

Mitochondrial permeability transition: a molecular lesion with multiple drug targets

Authors

Thomas Briston^{1*}, David Selwood², Gyorgy Szabadkai^{3,4,5}, Michael R Duchen³

¹ Neurology Innovation Centre, Hatfield Research Laboratories, Eisai Ltd., Hatfield, UK

² Wolfson Institute for Biomedical Research, Division of Medicine, University College London, London, UK

³ Department of Cell and Developmental Biology, Consortium for Mitochondrial Research, University College London, London, UK

⁴ Department of Biomedical Sciences, University of Padua, Padua, Italy

⁵ The Francis Crick Institute, London, UK

* Correspondence: thomas_briston@eisai.net (Thomas Briston)

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Abstract

Mitochondrial permeability transition, as the consequence of opening of a mitochondrial permeability transition pore (mPTP), is a cellular catastrophe. Initiating bioenergetic collapse and cell death, it has been implicated in the pathophysiology of major human diseases, including neuromuscular diseases of childhood, ischaemia-reperfusion injury and age related neurodegenerative disease. mPTP represents a major therapeutic target, as opening can be prevented by a number of compounds. However, clinical studies have so far been disappointing. We therefore address the prospects and challenges faced in translating *in vitro* findings to clinical benefit. We review the role of mPTP opening in disease, discuss recent findings defining the putative structure of mPTP and explore strategies to identify

novel, clinically useful mPTP inhibitors, highlighting key considerations in the drug discovery process.

Ruby anniversary: 40 years and finally some success

Mitochondria are essential for bioenergetic homeostasis and also play a principal role in regulating cellular Ca^{2+} homeostasis. Mitochondrial Ca^{2+} overload, especially when combined with oxidative or nitrosative stress and/or ATP depletion, triggers an abrupt increase in the permeability of the inner mitochondrial membrane (IMM) through the opening of a high-conductance voltage- and Ca^{2+} - sensitive channel, known as the mitochondrial permeability transition pore (mPTP) (Figure 1; Key Figure). mPTP opening causes the collapse of mitochondrial membrane potential and bioenergetic failure, coupled with the redistribution of low molecular weight solutes of less than 1500 Da across the IMM and mitochondrial swelling [1, 2].

Approximately 40 years have passed since the first biochemical description of the mitochondrial permeability transition in isolated mitochondria by Haworth and Hunter [3] and 30 years since the discovery that cyclosporin A (CsA) inhibits opening of the mitochondrial permeability transition pore (mPTP) *in vitro* [4]. Since then, mitochondrial dysfunction and persistent mPTP opening has emerged as a final common pathway to cell death underlying many disease states [5-9]. Specific pharmacological modulation of mPTP opening has long been sought. While the mPTP is clearly a druggable target, the development of inhibitors beyond the prototypical compound, CsA, itself limited by off-target effects and low therapeutic **efficacy** (see Glossary), has been slow and disappointing. However, in the last 5 years, multiple groups have identified several novel, chemically distinct small molecule inhibitors of the mPTP, opening avenues for drug discovery and development that will have wide therapeutic potential. Most notably, edaravone (Radicut™), a potent anti-oxidant and inhibitor of the permeability transition has been approved for the treatment of amyotrophic lateral sclerosis (ALS) [10] as well as there being early successes in the treatment of the collagenopathies [11-13].

Complex bio-architecture and regulation of the mPTP

The nature of the mPTP complex has long been debated and as yet no definitive biomolecular configuration has been agreed (Fully reviewed elsewhere [14]). Many candidates for the role of a pore forming inner membrane protein have been proposed, including the adenine nucleotide translocase (ANT) [15], the mitochondrial phosphate carrier (PiC) [16], the metalloprotease spastic paraplegia 7 (SPG7) [17], and either dimers, or an uncoupling channel within the c-ring of the F_1F_0 -ATP synthase [18, 19]. Outer membrane components, the voltage-dependent anion channel (VDAC) [15] and BCL-2 family members BAK/BAX [20] have further been suggested to play a role as modulators of pore opening. All of these hypotheses have been challenged, and loss- or gain-of function genetic studies have failed to validate each hypothesised candidate [21-24], although some are now understood to play potential regulatory roles [25, 26]. The only component that is unambiguously involved as a regulator of mPTP opening is the peptidyl prolyl *cis-trans* isomerase (PPIase), cyclophilin D (CypD, also known genetically as *Ppif* [Box 1]). Indeed, it is the role of CypD that confers sensitivity of pore opening to CsA, and much of the evidence supporting a role for the pore in disease models is supported by evidence of protection in CypD knockout mice (Table 1). Loss of CypD shifts the threshold for mPTP opening to a higher Ca^{2+} load, however mitochondria remain capable of permeability transition [27]. Despite this caveat, genetic ablation of CypD in the mouse has proven protective in a remarkable array of disease states in different tissues [27-34], although as yet, these data have failed to translate into clinical benefit (Box 2).

Recent data point to a role for the F_1F_0 -ATP synthase in pore formation. One model suggests that dimerisation and CypD mediated conformational changes of the F_1F_0 -ATP synthase complex give rise to the formation of the mPTP [18, 35]. CypD may electrostatically interact with the oligomycin sensitivity conferring protein (OSCP) subunit of the enzyme to potentiate or trigger mPTP opening [18]. This model has been recently refined by details of pH and Ca^{2+} -mediated regulation of the structure [36, 37], and opposes an alternative model, which suggests that the c-ring of the membrane spanning F_0 -component would constitute the pore forming unit without dimer formation [19]. Recent work has cast doubt on the F_1F_0 -ATP synthase hypothesis [22, 23]. He, Walker and colleagues demonstrated persistent mPTP opening in HAP1 cells selectively devoid of F_1F_0 -ATP synthase subunit c

(forming the c-ring), subunit b and the OSCP (both components of the peripheral stalk), together suggesting that the proton channel forming membrane components and peripheral stalk of the F₁F₀-ATP synthase are unlikely to be involved in mPTP formation [22, 23]. Furthermore, molecular dynamic simulations considering c-rings of different size and ion-conducting properties suggest that the c-ring, even if isolated from the F₁-domain is not the mPTP [38], raising more questions over structural conformation (for detailed reviews of mPTP structure see [14, 39]).

In the context of drug discovery, the absence of detailed bio-architecture makes traditional 'target-based' approaches (beyond CypD [Box 1]) to hit identification impossible. However, given the robust data set placing unregulated mPTP opening as a key event in major human diseases and evidence supporting a protective role for CypD ablation (Table 1), the accumulation of small-molecule mPTP modulators and modern molecular tool box, although optimistic, it is surely a matter of time before the true channel forming structures are unequivocally defined.

Disease relevance of mPTP opening: Lessons from *Ppif*^{-/-} models

The physiological role of CypD remains something of a puzzle, as *Ppif*^{-/-} mice are born healthy, in litters with correct Mendelian ratios and display increased anxiety and adult-onset obesity [27, 28, 40]. Beyond behavioural traits, the lack of significant phenotype suggests that CypD inhibition represents a valid therapeutic strategy to delay mPTP opening with little risk of toxicity or off-target effects. The most significant and robust evidence for a role of CypD and mPTP opening in disease derives from studies of **ischaemia-reperfusion injury** (see Glossary) in the heart and compelling data further support a role for CypD in the pathology of the Bethlem or Ullrich type muscular dystrophies, consequences of collagen VI -, delta-sarcoglycan- or the more severe laminin-2-deficiencies [31, 33]. Moreover, single studies suggest a disease-modifying role of CypD following intestinal ulceration and enteropathy associated with chronic use of the non-steroidal anti-inflammatory drug (NSAID) diclofenac [41] and bone loss associated with aging-related osteoporosis [42]. Accumulating evidence suggests that cytoprotection in neurodegenerative disease may be possible through therapeutic modulation of mPTP opening. Loss of CypD conferred neuroprotection across models of neurological disease, including Alzheimer's disease, MS

and ALS. CypD ablation improved learning and memory in mouse models of Alzheimer's disease expressing mutant amyloid precursor protein (mAPP) [34] and preserved axonal integrity in an experimental allergic encephalomyelitis (EAE) mouse model of MS [30] and delayed onset of ALS and prolonged survival in a mSOD1-G93A mouse model [43]. Together these observations, although supporting a role for mPTP opening in disease, reflect only the consequences of CypD knockout, and therefore must be interpreted as such. Despite these caveats, independent observations in multiple pre-clinical models argue that pharmacological modulation of mPTP opening may be beneficial across a range of important pathologies, most of which lack alternative current treatment options.

Tractability of the mPTP: The search for small molecule inhibitors

Expression of CypD and mPTP sensitivity across tissues

mPTP opening has been observed in mitochondria isolated from multiple tissues and species [44, 45], suggesting that the phenomenon may be universal across cell types, at least *in vitro* [46-48]. It has proven much more difficult to assess mPTP opening in mitochondria *in situ*, and mPTP opening has only recently been described *in vivo* using tritium-labelled 2-deoxyglucose ([³H]DOG) [49], a similar technique pioneered by the Griffiths and Halestrap *ex vivo* [6, 50]. Importantly, differences in the Ca²⁺ threshold for mPTP opening have been described in mitochondria from different tissues [44, 45, 51]. CypD expression appears to be key in determining the sensitivity to stress-induced mPTP opening but correlations between CypD protein expression patterns and tissue specific mPTP formation has not been studied comprehensively. It has been shown that CypD expression is higher in mitochondria isolated from the rat brain substantia nigra compared to striatum and cortex and to be higher in non-synaptic mitochondria and inter-neurons, potentially explaining the heterogeneous response to mPTP formation observed in brain mitochondria and suggesting regional differences in sensitivity [52]. Finally, CypD protein expression and activity may be tightly regulated through complex interactions with hematopoietic-substrate-1 associated protein X-1 (HAX-1) [53], and heat-shock protein-90 [54], further complicating therapeutic targeting.

Structural considerations

Considering that the channel forming units of the mPTP complex are not yet definitively identified, structural concerns for therapeutic targeting are limited to CypD. CypD is one of 17 cyclophilins in humans all of which share common architecture (61% to 86% homology), whereby two α -helices enclose eight antiparallel β -sheets. Catalytic activity and CsA-sensitivity are determined by a tryptophan (Trp-121) within a short α -helical turn between loops β 6 and β 7 [55]. 11 of the 17 cyclophilins are known to be CsA sensitive. PPlase domains contain two binding pockets (S1' and S2). "Gatekeeper" residues control substrate access to S2 [55]. The S2 pocket is defined by the residues T73, S81, K82, A103, T107, S110 and Q111 in CypA and by TGKATSQ in CypD other cyclophilins show more diversity in the S2 pocket. Structural distinctions between each cyclophilin are also observed between β 1- β 2 and α 2- β 8 loops outside of the active site and may be involved in modulation of activity via protein-protein interactions (Further reading see [56]). The BioGRID databased indicates 36 binding partners for CypD but whether they all interact with the so-called isomerase site is unknown. Complete elucidation of molecular regulation, signaling and structural characterisation of all cyclophilin family members will ultimately facilitate rational drug design. However, the observed structural diversity, particularly around the S2 pocket and "gatekeeper" regions, presents an attractive strategy with respect to achieving isomerase isoform selectivity, although no tool/reference compounds exist to model this hypothesis *in vitro*.

Discovery and development of small molecule mPTP inhibitors: difficulties in achieving selectivity and understanding mechanism

Inhibitors of CypD

To date, multiple small molecule mPTP inhibitors have demonstrated efficacy in preclinical disease models. However, without complete understanding of the molecular properties, interpretation of pharmacological data is complex, owing to: (1) **polypharmacology** (see Glossary) of CsA and derivatives; (2) mechanistic liabilities of compounds that desensitise mPTP opening rather than causing true inhibition, and (3) unknown mechanism of action of CypD-independent inhibitors (as discussed below). Instead, *Ppif*^{-/-} (CypD ablation) models afford a cleaner system to corroborate a role for mPTP perturbation in disease. Most mPTP inhibitors target CypD, with the best characterised being CsA (Figure 2A; Table 1). CsA is a

hydrophobic undecapeptide and non-selective cyclophilin inhibitor and immunosuppressant, binding a highly conserved groove and occluding the cyclophilin PPIase active site [57, 58]. In pre-clinical models of mPTP opening, CsA demonstrates a narrow effective concentration range, with cardioprotection observed at 0.2 μ M and benefit lost at concentrations above 0.4 μ M following substrate-free anoxia studied and in *ex vivo* ‘Langendorff perfused’ hearts [59, 60]. This narrow therapeutic window often confounds pre-clinical studies and will complicate clinical use as these precise concentrations may be hard to achieve (Box 2). *N*-methyl-isoleucine-4-cyclosporin (NIM-811) and *N*-methyl-D-alanine-3-*N*-ethyl-valine-4-cyclosporin (Debio025/alispovir and previously known as Unil025) are non-immunosuppressive, semi-synthetic analogues of CsA which lack the calcineurin binding domain [61, 62]. Both are potent, non-selective cyclophilin inhibitors and have demonstrated activity in multiple *in vivo* models of myopathy, including dystrophin mutant *sapje* zebrafish [13], *ColVI* mutant zebrafish [63], myopathic *Col6a1*^{-/-}, dystrophic *mdx* mice [64-66] as well as *in vitro* patient muscle-derived cells [13].

Sanglifehrin A (SfA) is a large molecular weight natural product, isolated from *streptomyces* sp., and is also a non-selective inhibitor of cyclophilins (Figure 2B), binding both CypD and CypA, and has significant immunosuppressive activity [67]. SfA is structurally unrelated to CsA and but also binds to the isomerase cleft [67]. SfA, CsA and analogues are limited by poor physicochemical properties (aqueous solubility, high molecular weight), polypharmacology and cytotoxicity (most notably nephrotoxicity). All are large molecules (CsA = ~1200 Da), with poor **blood brain barrier** (BBB; see Glossary) permeability, restricting their utility as neuroprotective agents in CNS disease and their therapeutic development potential for treating primary mitochondrial diseases.

A number of groups have identified non-peptidic small molecule inhibitors of CypD [68-70] (Table 1). *In silico* modelling and *in vitro* surface plasmon resonance (SPR) identified C-9, a 4-aminobenzenesulfonamide derivative, as binding CypD with high **affinity** (see Glossary) [70, 71]. C-9 inhibits CypD PPIase activity and delays A β - and Ca²⁺-induced mitochondrial swelling and A β -induced neuronal cell death [71]. Similarly, Shore *et al.* developed urea-based small molecule, active-site binding CypD inhibitors [68]. Compound 19 protected isolated mouse pancreatic acinar cells from necrotic cell death induced by tauroolithocholate

acid 3-sulfate [68] and demonstrated cytoprotection in models of UV-induced retinal degeneration, reducing oxidative damage and cytochrome c release [72].

Park *et al.* undertook a novel approach to hit identification, having generated an energy-based pharmacophore map based on the co-crystal structure of the CypD-CsA complex [69]. Hits were optimised using *in silico* pharmacokinetic analysis and molecular docking, and following biological validation and SPR, 2 compounds were identified with a K_D in the nanomolar range, which may serve as lead molecules for further development [69]. Biological and technical limitations of the PPIase activity assay, including use of non-natural substrate, speed of reaction, high spontaneous substrate turnover and potential for identifying inhibitors of chymotrypsin inhibitors, make large scale screening challenging and necessitate that binding affinity and the functional consequences must be measured in order to assess activity, and PPIase assays must therefore be complemented by alternative approaches, including assessment of binding using SPR, fluorescence polarisation, homogeneous time resolved fluorescence (HTRF), isothermal calorimetry (ITC) . Furthermore, CypD inhibitors shift the threshold for mPTP opening to a higher Ca^{2+} load, but do not completely suppress PTP opening [27]. Therefore, the development of mPTP inhibitors with a mechanism distinct from CypD inhibition is attractive.

CypD-independent inhibitors of mPTP opening

The first identified CypD-independent inhibitor, TRO-40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-ol), binds to the cholesterol binding site of the 18 kDa outer mitochondrial membrane translocator (TSPO; Figure 1; Key Figure; Table 1). Schaller *et al.* demonstrated that the TSPO ligand TRO-40303 delays oxidative stress-induced mPTP opening *in vitro* and following *in vivo* reperfusion injury [73]. However, in experiments using conditional *Tspo* knockout mice, Šileikytė *et al.* concluded that the TSPO had no role in mPTP formation, regulation or mPTP-mediated cardiac cell death [74], casting doubt on the prospect for TSPO ligands as a therapeutic paradigm for mPTP modulators. However, despite these data, TRO-40303 progressed to Phase II clinical trial, and maybe predictably, yielded disappointing results (Box 2).

A number of other compounds, including derivatives of *N*-phenyl-benzamides, cinnamic anilides, isoxazoles, PP11 analogues and the small molecule ER-000444793 (Table 1), have

been identified as novel CypD-independent inhibitors of mPTP opening. (*E*)-3-(4-fluoro-3-hydroxy-phenyl)-*N*-naphthalen-1-yl-acrylamide, a cinnamic anilide, with potency greater than CsA, inhibits Ca²⁺-, oxidative stress- and chemical cross-linker-induced mPTP opening [75]. From the same cinnamic anilide series, GNX-4975 has been proposed to bind the interface and stabilise the interaction between PiC and c-conformation of ANT [76], one model hypothesis for mPTP formation and providing the first details on potential compound mechanism [77]. Notably, F is able to cross the blood-brain barrier and has demonstrated efficacy in neurodegenerative disease models, slowing disease progression and increasing lifespan in a mouse model of motor neurone disease (ALS) expressing the G37R-human mutant superoxide dismutase (SOD)-1 [78].

Screening of 363,827 molecules from the NIH Molecular Libraries Small Molecule Repository in a mitochondrial Ca²⁺-induced swelling assay identified the isoxazole chemotype as a potential 'hit'. **Structure activity relationship** (SAR; see Glossary) around the scaffold, identified *N*-(3-chloro-2-methylphenyl)-5-(4-fluoro-3-hydroxyphenyl)isoxazole-3-carboxamide (compound 60), with equipotent inhibition to CsA in models of Ca²⁺- and chemical-induced mPTP opening, cross-species activity and improvements in muscle function and structure in congenital muscular dystrophy associated with a collagen VI mutation in a zebrafish model [12]. The isoxazole ML 404 (CID 72199308) was also identified through the NIH Molecular Libraries Program as a highly potent inhibitor of Ca²⁺-induced mPTP opening [79]. More recently, 3-(benzyloxy)-5-chloro-*N*-(4-(piperidin-1-ylmethyl)phenyl)benzamide (Compound 4), was identified following SAR studies based on the a *N*-phenyl-benzamide scaffold [80]. Compound 4 delays Ca²⁺- and oxidative stress-induced mPTP opening in isolated mitochondria, has favourable physicochemical properties, and the potential for blood-brain barrier and CNS penetration, however no biological validation in cell or animal models have been reported [80]. Compound 4 appears to target directly the mPTP, since it equally inhibits pore opening induced by Ca²⁺ and redox-sensitive thiol oxidation in the matrix or membranes. Moreover, its inhibitory effect on respiration and toxicity at higher micromolar concentrations indicates that it might target the OXPHOS machinery, and since the effect of oligomycin is not additive to that of Compound 4, it hints to the involvement of the ATP synthase. Finally, *N*-(2-benzylphenyl)-2-oxo-1H-quinoline-4-carboxamide (ER-000444793) was identified from a screen of 50,000 compounds which

delayed Ca²⁺-induced mPTP opening, independent of CypD binding and PPLase inhibition (Table 1) [81]. No further information on the potential target of ER-000444793 is known yet, although its effect on ATP synthesis has been ruled out.

A number of alternative approaches for mPTP inhibition have been reported, probably acting via inhibiting pathways upstream to mPTP opening, including modulation of mitochondrial redox state [10, 82, 83], ETC function [84] and Ca²⁺ influx (REF). The most significant achievement to date is the approval of Edaravone (Radicut™) [10] (Box 2), a potent anti-oxidant and inhibitor of mPTP opening and having demonstrated protection in multiple models [85, 86]. The mitochondrial targeted antioxidant (AntiOxBEN₃), targeted to mitochondria by conjugation with triphenylphosphonium (TPP⁺), delayed Ca²⁺-induced mPTP opening and protected against iron- and hydrogen peroxide-induced cytotoxicity [82]. A small molecule CypD-independent mPTP inhibitor DS44170716 has been described, which apparently reduces mitochondrial Ca²⁺ uptake but this appears to result from a loss of mitochondrial membrane potential and inhibition of respiration, raising safety concerns and significantly reducing therapeutic potential [84]. DS16570511 has been proposed as a novel inhibitor of the MCU, potentially inhibiting mPTP opening via inhibition of Ca²⁺ uptake [84]. Finally, screening of the Hoffmann-La Roche compound collection identified Ro-68-3400 as a potent mPTP inhibitor and as binding to VDAC1 [87], however these data have been questioned [88].

Although the mechanism of action is yet to be fully elucidated, all compounds discussed here inhibit mPTP opening through mechanisms distinct from CypD inhibition, together avoiding off-target effects on other cyclophilins and immunomodulation. On-target toxicity is unknown given the absence of a biomolecular target, however many have demonstrated a robust safety profile *in vitro*. Notwithstanding the recent data supporting a role for F₁F₀-ATP synthase in mPTP formation, compound 60, PP11 analogues (compound 10) and ER-000444793 are free of potentially detrimental effects on ATP synthesis. Altogether, well-defined HTS campaigns and a sensible critical path for compound discovery and development have together identified a chemically diverse collection of tool/reference molecules that are not restricted by mechanistic limitations of CypD inhibition. These compounds will initially provide chemically useful pharmacophores and help in understanding the exact bio-molecular architecture and regulation of the mPTP complex.

Lead optimisation iterations may improve potency and pharmacokinetic properties and potentially begin to pave the way to achieving greater therapeutic efficacy.

Strategies for the discovery of small molecule mPTP inhibitors

Target Validation, hit identification and delineation of a screening flow

Target Validation (TV; see Glossary) is the initial and most important stage of the drug discovery process and aims to establish whether a biomolecule (protein, nucleic acid) is worthy of significant investment and development. A **high-throughput screen (HTS)** (see Glossary) may or may not form part of initial TV. However, establishment and optimisation of a HTS compatible screening system as well as delineation and establishment of downstream/orthogonal **bioassays** (see Glossary) comprising the screening flow and critical path for hit identification are essential for any small molecule screening campaign (Figure 2C). The complex and uncertain nature of the mPTP complex restricts target-based screening strategies, as beyond CypD, there is still an uncertainty about ‘target’ biomolecules. Instead, phenotypic screening methodologies, using system-based bioassays must be adopted. Phenotypic screening is mechanism of action- and target-agnostic and instead looks to identify small molecule or biological modifiers of a cellular process. The choice of primary screening bioassay is determined by quality (reproducibility, variability and **assay window** (see glossary)) and can be calculated using a simple statistical measure, the **Z-prime score** (see Glossary) [89].

A trio of system-based bioassays, each assessing Ca²⁺-induced mPTP opening in isolated rodent liver mitochondria comprise the most widely used phenotypic screening strategy to address mPTP opening (Figure 2D-F). Delay in mPTP opening defines an active molecule, or ‘hit’ compound, however, compound-mediated depolarisation of the mitochondrial inner membrane, inhibition of the **mitochondrial Ca²⁺ uniporter** (MCU; see Glossary) or of the phosphate carrier (PiC) would each prevent electrophoretic Ca²⁺ uptake and Ca²⁺ sequestration, producing data consistent with inhibition of mitochondrial Ca²⁺-induced swelling (Box 3). To eliminate false positives, compounds are ideally profiled across three mPTP system-based assays in parallel or sequentially (Figure 2D-F and Box 3), and the data used to define the mechanism and these strategies have been used successfully to identify mPTP inhibitors, such as ER-000444973 [81]. Furthermore, together or individually defined

bioassays are used to delineate pharmacology, guide SAR and confirm cross species activity (using mitochondria isolated from human cell lines) (Figure X and Box 3).

Challenges in discovering and developing mPTP inhibitors

Pre-clinical translation

Lack of efficacy is the most significant reason for clinical failure of a candidate molecule, potentially stemming from poor translation of pre-clinical conclusions. A fundamental determinant for a successful phenotypic screen is the degree to which the bioassay reflects, and is able to predict, success in modulating the (patho-)physiological end-point of interest. Moffat *et al*, termed 'chain of translatability' to describe a shared mechanistic basis for the bioassay end-point and disease phenotype, qualities which are necessary if they are to have robust predictive power [90]. Given disappointing outcomes from CsA clinical trials (Box 2), pre-clinical models of reperfusion injury have failed to translate [91], leaving the problem of how to assess the clinical utility of mPTP inhibitors. Phenotypic screening approaches used for small molecule mPTP modulators are; (1) outside of the cellular context (i.e. in isolated mitochondria) and (2) do not reflect mechanisms underlying disease pathophysiology (i.e. not causal), therefore together decreasing the probability of successful translation. Given these observations, it will be necessary to have robust secondary bioassays to profile compounds in models with physiological relevance. E.g. an intact cell based assay, allowing specific activation of the mPTP, could further increase the breadth of available compounds acting not only the mPTP but also on its consequences on cell survival, a further step for translatability. Although a variety of cell based mPTP assays have been proposed, the lack of their robustness and specificity so far impeded their routine use for large scale compound screens. Furthermore, target identification following a phenotypic screen is challenging and hence, without clear target deconvolution it is difficult to predict tissue specific activity (based on protein expression) and safety (based on protein function). Further studies on putative pore components are needed in order to introduce single protein target based approaches above CypD.

Physiological function of the mPTP

Although mPTP opening is well established as a mechanism of cell death, transient, reversible opening has been linked to numerous physiological processes, including: (1) energy metabolism, (2) Ca^{2+} and redox signalling and (3) development [92-94]. The ATP synthasome, composed of F_1F_0 -ATP synthase, PiC and ANT, is an hetero-oligomeric, mitochondrial inner membrane super-assembly [95]. Evidence supports a role for each element of the complex in the formation or regulation of pore opening [18, 25, 26] and CypD binding [15, 16, 96]. Therefore it is plausible that physical and functional coupling of mPTP components may regulate mitochondrial metabolism [92] and observations suggest that CypD may control F_1F_0 -ATP synthase plasticity; its ability to oligomerise into synthasomes and to respond appropriately to bioenergetic demand [97].

Mitochondria are a central hub for cellular Ca^{2+} homeostasis [98] and take up Ca^{2+} from the cytosol during physiological Ca^{2+} signalling. It has been suggested that low-conductance mPTP opening and partial transmembrane depolarisation may contribute to mitochondrial Ca^{2+} efflux [93], although these observations have recently been questioned [99]. Furthermore, CypD ablation increases mitochondrial matrix Ca^{2+} , reprogramming cardiac metabolism, consistent with increased activity of Ca^{2+} -dependent dehydrogenases of the Krebs cycle [100].

During development, myocyte differentiation and maturation of mitochondrial structure and function are favored by mPTP closure. Indeed, myocyte differentiation can be pharmacologically and genetically accelerated through mPTP inhibition via regulation of ROS [94]. The physiological role for mPTP opening requires further investigation. However emerging data suggests that therapeutic targeting mPTP opening has to be carefully considered given accumulating evidence of a role in physiology (Box 1).

Opportunities for achieving selectivity and efficacy of mPTP inhibitors

To achieve selectivity for CypD, over distinct cyclophilins, Malouitre *et al.* took advantage of the fact that CypD is the only mitochondrially localised cyclophilin [101]. Mitochondrial targeting of CsA was achieved by conjugation of a lipophilic TPP⁺ cation at the sarcosine-3 position of the CsA ring. Theoretically, accumulation of a compound into mitochondria in

response to the transmembrane potential is expected to increase matrix concentration of the compound by up to a thousand-fold, reducing the dose required to reach the target and so reducing off-target effects. Indeed, transmembrane potential-driven electrophoretic uptake and matrix accumulation of mitochondrially-targeted CsA (mtCsA) decreased calcineurin binding demonstrated good selectivity for CypD over CypA and induced protection in a model of oxygen and glucose deprivation-induced necrosis in hippocampal neurones [57, 101, 102], suggesting pharmacological activity remains following substitution.

JW47 is a next generation mitochondrially targeted CsA and features a quinolinium cation in the place of TPP⁺, removing TPP⁺-induced toxicity and physicochemical restrictions. JW47 demonstrates less immunosuppressive activity compared to CsA and neuroprotection in a relapsing-progressive EAE model of MS [103]. *In vivo* efficacy of JW47 provides proof-of-principal for the selective targeting of CypD and use of a quinolinium cation as a mitochondrially targeting group. However, to date achieving small molecule, non-artificially targeted selectivity for CypD remains elusive.

Numerous novel biomolecule-targeting modalities have been described which may have application and benefit in achieving cyclophilin specificity and potency. PROteolysis TArgeting Chimeras (PROTACs) are bi-functional molecules that simultaneously recruit an E3-ubiquitin ligase and bind a target protein. PROTACs are composed of 2 distinct ligands, one to bind the target protein and one to recruit E3-ubiquitin ligase, separated by a linker. Forced proximity between the E3-ligase and exposed lysine residues on the bound target protein, drives ubiquitination and proteasomal targeting for degradation [104]. PROTACs are required only to bind the target molecule and need not inhibit the active site or prevent protein-protein interactions, providing an advantage of traditional small molecules, however may have limitations with respect to CNS penetration. Identification of target-binding moieties has been aided by development of **DNA encoded libraries** (see Glossary), hugely increasing chemical diversity and aiding ligand identification. Significant work has been done since conception, including applying click chemistry to improve cellular permeability, solubility and pharmacokinetics (CLIPTACs) [105]. It is now conceivable that targeted protein degradation may offer a novel strategy to target traditionally un-druggable proteins and limit protein aggregation.

RNA targeted therapeutics describes the use of oligonucleotides to modulate target biomolecule expression [106]. One example, single-stranded anti-sense oligonucleotides (ASOs) are chemically modified oligonucleotides of 18-20 nucleotides, which bind their cognate RNA sequence through Watson-Crick hybridization. Occupancy only- (inhibition of translation, steric interference) or occupancy-mediated degradation- (RNase H1 degradation of ASO-RNA complex) mechanisms reduce target protein expression and the technology is now optimised with improve stability, delivery and affinity [106]. Much like PROTACs, substantial target specificity can be achieved, as ASOs can be designed to regions of non-sequence homology. PROTACs and ASOs may provide opportunities to drug the undruggable, increasing specificity, reducing off-target effects and poly-pharmacology associated with traditional small molecules and potentially provides mechanisms to achieve cyclophilin specificity. While CypD is the only unambiguous mPTP regulator, these approaches will undoubtable have utility once further regulators or pore forming units are identified.

Conclusions and Future perspectives

Despite lack of supporting genetic data linking *Ppif* perturbation with disease, evidence for a role of the mPTP as a final common path to cell death in a number of major human diseases is extremely compelling [5-9]. Loss-of-function of CypD is likely beneficial and so unlikely to be identified from genome wide studies. mPTP appears to play a major part in the pathophysiology of diseases for which current therapeutic approaches are very limited and inadequate – MS, AD, PD, stroke, ischaemia-reperfusion injury in the heart, brain and kidney and hereditary neuromuscular diseases of childhood together representing an astonishing human and financial burden. It surely follows inevitably that finding a pharmacological solution could have an astonishing impact on healthcare and fulfil a huge unmet clinical need. These considerations provide very strong strategic and commercial arguments to devote significant effort in the search for an effective pharmaceutical solution to target the pore. That this has proven so difficult remains a puzzle, as does the difficulty in establishing the molecular identity of the pore itself (see Outstanding Questions), but both are hopefully just a matter of time.

Disclosures

T.B is an employee of Eisai Ltd.

Text Boxes

Box 1: Cyclophilin D: the only accepted positive regulator of mPTP opening

An abundance of pharmacological and genetic evidence recognises cyclophilin D (CypD) as the only, universally accepted protein modifier and key positive regulator of mPTP opening [27]. CypD (encoded by the *Ppif* gene), a mitochondrial matrix localised 21kDa cyclophilin, catalyses the *cis-trans* isomerisation of peptidyl-prolyl bonds in target proteins [107]. CypD PPIase enzymatic activity is necessary for positive regulation of pore opening [27], abolition of which affords the mechanism for CsA-mediated mPTP inhibition [4]. Genetic or pharmacological ablation of CypD increases the threshold for Ca^{2+} - and ROS-induced mPTP opening [27, 28]. However, at Ca^{2+} concentrations greater than the physiological threshold, despite CypD inhibition, pore opening can still proceed, suggesting a de-sensitising, regulatory role of CypD and not that of a structural component.

CypD has been observed to bind multiple putative mPTP components, including VDAC and ANT [15, 108], PiC [16] and SPG7 [17]. Furthermore, regulatory, non-structural interactions implicate CypD as a signalling hub between multiple functionally distinct proteins and potential mPTP modulators, including p53 [109], complement C1Q binding protein (C1QBP) [110], amyloid beta ($A\beta$) [9] and mitochondrial chaperones [54]. Furthermore, CypD has been identified in mitochondrial-associated membrane (MAM) fractions, within a VDAC1/Grp75/IP3R complex [111] (Figure 1; Key Figure). Recent models suggest a functional interaction between CypD and the lateral stalk of the F_1F_0 -ATP synthase, whereby CypD binding decreases enzymatic activity of the ATP synthase complex, in an event favoured by inorganic phosphate (Pi) and inhibited by CsA [96]. The oligomycin-sensitivity conferring protein (OSCP) subunit of the F_1F_0 -ATP synthase complex has been proposed as the CypD binding site [18, 112], however these data have recently been challenged [22]. CypD is also post-translationally modified, with *S*-nitrosylation [113], phosphorylation [114] and acetylation [115] having been proposed to modulate CypD activity. Global knockout of the mitochondrial Ca^{2+} uniporter (MCU), increases the phosphorylation of CypD and its

association with the F₁F₀-ATP synthase, reducing Ca²⁺ retention capacity and contributing to the lack of cardioprotection observed following ischemia-reperfusion injury [116]. The complex interactome and post-translational modifications (PTMs) of CypD necessitate further studies, however given the clear relationship between CypD ablation and increased tolerance to mitochondrial stressors, CypD modulation, either via direct inhibition or regulating protein-protein interaction and PTMs represents a valid therapeutic strategy.

Box 2: mPTP inhibitors in the clinic

Inconsistencies in CsA trials: A number of small clinical studies have explored the efficacy of CsA in improving cardiac outcomes following myocardial infarction-induced reperfusion injury. The first, small pilot phase II study and limited follow up in patients randomised to CsA treatment at time of PCI, identified significantly reduced infarct size during and improvements in cardiac functions [117, 118]. A number of small trials since, have also identified benefit in CsA treatment in post-operative myocardial injury [119, 120], however others concluded there are no clinical benefits of pre-thrombolytic CsA administration [121]. A larger phase II open label study, the CYCLE (CYClosporinE A in Reperfused Acute Myocardial Infarction) trial, also revealed no significant changes in primary or biochemical secondary end-points following CsA treatment up to 6 months follow up [122]. Larger phase III trials, such as CIRCUS (Cyclosporine to ImpRove Clinical oUtcome in STEMI patients) observed no significant difference in primary or secondary end-points, including all-cause mortality, ST elevation and infarct size [91], although long coronary occlusion times, high blood glucose at admission, and pharmacological limitations associated with CsA may go some way to explaining clinical failings [123]. However consistent with previous studies, nor could CsA reduce acute renal injury following CABG surgery in the CiPRICS (Cyclosporine to Protect Renal Function in Cardiac Surgery) study [124].

The disappointment of TRO40303: The MITOCARE study demonstrated no effect of TRO400303 in limiting myocardial injury following reperfusion between treatment and placebo [125] or effecting pro-inflammatory cytokines at 72 hours following PCI [126]. These data may have been predicted given data dismissing a role for TSPO in mPTP formation [74]. The history of mPTP inhibitors in the clinic has been disappointing.

Inconsistencies in trial design, notably the point of compound administration, indication, pharmacokinetic and pharmacodynamic properties polypharmacology of CsA and poor understanding of TSPO biology, may potentially reconcile heterogeneity in clinical measures [127, 128].

Clinical success of edaravone: The first phase III studies edaravone demonstrated reduction of revised ALS functional rating scale (ALSFRS-R) over placebo group and efficacy in ALS, however only in early-stage disease [10]. These data were confirmed in a second randomized, double-blinded, placebo-controlled trial [129]. Based on these successful outcomes in 2015 edaravone was approved for ALS in Japan in 2015. Indeed, the success of edaravone and data linking persistent mPTP opening as a key pathophysiological mechanism in cell death across multiple disease states, including neurodegenerative diseases [9, 34, 69, 78, 103] and muscular dystrophies [7, 11, 130], opens the door to targeting distinct clinical indications.

Box 3: Assessment of mPTP opening *in vitro* using isolated mitochondria

1. **mPTP detection by measurement of mitochondrial transmembrane potential:** The mitochondrial transmembrane potential is the electric component of the proton motive force [131], measured as voltage differential between the net positively charged, acidic mitochondrial matrix and the inter membrane space (IMS). Serial reduction of complexes I, III and IV of the electron transport chain (ETC), generates energy to pump protons from the matrix, across the inner mitochondrial membrane, to the IMS, generating an electrochemical gradient. Protons pass back down this gradient, through the F_1F_0 -ATP synthase (complex V), in a mechanism coupled to ADP phosphorylation.

Tetramethylrhodamine methylester (TMRM) is a fluorescent, cationic and lipophilic dye. TMRM, according to the negative potential of the IMS, is partitioned largely to the mitochondrial matrix, due its positive charge. Mitochondrial accumulation is strictly dependent on mitochondrial inner membrane potential and at low concentration ($[TMRM]_{extracellular} = 1-20 \text{ nM}$), generates fluorescence proportional to the voltage gradient across the IMM [131]. Under these conditions (called TMRM redistribution mode), loss of inner membrane potential, as a

consequence of ETC inhibition, ETC uncoupling or decreased supply of reducing equivalents, yields a decrease in fluorescence (Figure IA). At high concentration, ($[TMRM]_{extracellular} \geq 100 \text{ nM}$; determined empirically for different cell types), fluorescence of mitochondrially localised dye is quenched. Under these conditions, any disruptions to the transmembrane potential yields an increase in fluorescence as quenching is relieved as dye escapes the mitochondria (Figure IB), a process known as de-quenching [132]. Thus, using TMRM redistribution, mPTP will be detected as loss of mitochondrial TMRM fluorescence, while in de-quenching mode a transient increase of TMRM fluorescence indicated mPTP formation.

2. ***In vitro* assessment of mPTP opening using isolated mitochondria:** Mitochondrial Ca^{2+} uptake is transmembrane potential dependent, and under physiological conditions mitochondria can accumulate a large amount of Ca^{2+} without exceeding the threshold for mPTP opening, due to Ca^{2+} buffering in the matrix [133]. At extremes of cellular Ca^{2+} increases, mitochondrial buffering capacity is exceeded and the mPTP opens.
 - a. *Ca²⁺-induced mitochondrial swelling:* Following mPTP opening, mitochondria become swollen due to disruptions in ionic and solute balance. Swollen mitochondria show reduced absorbance at 520-540 nm as measured by spectrometry. mPTP inhibitors delay the observed decrease in absorbance.
 - b. *Mitochondrial Ca²⁺ retention capacity:* Electrophoretic mitochondrial Ca^{2+} uptake is mediated through the mitochondrial calcium uniporter (MCU). Repeated addition of small concentrations of Ca^{2+} to a mitochondrial suspension containing a mitochondrially impermeable, low affinity Ca^{2+} dye (i.e. Fluo-4FF pentapotassium salt) provides a sensitive measure of mitochondrial Ca^{2+} uptake and propensity to undergo mPTP opening. mPTP inhibitors allow mitochondria to retain greater amount of Ca^{2+} .
 - c. *Ca²⁺-induced transmembrane depolarisation:* Mitochondrial Ca^{2+} uptake is transmembrane potential dependent. Excess mitochondrial matrix Ca^{2+} disrupts the ionic gradient and causes mPTP opening and mitochondrial transmembrane depolarisation. Ca^{2+} -induced redistribution or de-quenching of TMRM is measured using a fluorescence spectrometry. mPTP inhibitors

delay the Ca²⁺-induced changes in TMRM fluorescence. Furthermore, measurement of TMRM fluorescence prior to Ca²⁺ addition identifies compound-induced mitochondrial toxicity.

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Figure Legends

Figure 1; Key Figure: Physiological and pharmacological regulation of the mPTP complex.

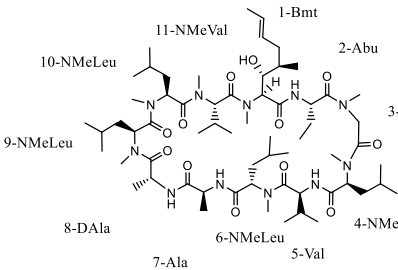
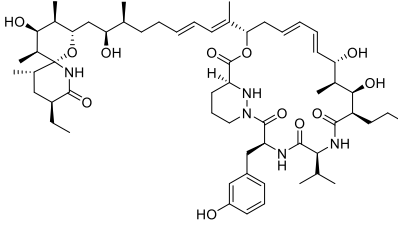
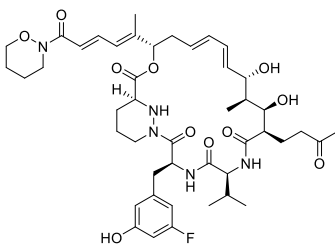
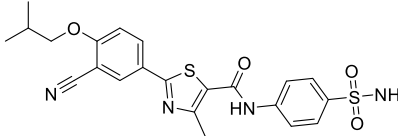
The mPTP is a proteinaceous complex, of unresolved bio-architecture. Although high matrix Ca^{2+} is the principal triggering event for mPTP opening, a number of physiological factors and pharmacological agents can influence probability of pore opening. In addition to extremes of Ca^{2+} , mPTP opening is further shaped by the cellular environment; low pH, adenine nucleotides and Mg^{2+} negatively regulate pore opening and conversely Ca^{2+} overload, adenine nucleotide depletion and reactive oxygen species (ROS) promote channel opening [6, 134, 135]. Small molecule inhibitors have been described as having both CypD-dependent and –independent mechanisms. Abbreviations: $\text{A}\beta$; amyloid beta, C1QBP; complement C1q binding protein, ETC; electron transport chain, MCU; mitochondrial Ca^{2+} uniporter

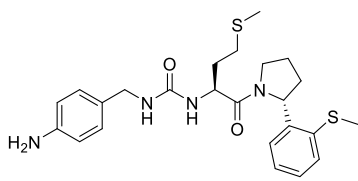
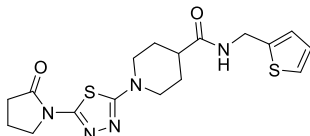
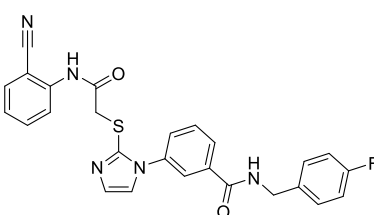
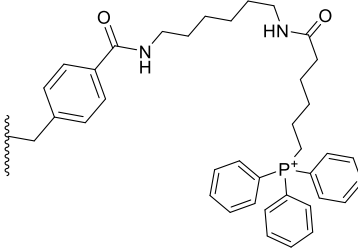
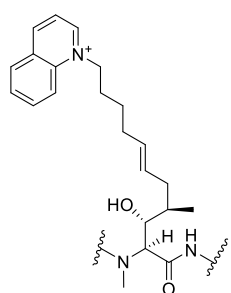
(Box 2) Figure I: Measurement of mitochondrial transmembrane potential using TMRM. (A) At low concentration, transmembrane potential depolarisation results in TMRM re-distribution. (B) At high concentration, transmembrane potential depolarisation results in relief of dye quenching and increased fluorescence. To ensure correct interpretation and meaningful data, careful consideration of experimental hypothesis and bioassay conditions is necessary to ensure correct use of membrane potential dyes.

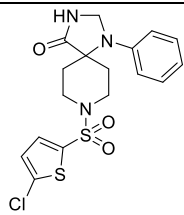
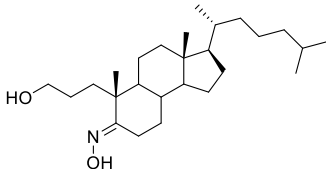
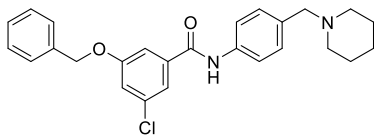
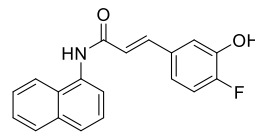
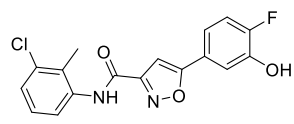
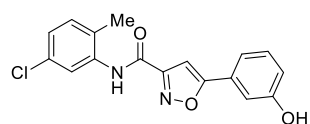
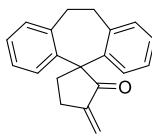
Table 1: Phenotype of *Ppif*^{-/-} in disease models

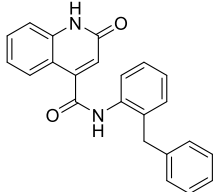
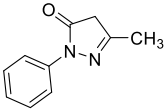
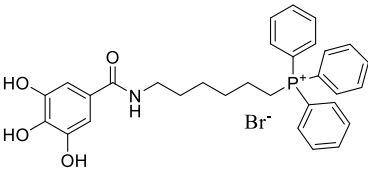
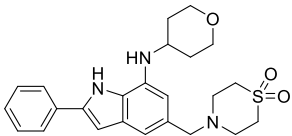
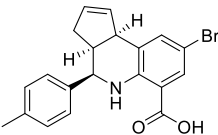
Disease model	Phenotype of <i>Ppif</i> ^{-/-}	Reference
Aging	(1) ↓ F ₁ F ₀ -ATP synthase enzymatic decline (2) ↓ F ₁ F ₀ -ATP synthase uncoupling (3) maintained respiratory control ratio (RCR)	[136]
Aging; osteoporosis	(1) normal/rescued trabecular bone volume and cortical thickness (2) resistance against age-related bone loss	[42]
Amyotrophic lateral sclerosis; mSOD1-G93A	(1) ↑ survival (2) delayed onset of disease	[43]
Alzheimer's disease; mutant amyloid precursor protein (APP)	(1) ↑ spatial learning and memory	[34]
Enteropathy; diclofenac-induced	(1) ↓ mucosal injury; (2) normal/rescued serum alkaline phosphatase	[41]
Multiple sclerosis; experimental allergic encephalomyelitis (EAE)	(1) Clinical improvement [↓ EAE score]; (2) axonal preservation	[30]
Muscular dystrophy; collagen VI deficiency	(1) ↓ myofibre degeneration; (2) ↑ mitochondrial function; (3) normalized apoptosis; (4) ↓ permeability of skeletal muscle fibres	[33]
Muscular dystrophy; laminin-2 deficiency	(1) ↑ survival; (2) ↑ life span; (3) ↑ total fibre number; (4) ↑ body weight	[31]
Muscular dystrophy; sarcoglycan deficiency	(1) ↓ myofibre degeneration (2); ↓ cardiac fibrosis; (3) normalised muscle mass; (4) ↓ inflammation	[31]
Myocardial infarction: ischaemia-reperfusion injury	(1) ↓ infarct size; (2) ↓ LDH release	[27, 28]
Renal ischaemia	(1) ↓ tubular cell necrosis; (2) ↓ tubular dilatation; (3) ↓ erythrocyte congestion	[32]
Stroke: acute middle cerebral artery occlusion and reperfusion	(1) neuroprotection; (2) ↓ infarct size	[29]

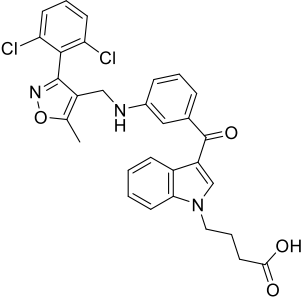
Table 2: mPTP inhibitors, including macrocyclic CypD inhibitors, small molecule CypD inhibitors and small molecule inhibitors of the mPTP working by non-CypD mechanisms. ND: not disclosed, ETC: electron transport chain.

Compound Name	Chemical class	Structure	Target	Comment	Reference
Cyclosporin A	Natural product		CypD	Failed Phase III clinical trial in cardiac reperfusion injury,	[4]
NIM-811	CsA-derivative	4-NMelleCsA	CypD	Equipotent to CsA	[61]
Debio025	CsA-derivative	3-NMeDAla,4-NEtValCsA	CypD	Greater potency than CsA in swelling assay (10-fold)	[62]
Sanglifehrin A	Natural product		CypD	PPlase inhibition $K_i = 2.2$, equipotent to CsA	[67]
NVP019	Sanglifehrin analogue		CypD	Structure from Neurovive presentation	[137]
C-9	4-amino benzene sulfonamide		CypD	PPlase, binding	[71]

Compound 19	Urea-based		CypD		[68]
Compound 7 Chem Div ID: E017-0165	Thiadiazole		CypD	Very low Rmax reported for SPR binding data	[69]
Compound 29 Chem Div ID: F985-0666	Imidazole		CypD	Very low Rmax reported for SPR binding data	[69]
mtCsA	Mitochondrially targeted CsA. 3-Sar substituted		CypD		[101]
JW47	Mitochondrially targeted CsA, 1-Bmt substituted		CypD	Protective in relapsing-progressive EAE model of MS	[103]
Compound 10	1,3,8-triazaspiro[ATP synthase c subunit	oligomycin/PP11 analogues target c subunit of ATP synthase	[138]

	4.5]decane				
TRO-40303	Steroid analogue		TSPO(+)	Failed to limit myocardial injury following reperfusion in Phase II study	[73]
Compound 4	N-phenylbenzamide		ND		[80]
Compound 22	Cinnamic anilide		ND		[75]
GNX-4975	Cinnamic anilide	Structure unclear from publication but belongs to same class as compound 22 above (maybe identical).	Stabilisation of PiC and c-ANT interaction	2 fold increase in lifespan G37R-human mSOD-1 mouse	[76, 78]
N-(3-chloro-2-methylphenyl)-5-(4-fluoro-3-hydroxyphenyl)isoxazole-3-carboxamide	Isoxazole		ND	Improved muscle function and structure in mutant collagen VI zebrafish	[12]
ML404 (CID 72199308)	Isoxazole		ND	Similar to compounds disclosed in [12]	[79]
Ro-68-3400	Dihydrospiro[cyclopentane-		VDAC1		[87]

	1,5'-dibenzo[a,d][7]annulen-2-one				
ER-000444793	Quinolone		ND		[81]
Edaravone (Radicut™)	dihydropyrazol-3-one		Anti-oxidant	Enol form linked to activity, approved for ALS	[85, 86]
AntiOxBEN ₃	TPP-linked antioxidant		Anti-oxidant		[82]
NecX, NecroX-7,	7-aminobenzimidazole		Potent reactive oxygen scavenger		[83]
DS44170716	Tetrahydroquinoline	 Plus enantiomer	ETC inhibitor		[84]
DS16570511	Substituted 1H-indol-1-yl)but		MCU inhibitor		[139]

	anoic acid				
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Glossary

Affinity: Ability of a ligand to bind a biomolecule.

Assay window: Scale of the change in an assay read-out defined by the positive and negative controls (i.e. difference between the mitochondrial calcium uptake capacity in the presence of DMSO (negative control) and CsA (positive control)).

Blood brain barrier; BBB: selectively permeable biological membrane separating the central and peripheral systems. BBB is an endothelial cell layer surrounding microvasculature that prevents the movement of molecules from the blood to the brain, protecting the central nervous system from chemicals and micro-organisms.

Bioassay: analytical method designed and optimised to test a biological hypothesis.

DNA-encoded library: a mechanism to massively increase scale of chemical matter screened against a drug target. Compounds are covalently bound to short unique DNA oligonucleotides which are then screened against an immobile protein target. Bound compounds are DNA sequenced allowing elucidation of chemical structure.

Efficacy: ability of a ligand to induce anticipated biological effect.

HTS; High throughput screen: process in drug discovery, often utilising automation and robotics, to simultaneously screen thousands of compounds against a target biomolecule to induce anticipated effect in selected bioassay.

Ischaemia-reperfusion injury: pathology caused subsequent to vascular reperfusion following a period of ischaemia. Reperfusion triggers a cascade of pathophysiological signalling, neutralising acidic pH, restoring mitochondrial transmembrane potential and

triggering mitochondrial Ca²⁺ uptake and ATP synthesis. Together, mitochondrial Ca²⁺ overloading, adenine nucleotide depletion and ROS generation, create the perfect environment for mPTP opening and necrotic cell death.

MCU complex; mitochondrial calcium uniporter complex: protein complex regulating cellular Ca²⁺ homeostasis and dynamics. The MCU complex comprises a low affinity Ca²⁺ inner mitochondrial membrane transmembrane channel, the MCU, and regulators MICU1 and MICU2, which together facilitate the electrophoretic movement of Ca²⁺ from the cytoplasm to the mitochondrial matrix.

Pharmacodynamics: study of ‘how a drug acts on an organism’ and aims to understand how the biochemical and physiological effects are associated with compound structure.

Pharmacokinetics: study of ‘how an organism acts on a drug’, typically divided in to understanding drug absorption, distribution, metabolism within the body and excretion and clearance from the body.

Polypharmacology: activity of compounds against multiple biomolecules.

SAR; Structure-activity relationship: Relationship between chemical structure and biological activity. SAR is an iterative cycle of analysis of biological activity following modifications to chemical groups, to allow identification of chemical space responsible for eliciting desired biological effect.

Target Validation (TV): TV necessitates the identification, evaluation and tractability assessment of a ‘target’ biomolecule. Human biology, genetics, protein expression and biochemical evidence all inform on biomolecule identification. In-house confirmation of literature data and assessment of available reference/tool compounds in disease-relevant **bioassays** (see Glossary) will confirm tractability and the relationship with a disease phenotype, together contributing to achieving TV.

Z-Prime score: Measure of assay quality using the equation: $Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{\mu_p - \mu_n}$, taking into account the standard deviation (σ) and means (μ) of the positive (p) and negative (n) controls. A higher Z-prime score suggests greater assay quality.

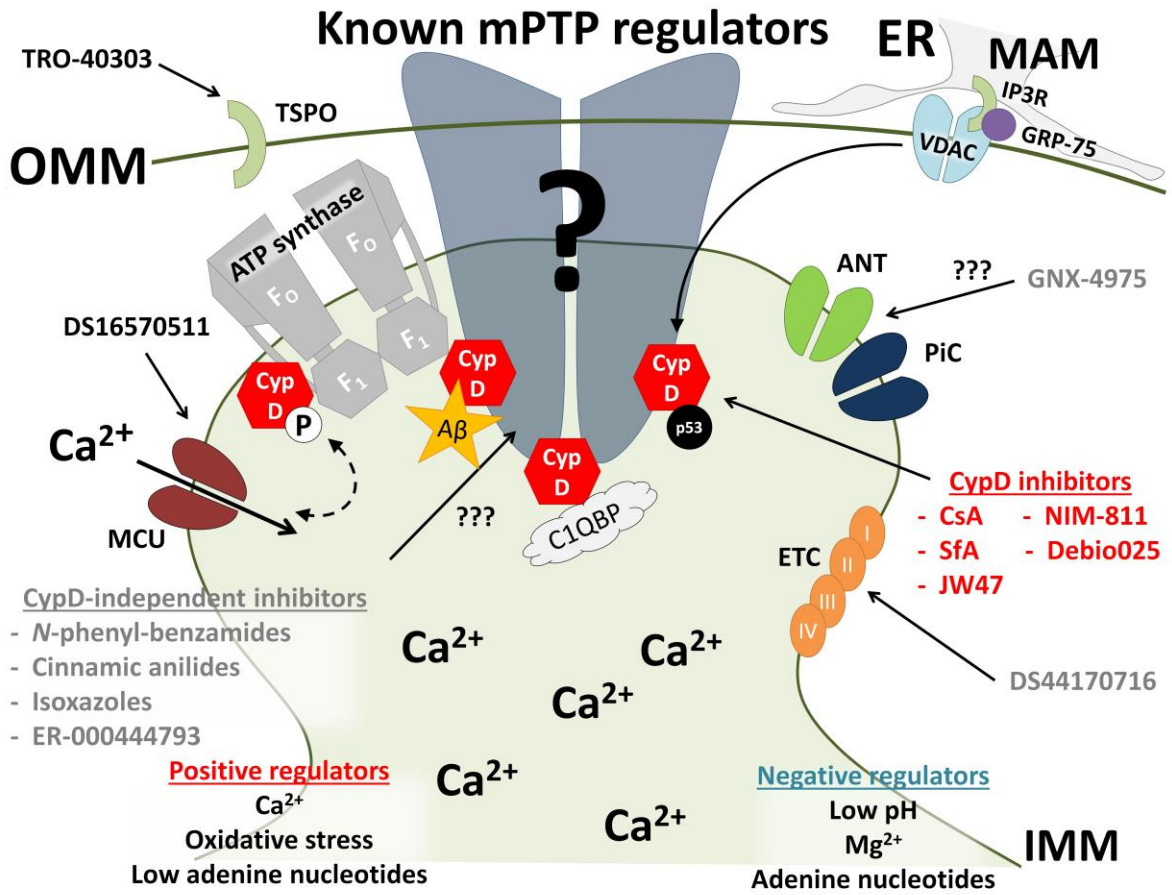
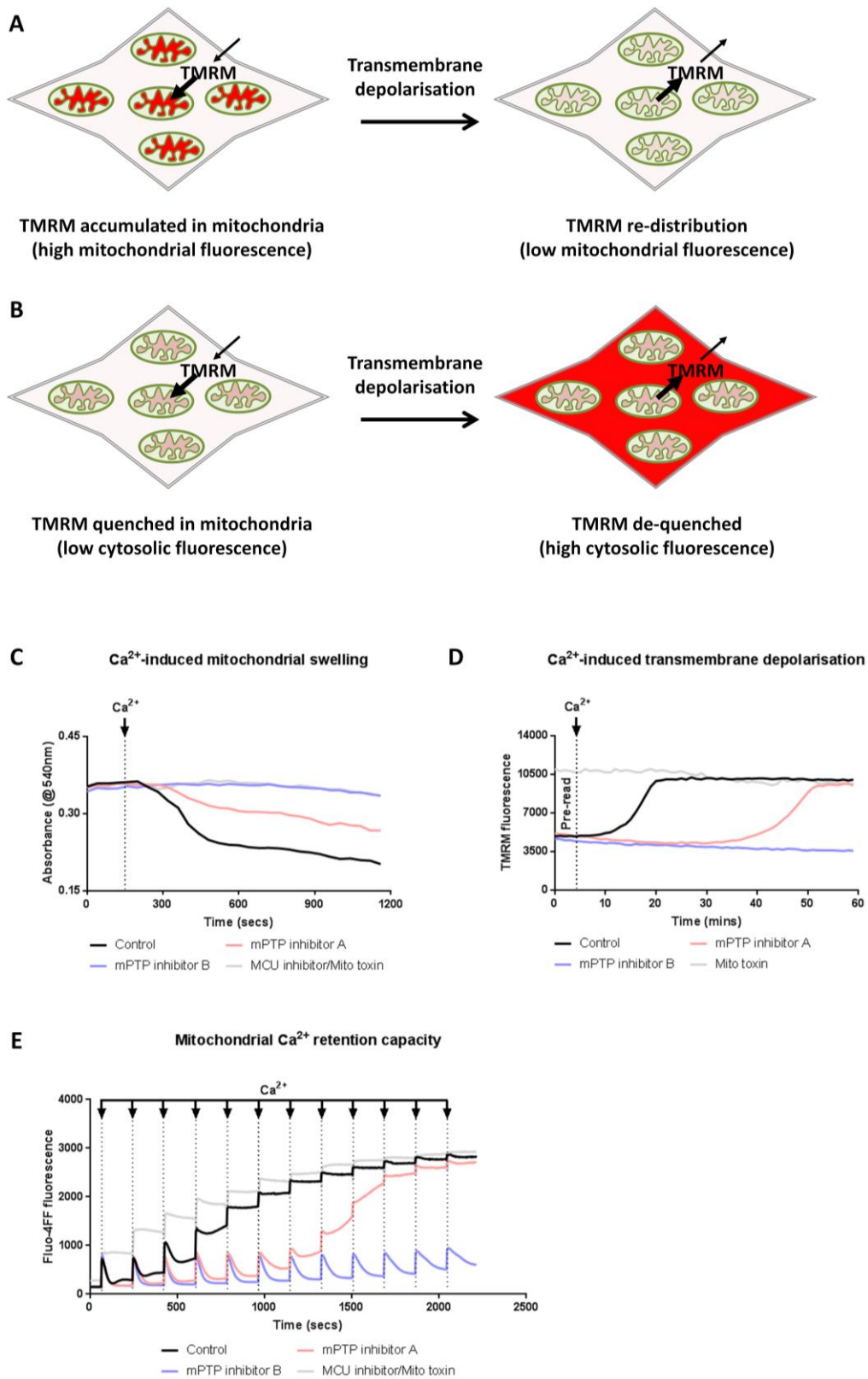


Figure 1. Key Figure



(Box 2) Figure II: