural antioxidants or pharmacological agents inhibit mPTP opening and display cardioprotective effects in different in vitro and ex vivo model of ischemia-reperfusion.\(^\text{104-107}\) However, the translation of these successful laboratory results to the clinical setting remains inconsistent.\(^\text{108-110}\) It should be noted that a recent clinical trial associating N-acetyl-cysteine with nitrate therapy showed reduced infarct size in STEMI patients undergoing PCI.\(^\text{111}\) Adlam et al. demonstrated that targeting antioxidant to mitochondria can reduce reperfusion injury but that the protective effect is lost when the antioxidant agent did not accumulate into mitochondria.\(^\text{112}\) This can explain the absence of effect of antioxidants since a lot of these drugs exhibit low bioavailability and low selectivity. Thus, they do not reach mitochondria or require high dosage to reach mitochondria in vivo. In this case, high doses of antioxidant agents, such as polyphenols for instance, can act as prooxidants\(^\text{113}\), leading to cellular dysfunction and cell death. The challenge is, therefore, to target antioxidants to mitochondria and to reduce the deleterious consequence of off-target subcellular localization.

Several approaches have been reported (see reviews \(^\text{86,101,114,115}\)) and here we just mention them briefly. The first one uses the high selective mitochondrial membrane potential as a selective system to deliver molecules to the organelle. Lipophilic cations are conjugated to antioxidant compounds which are sufficiently lipophilic to cross lipid bilayer membranes. These substances can be selectively taken up into the mitochondria by the large, negative inside, inner membrane potential.\(^\text{116}\) A disadvantage of this strategy is that the molecules need a certain level of membrane potential and thus viable mitochondria to accumulate inside the organelle. These drugs include alpha tocopherol (MitoVit E), ubiquinone (MitoQ), SNO (MitoSNO), tempol (MitoTempol), resveratrol (Mito-resveratrol) and plastoquinone (SkQs compounds). A number of experimental studies indicate that these drugs could constitute a valid approach to limit cardiac reperfusion-injury.\(^\text{112,117-119}\) The second mitochondrial delivery drug system are peptides engineered to facilitate the crossing through the mitochondrial inner membrane. These peptides are mitochondrial penetrating peptides\(^\text{120}\), hemigramicidin-TEMPO conjugates\(^\text{121}\) and Szeto-Schiller (SS) peptides.\(^\text{122}\) SS-peptides are today the most promising peptides to reduce ischemia-reperfusion injury. SS-peptides include a sequence motif targeted to mitochondria and enter the mitochondria in a potential-independent manner. Their antioxidant effect is due to the presence of basic amino acids, tyrosine and dimethyltyrosine, which are effective at scavenging ROS. These peptides reduce mitochondrial ROS production, inhibit mPTP opening and prevent the release of cytochrome c from mitochondria.\(^\text{123}\) They were shown to reduce infarct size in rats when they were administrated before ischemia\(^\text{124}\) or at reperfusion.\(^\text{122}\) One of these peptides was also shown to stabilize the interaction between cardiolipine and cytochrome c and to improve oxidative
phosphorylation. This effect can also contribute to the anti-ischemic effect of these drugs. However, in a recent clinical trial, administration of this peptide at the time of PCI failed to reduce infarct size.126

It should be kept in mind that ROS also exert physiological role. Therefore, we cannot disregard the importance of physiological low concentrations of ROS which are necessary for signaling processes. Determining the threshold separating the physiological from the pathological level of ROS remains a major objective which could help to develop antioxidant agents efficient during myocardial ischemia-reperfusion.

3. Pharmacological control of mPTP opening

mPTP is thought to be a multiprotein complex which forms and opens under conditions that prevail at the time of reperfusion such as Ca\(^{2+}\) overload, oxidative stress, adenine nucleotide depletion, high phosphate concentrations or membrane depolarization. mPTP was originally thought to form at contact sites between inner and outer membranes, involving the Voltage Dependent Anion Channel, the Adenine Nucleotide Transporter and the Phosphate Carrier. However, genetic ablation of those putative components successively ruled out their participation in the structure of the pore. Recently, a new hypothesis proposes that ATP synthase might form the core of the pore, either by dimerization or by detachment of the c subunit. Nevertheless, a common agreement considers that opening of the mPTP is under the control of CypD. CypD is a soluble matrix protein which catalyses or stabilizes the open state of mPTP. The crucial role of CypD has been demonstrated by the deletion of the gene in mice, allowing mitochondria to sustain higher Ca\(^{2+}\) concentrations by desensitizing mPTP.129,130

3.1. Targeting CypD

3.1.1. Cyclosporin A and its derivatives

The first demonstration of the impact of CypD inhibition on pore opening was made by the observation that cyclosporin A (CsA), an immunosuppressant agent targeting all cyclophilins, inhibits mPTP opening. CsA binds tightly to cyclophilins and inhibits peptidyl-prolyl-cis-trans-isomerase (PPIase) activity. In mitochondria, interaction of CsA and CypD results in inhibition of PPIase activity and inability of CypD to bind to membrane proteins. This results in pore closure. Since the observations made by Crompton and colleagues, CsA has been proven to be protective in several in vitro, ex vivo and in vivo models although several failures were reported in larger animals (for a review, see132). A proof of concept clinical trial demonstrated that administration of 2.5 mg/kg at the time of PCI is cardioprotective. This was associated with reductions in biomarker release and adverse remodeling at 6 month post PCI. Based on these encouraging results, CIRCUS (Cyclosporin to ImpRove Clinical oUtcome in STEMI patient), a phase III placebo-controlled trial including 975 patients failed to demonstrate any beneficial effect of CsA administration on every endpoint. Another phase III clinical trial, CYCLE, also failed to recapitulate the beneficial effects of CsA. These negative results have been extensively commented elsewhere (e.g.137-139) and raise major concerns toward the clinical use of CsA in mPTP based pathologies.

Enhancing mitochondrial biodisponibility might help to counteract the side effects observed with CsA and could enlarge its therapeutic window. To this end, a mitochondrial targeted CsA (mtCsA) designed to minimize non CypD interactions in cells was developed by the Crompton’s group. They combined CsA with a triphenylphosphonium cation to specifically target mitochondria. This improved
cytoprotection in a simulated ischemia-reperfusion model by lowering the maximal concentration needed to inhibit mPTP opening and decreasing cellular toxicity. Following the same principle, JW47, a CsA derivative coupled with a quinolinium cation demonstrated reduced cellular toxicity and afforded significant protection in a mouse model of encephalomyelitis by selectively inhibiting CypD. Recently, the use of nanoparticles to address CsA to ischemic myocardium yielded promising results. Indeed, encapsulated CsA accumulated in the mitochondria of the ischemic area, resulting in a decrease in infarct size in mice at lower concentrations than CsA alone.

CsA has also been modified to provide derivatives devoid of immunosuppressant activity by modifying the residues normally interacting with calcineurin. This is the case for NIM811 (N-methyl-4-isoleucine-CsA) which inhibits mPTP opening and exerts protective effect in various models of diseases where mPTP plays a major role such as post-cardiac arrest syndrome in rabbits, traumatic brain injury in rats and mice or cardiac and liver ischemia-reperfusion. Debo-025 (N-Me-Val-4-CsA, alisporivir) is another CsA derivative lacking the immunosuppressant activity which demonstrated cardioprotective effects.

Other cyclic polypeptides unrelated to CsA have been described as mPTP inhibitors. Sanglifehrin A (SfA) is a cyclic polypeptide which exerts immunosuppressant activity independently from calcineurin interaction. As compared to CsA, SfA binds differently on CypD and shows important difference in its mechanism of action. Indeed, SfA inhibits PPIase function but does not inhibit the binding of CypD to mitochondrial membranes and especially to ANT. SfA inhibits mPTP opening as potently as CsA, suggesting that inhibiting PPIase activity, rather than CypD binding to the mPTP components, is the key event in this mPTP inhibition strategy.

Antamanide is a cyclic decapeptide derived from the fungus Amanita phalloides which has been described to exert immunosuppressant activity by interacting with CypA but not calcineurin. In this context, it has been supposed that antamanide could also inhibit CypD and therefore block mPTP opening. Antamanide antagonizes mPTP opening in vitro by inhibiting CypD PPIase activity although it displays a ten times lower affinity for CypD than CsA.

Nevertheless, all of these drugs have limitations which hamper their clinical use. Indeed, they have severe side effects including nephrotoxicity, neurotoxicity and hepatotoxicity which limit their use in vivo.

3.1.2. New small molecules inhibiting CypD

Recently, the discovery of new non peptidic small-molecules inhibitors of cyclophilins (SMCypI) unrelated to CsA or SfA have been described. These compounds were designed by fragment based drug discovery and aimed at inhibiting cyclophilins PPIase activity with submicromolar activity. These inhibitors are non-toxic small molecules devoid of immunosuppressive effects. In addition, one can imagine coupling them to lipophilic cations to develop their mitochondrial selectivity similarly to previous antioxidant agents, to bypass the other cellular cyclophilins and increase their selectivity for CypD. Based on this molecular scaffold, two groups developed small inhibitors of CypD which demonstrated cytoprotection in cellular models of acute pancreatitis and UV-associated retinal degeneration by inhibiting mPTP opening although they have not been tested in vivo yet. The cardioprotective properties of these molecules remains currently under investigation.

Another group identified the 4-aminobenzenesulfonide scaffold as a CypD inhibitor. Their compound, C-9, binds to CypD and inhibits PPIase activity with a micromolar IC50. This allowed the inhibition
of Ca\textsuperscript{2+} induced mitochondrial swelling. In vitro, C-9 prevented mitochondrial dysfunction induced by Aβ peptide in neuronal cells as demonstrated by the restoration of ATP content and cytochrome c oxidase function. Moreover, C-9 did not exert toxic effect on cellular cultures even at high concentrations (100 µM).

As CypD is only a modulator of mPTP opening, its inhibition decreases the susceptibility of opening rather than totally blocking it. Indeed, in mitochondria devoid of CypD, pore opening can still be observed at higher Ca\textsuperscript{2+} concentrations. Hence, inhibiting CypD may not be sufficient to afford protection in case of severe stimuli, emphasizing the needs for other targets as well.

### 3.2. mPTP inhibitors that do not interact with CypD

As previously described, CypD inhibition displays limitations and a number of groups try to identify inhibitors acting through other modulators of the pore or directly interacting with a pore component although the exact structure of the pore remains elusive.

#### 3.2.1. TSPO ligands

An example is the 18 kDa translocator protein (TSPO), an outer membrane protein which has been shown to interact with putative components of the mPTP.\textsuperscript{160} Several studies have demonstrated that TSPO ligands, such as SSR180575 and 4’-chlorodiazepam, exert cardioprotective effects.\textsuperscript{161,162} This effect was associated with a limitation of the permeability of mitochondrial membrane to cytochrome c and Apoptosis Inducing Factor. 4’-chlorodiazepam also increased the resistance of mitochondria to Ca\textsuperscript{2+}-induced mPTP opening. A similar profile was observed with TRO40303. TRO40303 is a ligand which was originally selected using a cell-based screening assay aimed to identify small molecules that maintain survival of trophic factor-deprived rat motor neurons.\textsuperscript{163} TRO40303 binds specifically to the cholesterol site of TSPO and exhibits cytoprotective properties in various cell types. TRO40303 was shown to inhibit mPTP opening in isolated cardiomyocytes.\textsuperscript{164} It has to be noted that TRO40303 does not decrease mitochondrial sensitivity to Ca\textsuperscript{2+} as assessed by the measurement of mitochondrial Ca\textsuperscript{2+} retention capacity, indicating that it might inhibit mPTP opening by an indirect mechanism of action. This is in line with the data of Sileikyte and co-workers who demonstrated that TSPO is not required to regulate mPTP opening in TSPO KO mouse heart.\textsuperscript{165} However, a new indirect mechanism was recently described. Paradis et al. (2013) showed that the reperfusion of an ischemic myocardium was associated with an accumulation of cholesterol into mitochondria and a concomitant strong generation of auto-oxidized oxysterols resulting from the oxidation of cholesterol by ROS.\textsuperscript{166} The TSPO ligands 4’-chlorodiazepam and TRO40403 abolished the mitochondrial accumulation of cholesterol and the formation of oxysterols, reduced oxidative stress and prevented mPTP opening at reperfusion.\textsuperscript{166} They remained efficient in hypercholesterolemic conditions.\textsuperscript{167} This new and original mechanism may contribute the cardioprotective properties of TSPO ligands.

*In vivo*, administration of TRO40303 reduced reperfusion injury in rats\textsuperscript{164,168} but the cardioprotective effect of the drug was not confirmed in a clinical trial (MITOCARE) which demonstrated no benefit in patients when TRO40303 was administrated at the time of PCI.\textsuperscript{169}

#### 3.2.2. Ubiquinones

Several ubiquinones have been described as mPTP inhibitors while others are mPTP inducers. Indeed, mPTP might possess a quinone binding site which controls the Ca\textsuperscript{2+} sensitivity.\textsuperscript{170} The most potent ubiquinone Ub0 increases the Ca\textsuperscript{2+} retention capacity in a
larger extent than CsA suggesting that ubiquinones do not act by inhibiting CypD. Another argument is that Ub5 is able to antagonize the inhibition afforded by Ub0 but not that afforded by CsA. Inhibition by ubiquinones is not limited to Ca\textsuperscript{2+}-induced mPTP but is also observed with other inducers such as atractyloside, oxidative stress and mitochondrial depolarization.\textsuperscript{170} The exact mechanism of action by which ubiquinones inhibit mPTP opening is not known but, as they also interfere with the electron transport chain, it has been suggested that ubiquinones act as electron acceptor and exert antioxidant effect.\textsuperscript{171} Nevertheless, the most active ubiquinone Ub0 has never been tested \textit{in vivo}, except as a mitochondrial targeted ubiquinone, mitoQ, which is cardioprotective by limiting oxidative stress.\textsuperscript{112} Ub10, which is as potent as CsA, has been previously described to improve cardiac functional recovery in an isolated perfused rabbit heart model of ischemia-reperfusion\textsuperscript{172} but the authors did not mention the inhibition of mPTP as the protective mechanism involved. The preservation of ATP observed may suggest that mitochondria remain functional at the time of reperfusion which is consistent with pore closure. Cardioprotective effects of Ub10 have been extensively studied in the past, including a clinical trial demonstrating the benefit of Ub10 administration following myocardial infarction.\textsuperscript{173}

3.2.3. Cinnaaic anilides
High throughput screening of commercially available small molecules libraries, based on isolated mitochondrial swelling, has led to the discovery of at least four classes of new, direct, low molecular weight mPTP inhibitors. The first class of these compounds was reported in 2014 by Fancelli and colleagues who demonstrated the potency of cinmic anilides derivatives.\textsuperscript{174} Cinnamic anilides inhibited mitochondrial swelling induced by various mPTP known inducers such as Ca\textsuperscript{2+} overload, oxidative stress and chemical cross linkers in isolated mitochondria. The compounds enhanced mitochondrial Ca\textsuperscript{2+} retention capacity more efficiently than CsA. The higher Ca\textsuperscript{2+} retention capacity observed suggests a CypD independent mechanism of action and this was confirmed by the additive effects produced by the association of CsA and cinamic anilides. \textit{In vivo}, treatment with cinamic anilides at the time of reperfusion was able to limit infarct size in a rabbit model of ischemia-reperfusion but there was no difference with CsA.\textsuperscript{172} GNX-4728, a cinnamic anilide derivative also demonstrated protective effect in a mouse model of amyotrophic lateral sclerosis as it slowed the disease progression and improved motor function by limiting mPTP-mediated neurodegeneration.\textsuperscript{175} A possible explanation of the mechanism of action of cinmic anilides was proposed by Richardson and Halestrap.\textsuperscript{176} Their data suggest that these drugs might inhibit mPTP opening by interacting at the Ca\textsuperscript{2+} binding site between the Adenine Nucleotide Translocase and the inorganic phosphate carrier and thereby stabilizing the “c” conformation which favors mPTP closure.

3.2.4. Isoxazoles
Screening of the NIH Molecular Libraries Small Molecule Repository using Ca\textsuperscript{2+}-induced mitochondrial swelling followed by structure-activity relationship optimization led to the identification of isoaxoxoles as powerful mPTP blockers with picomolar inhibitory activity.\textsuperscript{177} They inhibited mPTP in both mouse liver mitochondria and Hela cells demonstrating that their effect is not species-specific. Isoxazoles do not target CypD as mitochondria treated with isoaxoxoles exhibited at least a three-fold higher Ca\textsuperscript{2+} retention capacity than with CsA and association of isoaxoxoles and CsA resulted in a synergistic effect. According to the most recent hypothesis on mPTP structure, F\textsubscript{1}F\textsubscript{0}-ATP Synthase might form the core of the pore. Effect of isoaxoxoles on F\textsubscript{1}F\textsubscript{0}-ATP
Synthase was thereby assessed and the authors demonstrated that isoxazoles do not interfere with mitochondrial respiration suggesting that isoxazoles do not target F₁F₀-ATP Synthase. The most efficient analogue demonstrated beneficial effect in a zebra fish collagen VI myopathy model as it improved motor function and muscle structural organization. However, the use of isoxazoles in murine and larger animal models is hampered by the instability of the compounds which are rapidly degraded.

3.2.5. Benzamides

Along with isoxazoles, Roy and colleagues identified benzamide scaffold as a potential mPTP blocker by high throughput screening and further derived the hit compounds to increase their mPTP inhibitory effect. Phenylbenzamides were able to inhibit mitochondrial swelling induced by various mPTP inducers and increased Ca²⁺ retention capacity as potently as isoxazoles but in a greater extent than CsA. This effect was still present in mitochondria isolated from CypD null mice, demonstrating a CypD-independent mechanism of action. However, in vivo use of benzamides is hampered by their cellular toxicity as they decrease inner membrane potential and ATP synthesis.

3.2.6. ER-000444793

Finally, another group identified the compound ER-000444793 which was shown to delay mitochondrial depolarization in response to Ca²⁺ overload and to increase Ca²⁺ retention capacity, indicating that ER-000444793 inhibits mPTP opening. Experiments performed on purified CypD demonstrated that ER-000444793 inhibiting properties do not rely on inhibition of CypD enzymatic function.

Taken together, these recent data are rather encouraging after the CIRCUS and CYCLE trials failure, showing that there is still a place for new mPTP inhibitors. These data demonstrate that mPTP can be efficiently inhibited by other mechanism independent from CypD. Such compounds could also bring new insights in elucidating the exact mPTP molecular identity. However, the lack of knowledge of the mPTP structure remains the main obstacle to develop novel mPTP inhibitors.

4. Pharmacological control of mitochondrial outer membrane permeability

Although mPTP opening is a well established mechanism mediating cell death during myocardial ischemia-reperfusion, clinical trials aiming to inhibit mPTP failed to demonstrate positive results. As mentioned above, several explanations have been proposed to explain these failures but a possibility is that mPTP inhibition is not sufficient per se to improve clinical outcome. Indeed, other mechanisms which can permeabilize mitochondrial membrane in the absence of mitochondrial depolarization and release proapoptotic agents from the mitochondrial intermembrane space have been described. They include the formation of a channel by the proapoptotic members (Bax/Bak) of the Bcl-2 family proteins which control mitochondrial membrane permeabilization. Mouse models of this family of proteins have revealed their importance during myocardial ischemia-reperfusion. Over-expression of Bcl-2 in mice, Bax deletion or interference with Bax activation attenuate apoptosis and reduce infarct size, while reduction of Bcl-2 levels suppresses the protection afforded against injury. Therefore, targeting Bcl-2 family proteins or inhibiting the MAC channel appears attractive to develop anti-ischemic agents which could act through a gain of antiapoptotic or a loss of proapoptotic function. Several molecules that inhibited cytochrome c release and apoptosis triggered by induction of the Bax channel were
identified\textsuperscript{190,191} but they were not investigated in animal models.

Similarly, Hetz et al.\textsuperscript{192} identified two small Bax channel inhibitors which prevented cytochrome c release, mitochondrial depolarization and blocked apoptosis of neurons in an animal model of ischemic brain injury but to our knowledge these molecules have not been evaluated in animal models of myocardial ischemia-reperfusion. More recently, a promising approach used a chemical inhibitor of dynamin-related protein (Drp1) which regulates Bax/Bak mitochondrial outer membrane permeability.\textsuperscript{193} This inhibitor, the mitochondrial division inhibitor 1, afforded neuroprotection \textit{in vitro} and \textit{in vivo}\textsuperscript{194} and was effective to protect against myocardial ischemia-reperfusion injury only when administrated before the induction of ischemia.\textsuperscript{195} Using a nanoparticle delivery system which improved the delivery of the drug to the myocardium, Ishikita et al.\textsuperscript{196} demonstrated that the intravenous administration of the mitochondrial division inhibitor 1 \textit{in vivo} at the time of reperfusion reduced ischemia-reperfusion injury in wild-type but also in CypD knock-out mice.

Another candidate for mitochondrial membrane permeabilization is the voltage-dependent anion channel (VDAC). VDAC is the main permeability pathway for metabolites through the mitochondrial outer membrane\textsuperscript{197} and is important for communication between the mitochondria and the rest of the cell. This probably explains the large number of proteins and small molecules interacting with this channel.\textsuperscript{198} VDAC interacts with pro- and anti-apoptotic proteins of the Bcl-2 family members and one of its isoforms (VDAC1) has been involved in the mitochondrial release of proapoptotic proteins independently of mPTP opening\textsuperscript{199-201} whereas VDAC2 attenuates cell death\textsuperscript{202} in part by binding and inactivating the Bcl-2 family member Bak.\textsuperscript{203} This highlighted VDAC as a potential therapeutic target in ischemia-reperfusion. According to this idea, a cell permeable peptide corresponding to the BH4 domain of Bcl-XL, which had been reported to close VDAC and to prevent the VDAC-mediated release of cytochrome c\textsuperscript{204}, was shown to attenuate ischemia-reperfusion injury in rat hearts\textsuperscript{205}. However, the studies on VDAC did not lead to more therapeutic development in ischemia-reperfusion probably because a lot of questions remain concerning the function and the regulation of the channel \textit{in vivo}.

5. Inhibition of mitochondrial membrane permeability: from basic science to effective clinical therapy.

Since the identification of the cardioprotective effect of preconditioning by Murry et al.\textsuperscript{11}, three decades of basic science allowed to demonstrate the central role of mitochondrial channels in the induction of myocardial injuries and to propose pharmacological agents or conditioning strategies for reducing infarct size. Numerous animal model studies have clearly demonstrated the potentiality of these approaches to reduce the extent of myocardial injury and clinical pilot studies displayed promising results\textsuperscript{53,135}. However, larger multicentric studies annihilated the encouraging results generated by these proof-of-concept trials and, for example, no protective effect could be observed with CsA\textsuperscript{135,136} or the delta-PKC inhibitor decasertib\textsuperscript{54}. In the same way, the TSPO ligand TRO40303 which displayed cardioprotective effects in animal models was ineffective in a clinical study.\textsuperscript{169} Up to now, the translation of pharmacological or other approaches issued from experimental studies into clinical application remains negative.\textsuperscript{46,206}

Different reasons can be evoked to explain these failures\textsuperscript{138,139} such as pharmacokinetic and pharmacodynamic issues in STEMI patients, patient recruitments but also the concomitant use of other drugs during PCI that possess \textit{per se} cardioprotective effects.
such as the anesthetic propofol or the P2Y12 platelet inhibitors.

Given that a number of different pharmacological interventions limiting mitochondrial membrane permeability have demonstrated positive results in preclinical studies (Figure 1) and that multiple factors are involved in its induction, the combination of pharmacological strategies acting on these different targets appears as a realistic approach to reduce myocardial reperfusion injury. A good example is the recent clinical trial associating an antioxidant, N-acetylcysteine, with nitrate therapy.¹¹¹

![Diagram of pharmacological strategies](image-url)

**Figure 1: Pharmacological strategies aimed at limiting mitochondrial membrane permeabilization during myocardial ischemia-reperfusion.**

Schematic representation of pharmacological strategies targeting mitochondrial membrane permeabilization either by limiting the formation of Mitochondrial Apoptosis-induced Channel (MAC/Bax) or by inhibiting the opening of the mitochondrial permeability transition pore (mPTP).

CypD: Cyclophilin D; ROS: Reactive oxygen species; RISK: Reperfusion Injury Salvage Kinase; SAFE: Survivor Activating Factor Enhancement; MCU: Mitochondrial Calcium Uniporter; SMCypl: small-molecule cyclophilins inhibitors

→: induction

⊥: direct (plain) or indirect (dotted) inhibition
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