A mitochondrial-targeted cyclosporin A with high binding affinity for cyclophilin D yields improved cytoprotection of cardiomyocytes

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Mitochondrial CyP-D (cyclophilin-D) catalyses formation of the PT (permeability transition) pore, a key lesion in the pathogenesis of I/R (ischaemia/reperfusion) injury. There is evidence [Malouitre, Dube, Selwood and Crompton (2010) Biochem. J. 425, 137–148] that cytoprotection by the CyP inhibitor CsA (cyclosporin A) is improved by selective targeting to mitochondria. To investigate this further, we have developed an improved mtCsA (mitochondrial-targeted CsA) by modifying the spacer linking the CsA to the TPP+ (triphenylphosphonium) (mitochondrial-targeting) cation. The new mtCsA exhibits an 18-fold increase in binding affinity for CyP-D over the prototype and a 12-fold increase in potency of inhibition of the PT in isolated mitochondria, owing to a marked decrease in non-specific binding. The cytoprotective capacity was assessed in isolated rat cardiomyocytes subjected to transient glucose and oxygen deprivation (pseudo-I/R). The new mtCsA was maximally effective at lower concentrations than CsA (3–15 nM compared with 50–100 nM) and yielded improved cytoprotection for up to 3 h following the pseudo-ischaemic insult (near complete compared with 40%). These data indicate the potential value of selective CyP-D inhibition in cytoprotection.

Key words: cyclophilin D (CyP-D), cyclosporin, ischaemia, mitochondrial targeting, reperfusion injury (RI), triphenylphosphonium (TPP+).

INTRODUCTION

Myocardial ischaemia is a major cause of death and disability. Minimizing tissue injury requires early restoration of blood flow. After prolonged ischaemia, however, still surviving cells become adversely affected by restored blood supply and succumb on reperfusion. RI (reperfusion injury) is a complicating factor (angioplasty, thrombolysis and bypass surgery) and in organ transplantation.

Considerable evidence implicates the PT (permeability transition) pore model of necrotic cell death [1,2] in RI. The non-selective PT pore forms in the mitochondrial inner membrane under the synergistic influence of high Ca2+ concentration [3], oxidative stress [1] and depleted adenine nucleotides [2], factors common to RI. The resulting collapse of the proton electrochemical gradient allows the ATP synthase to operate in reverse, catalysing rapid ATP hydrolysis [1] and leading to irreversible cell injury. The molecular identity of the PT pore is controversial. Reconstitution studies indicate that the pore forms from a malfunctioning adenine nucleotide translocase [4–6], but additional, or alternative, transport proteins, e.g. the phosphate carrier [7], have also been considered. It is generally agreed, however, that PT pore formation is catalysed or stabilized by CyP (cyclophilin)-D, an intra-mitochondrial peptidylprolyl cis–trans-isomerase. CyP-D is regulated by acetylation, which potentiates its binding to the adenine nucleotide translocase and lowers the Ca2+ threshold for PT pore formation [8]. The CyP inhibitor CsA (cyclosporin A) blocks pore formation and gives partial protection in isolated cardiomyocyte [9], perfused heart [10] and mouse [11] models of RI. PT pore opening is promoted by CyP-D overexpression [12] and is attenuated by CyP-D knockout [13]. Genetic ablation of CyP-D reduces infarct size in heart [14] and other tissues (brain [15] and kidney [16]), and is also protective in models of degenerative diseases, i.e. Alzheimer’s disease [17], multiple sclerosis [18], muscular dystrophy [19] and motor neuron disease [20].

Thus the PT pore appears to be an intrinsic feature of RI and many other diseases leading to lethal cell injury, and, in controlling PT pore formation, CyP-D offers a potential target for pharmacological intervention. However, CsA may exert variable effects on cell viability owing to its interaction with cytosolic CyP-A and other non-mitochondrial CyPs. Recently, we have shown that targeting CsA to mitochondria, thereby minimizing extra-mitochondrial interactions, improves cytoprotection in an isolated neuronal model of ‘energy failure’ [21]. In the present paper, we report the synthesis of a modified mtCsA (mitochondrial-targeted CsA) with a much-improved CyP-D-binding affinity over the prototype, and show that it yields better cytoprotection than CsA in an isolated cardiomyocyte model of RI.

EXPERIMENTAL

Synthesis of cyclosporin analogues

A scheme of the syntheses is given in Figure 1. Compound 2, SMBrz-CsA ([sarcosine-3-(4-methylbenzoate)]-CsA), was used as a key intermediate in the synthesis of the alkyl-linked TPP+ (triphenylphosphonium) analogue (compound 5), as described previously [21] and in the new alkyl linked rosamine (compound 6) and ether-linked TPP+ (compound 9) analogues as...
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RESULTS AND DISCUSSION

A mitochondrial-targeted cyclosporin with improved CyP-D binding affinity

CsA (compound I, Figure 1) is a lipophilic cyclic undecapeptide that inhibits the PPIase activity of CyPs. CsA inhibition of the mitochondrial isoform CyP-D prevents PT pore formation and partially protects against RI in experimental models (as described in the Introduction). Recently, we reported that neuroprotection by CsA is limited by counter-protective interactions with extra-mitochondrial CyPs and that cytoprotection is improved when CyP-D is inhibited selectively [21]. CyP-D selectivity was achieved with a mitochondrial-targeted CsA, here designated mtCsA1 (compound 5, Figure 1) in which residue 3 of the CsA ring was conjugated via a linker to the lipophilic TPP+ cation. The positively charged mtCsA1 was accumulated electrophotorectly by mitochondria in response to the negative-inside inner membrane potential, enabling selective inhibition of mitochondrial CyP-D in cells. However, the high binding affinity of CyP-D for CsA was decreased substantially by the modifications in mtCsA1, and more mtCsA1 was required for PT pore blockade than CsA itself. The first aim of the present study was therefore to investigate whether the intrinsically high binding affinity of CyP-D for CsA could be retained within this basic strategy (CsA–linker–TPP+) of targeting CsA to mitochondria.

The synthesis of the required analogues (Figure 1) was accomplished by adaptation of our previously described method for synthesis of mtCsA1 [21]. SMBz-CsA (compound 2) was coupled in solution to Fmoc-monoprotected diamine using the standard peptide coupling reagent PyBOP to afford the corresponding Fmoc-protected amide intermediate 3. This intermediate was deprotected by reaction with piperidine in DMF to give amine 4 and coupled in solution to rosamine acid [22] with PyBOP and triethylamine to produce target compound 6. The ether-linked compounds 7–9 were prepared using similar methods.

CyP-binding affinities for CsA analogues were determined from PPIase inhibition, and are reported as inhibitor (dissociation) constants in Table 1. CyP-D bound the prototype mtCsA compound, mtCsA1, with an affinity (Ki = 93 nM) approximately 1/30 of that for CsA (Ki = 3 nM). Some loss of binding affinity in mtCsA1 might be anticipated. The CsA ring half inserts into the CyP active site with residues 9–11 and 1–3 interacting with the CyP and residues 4–8 remaining exposed to the solvent [24]. Hence position 3 additions, as in mtCsA1, might sterically hinder binding to CyP-D. Yet the additions at position-3 that form the linker caused relatively modest decreases in binding affinity (Ki values: compound 2, 7 nM; compound 4, 15 nM). The major loss of affinity occurred with Fmoc (Ki value for compound 3, 160 nM) and TPP+ (Ki value for mtCsA1, 93 nM). Addition of rosamine to the linker, as an alternative (mitochondrial-targeting) lipophilic cation to TPP+, also decreased binding affinity considerably (Ki value for compound 6, mtCsA2, 202 nM). These findings indicate that the large loss of binding affinity is not due specifically to TPP+, since addition of other bulky groups caused losses at least as great, or to modification at position 3. Rather, it seems that these bulky groups are not effectively separated from the CsA by the linker, possibly as a result of hydrophobic collapse in which the linker–TPP+ (−Fmoc, −rosamine) folds back on itself to maximize hydrophobic interactions, thereby stabilizing the TPP+ (Fmoc, rosamine) in close proximity to the CsA. We therefore investigated the use of a shortened linker containing an ether linkage to reduce hydrophobicity.

The ether-linked derivatives are shown as compounds 7–9 (Figure 1). Remarkably, CyP-D bound the new Fmoc and TPP+ derivatives with an affinity close to that for CsA (Ki values:
Inhibition by CsA derivatives of CyPs, calcineurin and the PT pore

| Compound | W | R | $K_i$ (nM) | CyP-D | CyP-A | Calcineurin inhibition (%) | $I_{50}$ for PT (pmol/mg of protein) | CyP-A inhibition at $I_{50}$ (%) |
|----------|---|---|------------|-------|-------|--------------------------|-----------------------------------|---------------------------------|
| 1, CsA   | – | – | 3 (0.992)  | 4 (0.987) | 74 | 125 + 10 | 60 + 7 |
| 2, SMBz-CsA | – | – | 7 (0.954)  | 8 (0.958) | 3 | 286 + 16 | – |
| 3        | (CH$_2$)$_2$ Fmoc | 160 (0.955) | – | – | – | – | – |
| 4        | (CH$_2$)$_2$ H | 15 (0.951) | – | – | – | – | – |
| 5, mtCsA1 | (CH$_2$)$_2$ CO((CH$_2$)$_n$PPh$_2$)$^{+}$ | 93 (0.993) | 115 (0.975) | 5 | 353 ± 27 | 9 ± 1 |
| 6, mtCsA2 | (CH$_2$)$_2$ Rosamine | 202 (0.990) | 213 (0.995) | – | 770 ± 32 | 21 ± 6 |
| 7        | 0 | Fmoc | 6 (0.959) | – | – | – | – |
| 9, mtCsA3 | 0 | CO((CH$_2$)$_n$PPh$_2$)$^{+}$ | 5 (0.971) | 8 (0.982) | 4 | 29 ± 3 | 1 ± 1 |

Mitochondrial accumulation of mtCsA compounds

The capacity of mitochondria to accumulate mtCsA compounds was evaluated by comparing inhibition of the CyP-D-dependent PT pore and inhibition of extra-mitochondrial CyP-A in a system comprising isolated mitochondria and added recombinant CyP-A. PT pore opening was triggered by addition of Ca$^{2+}$ and monitored from the resultant mitochondrial swelling (Figure 2A) [21]. Ca$^{2+}$ uptake is electrophoretic and, when rapid, depresses $\Delta\phi_m$ (inner membrane potential) [12]. As dissipation of $\Delta\phi_m$ would compromise electrophoretic accumulation of mtCsA compounds, Ca$^{2+}$ was infused slowly over several minutes to limit the rate of Ca$^{2+}$ influx and to prevent loss of $\Delta\phi_m$ arising from rapid Ca$^{2+}$ flux [21]. Extramitochondrial CyP-A activities were determined in parallel (identical) incubations (see the Experimental section).

Representative time courses of PT pore opening, showing mtCsA3 inhibition, are given in Figure 2(A); equivalent time courses were obtained for all CsA compounds analyzed ($I_{50}$ values, Table 1). To compare inhibition by the different compounds, rates were calculated from the time period between first detection of PT pore opening in the absence of inhibitor (vertical arrow, Figure 2A) and the attainment of a 0.24 unit change in absorbance (horizontal arrow; corresponding to an approximate half-maximal absorbance change). Approximately 29 nM total mtCsA3 produced 50% inhibition of PT pore opening, but no detectable inhibition of CyP-A (Figure 2B).

A total mtCsA3 concentration of 200 nM yielded just 17% inhibition of CyP-A, indicating an extra-mitochondrial free mtCsA3 concentration of approximately 1.6 nM (calculated from $K_i = 8$ nM, Table 1), i.e. that nearly all mtCsA3 had been accumulated by mitochondria, with negligible amounts remaining outside for inhibition of CyP-A.

In agreement with previous data [21], CsA itself displayed no selectivity for the PT pore over extra-mitochondrial CyP-A (Figure 2B); this would be expected as CsA is bound by CyP-D and CyP-A with similar affinities (Table 1) and, being electroneutral, would equilibrate to the same free concentrations on either side of the mitochondrial inner membrane. To compare selectivities by mtCsA compounds, Table 1 (rightmost column) gives the CyP-A inhibition at 50% inhibition of the PT. mtCsA selectivity for the PT increased with increased binding affinity for the target CyP-D (mtCsA3 > mtCsA1 > mtCsA2).

Non-specific binding of mtCsA compounds in mitochondria

In line with mitochondrial accumulation, considerably less mtCsA3 than CsA was needed for PT pore inhibition ($I_{50}$, compund 7, 6 nM; compound 9, 5 nM). Although addition of alkyl linker and TPP$^+$ to compound 2 caused a 13-fold decrease in CyP-D-binding affinity, the addition of ether linker and TPP$^+$ caused no loss. The new mtCsA (compound 9) is designated mtCsA3.

CsA binding to the cytosolic isoform CyP-A can also influence cell viability (described below) and, although mtCsA compounds are designed to minimize interactions with CyP-A by means of accumulation out of the cytosol into mitochondria, CyP-A data were also obtained (Table 1). CyP-A bound CsA and the mtCsA derivatives with a similar affinity as CyP-D, conforming to the close similarity between the CsA-binding sites in the two isoforms [24,25]. In addition, the CyP-A–mtCsA complex inhibits the protein phosphatase calcineurin [26]. To compare calcineurin inhibition by CyP-A–mtCsA complexes, the concentrations of CsA and derivatives were chosen to give the same concentration (720 nM) of their respective complexes with CyP-A (calculated from $K_i$ values), and we confirmed that, when added alone, these concentrations of CyP-A, CsA and derivatives did not inhibit calcineurin ([21], results not shown). Table 1 shows that, although the CyP-A–mtCsA complex inhibits calcineurin phosphatase activity, the CyP-A complexes with the mtCsA compounds did not. The additions at position 3 of CsA in the derivatives presumably prevent binding of the CyP-Csa (derivative) complexes by calcineurin which is known to involve contacts between calcineurin and positions 3–7 of the CsA ring [24,26].

Table 1  Inhibition by CsA derivatives of CyPs, calcineurin and the PT pore

| Compound | W | $K_i$ (nM) | CyP-D | CyP-A | Calcineurin inhibition (%) | $I_{50}$ for PT (pmol/mg of protein) | CyP-A inhibition at $I_{50}$ (%) |
|----------|---|------------|-------|-------|--------------------------|-----------------------------------|---------------------------------|
| 1, CsA   | – | 3 (0.992)  | 4 (0.987) | 74 | 125 + 10 | 60 + 7 |
| 2, SMBz-CsA | – | 7 (0.954)  | 8 (0.958) | 3 | 286 + 16 | – |
| 3        | (CH$_2$)$_2$ Fmoc | 160 (0.955) | – | – | – | – | – |
| 4        | (CH$_2$)$_2$ H | 15 (0.951) | – | – | – | – | – |
| 5, mtCsA1 | (CH$_2$)$_2$ CO((CH$_2$)$_n$PPh$_2$)$^{+}$ | 93 (0.993) | 115 (0.975) | 5 | 353 ± 27 | 9 ± 1 |
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| 7        | 0 | Fmoc | 6 (0.959) | – | – | – | – |
| 9, mtCsA3 | 0 | CO((CH$_2$)$_n$PPh$_2$)$^{+}$ | 5 (0.971) | 8 (0.982) | 4 | 29 ± 3 | 1 ± 1 |
29 pmol/mg compared with 125 pmol/mg; Table 1). However, in the same experimental system, more mtCsA1 was required \((I_{50}, 353 \text{ pmol/mg})\), despite its being accumulated by mitochondria [21]. This suggests that the bulk of mitochondrially accumulated mtCsA was bound non-specifically. This was also the case with the other alkyl-linked derivative, mtCsA2 \((I_{50}, 770 \text{ pmol/mg})\). In this connection, Scatchard analyses reveal that a small amount of mitochondrial CsA is partitioned into membrane phospholipids (approximately 15 % at the \(I_{50}\) [27]). The amount of membrane-partitioned mtCsA would be expected to increase in direct proportion to the free mtCsA concentration in the mitochondrial matrix. In turn, the matrix free mtCsA concentration at the \(I_{50}\) will increase in proportion to the \(K_{i}\) value of CyP-D for that mtCsA. This would lead to a linear relationship between the amount of mitochondrial mtCsA yielding 50 % PT inhibition (intra-mitochondrial \(I_{50}\)) and the free mtCsA concentration giving 50 % CyP-D inhibition \((K_{i} \text{ for CyP-D})\). Intra-mitochondrial \(I_{50}\) values were obtained by correcting the \(I_{50}\) values (which refer to total mtCsA) for the relatively small amounts of extra-mitochondrial mtCsA (legend, Figure 3), and these yielded the predicted linear relationship with \(K_{i}\) (Figure 3). Clearly, differences in lipid solubility between the mtCsA compounds would produce deviations from linearity, but any such deviations appear to be small. It is probable, therefore, that non-selective membrane binding of the mtCsA compounds was determined largely by the SMBz-CsA moiety common to all, with the remaining parts of the linkers exerting relatively little influence. Cationic mitochondrial-targeting groups, e.g. TPP +, conjugated to lipophilic molecules appear to lie on the inner surface of the inner membrane with the lipophilic moiety in the membrane interior [28].

The ordinal intercept in Figure 3 (at theoretical infinite CyP-D affinity for mtCsA, when all added mtCsA is bound to CyP-D) corresponds to the amount of mtCsA bound to CyP-D at the intra-mitochondrial \(I_{50}\). From this it is clear that, although the fraction of mitochondrially accumulated mtCsA1 and mtCsA2 actually bound to CyP-D was relatively small (3 and 1.5 % respectively at the \(I_{50}\)) a far greater proportion of mtCsA3 (>50 %) was CyP-D-bound.

Protection by mtCsA3 in cardiomyocytes

The cytoprotective properties of the improved mtCsA compound were evaluated in isolated heart cells subjected to simulated I/R (ischaemia/reperfusion). The cells were first exposed to glucose-free anoxia for 4 h and then aerobically with glucose and 50 \(\mu\)M t-butyl hydroperoxide (reoxygenation). When included, CsA and derivatives were added at the beginning of anoxia. \(\text{A-C}\) Cell necrosis during reoxygenation in the presence and absence of 50 nM CsA, 15 nM mtCsA3 and 400 nM SMBz–CsA, as indicated, was determined from nuclear staining with ethidium homodimer. Insets in \(\text{A}\) show cells dual-stained with Hoechst 33342 (H) and ethidium homodimer (E) after 3 h of reoxygenation. \(\text{B and E}\) Necrosis after 3 h of reoxygenation with the indicated concentrations of CsA, mtCsA3 and SMBz–CsA. Results are means ± S.E.M. for four cell preparations, >200 cells counted per preparation. \(\ast P < 0.05\) with respect to no addition.

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The cytoprotective properties of the improved mtCsA compound were evaluated in isolated heart cells subjected to simulated I/R (ischaemia/reperfusion). The cells were first exposed to glucose-free anoxia to mimic ischaemia; in practice, cells could be maintained under these conditions for approximately 4 h with little necrosis (<8 %). The myocytes were then reoxygenated in the presence of glucose and 50 \(\mu\)M t-butyl hydroperoxide, and this produced progressive cell necrosis amounting to approximately 50 % after 5 h (Figures 4A–4C). t-Butyl hydroperoxide was included to boost oxidative stress, a critical component of RI [29] and of PT pore opening in cardiomyocytes [1,30]. Other experiments (results not shown) confirmed that necrosis was substantially reduced on omission of either t-butyl hydroperoxide (<15 % necrosis after 5 h) or the period of glucose-free anoxia (no increase in necrosis during 5 h of reoxygenation).

After 3 h of reoxygenation, CsA gave a maximal cytoprotection of approximately 38 % at 50–100 nM (Figure 4D). Increased CsA concentration to 200 nM decreased cytoprotection and, in a separate series of paired experiments, 1 \(\mu\)M CsA gave no protection at all (controls, 25 ± 4 % necrosis; CsA tests, 25 ± 5 %; \(n = 4\), \(P = 0.86\)). This agrees with the reversal of cytoprotection with increased CsA concentration observed.
previously in isolated adult rat cardiomyocytes [9], isolated rat hippocampal neurons [21] and perfused rat heart [10]. In comparison, mtCsA3 delayed the onset of necrosis completely at 3–15 nM (Figure 4E). However, protection was reversed at higher concentrations and when reoxygenation was prolonged beyond 3 h (Figure 4B).

The increased maximal cytoprotection gained by mitochondrial targeting most logically reflects avoidance of counter-protective actions of CsA external to mitochondria. These might include calcineurin inhibition by the CyP–CsA complex and/or inhibition (PPlase) of CyP-A (cytosol) or other extra-mitochondrial CyPs. To evaluate the contribution of these factors to the enhanced protection by mtCsA3, we used SMBz–CsA (compound 2, Figure 1). Like mtCsA3, SMBz–CsA has similar binding affinities for CyP-D and CyP-A and, in complex with CyP-A, does not inhibit calcineurin (Table 1). Unlike mtCsA3, however, SMBz–CsA lacks the mitochondrial-targeting TPP+ cation and is not accumulated by mitochondria, and, being freely permeable across the mitochondrial inner membrane, inhibits both CyP-D and CyP-A in cells [21]. Thus the cellular inhibition profile of SMBz–CsA (inhibiting CyP-D and CyP-A) is distinct from that of CsA (inhibiting CyP-D, CyP-A and calcineurin) and mtCsA (inhibiting CyP-D) [21]. As shown in Figure 4(D), maximal cytoprotection by SMBz–CsA (65%) was greater than that given by CsA (38%), indicating that avoidance of calcineurin inhibition may contribute to the increased potency of mtCsA3 with respect to CsA, as shown previously for mtCsA1 in neuronal cells following glucose-free anoxia [21]. However, maximal protection by the SMBz–CsA was less than that given by mtCsA3, suggesting that avoidance of inhibition of CyP-A or other extra-mitochondrial CyPs also contributes to the enhanced cytoprotection gained by mitochondrial targeting. In this connection, both CyP-A and CyP-B (sarcoplasmic reticulum) counteract oxidative stress [23,31] and inhibition of these PPlases may be counter-protective when oxidative stress is a critical element in the pathogenesis, as in the present regime.

In conclusion, we have developed a mitochondrial-targeted CsA with a binding affinity for CyP-D close to that of CsA itself. These two features, intra-mitochondrial selectivity for CyP-D and mitochondrial targeting, are designed to minimize non-CyP-D interactions in cells. In a model system, the mtCsA counters R1 more effectively than CsA, underlining the potential improvement in cytoprotection to be gained by selective CyP-D inhibition. MtCsA3 is effective over a discrete concentration range (Figure 4E) and, to be useful therapeutically, will need to establish intra-mitochondrial concentrations within the effective range before PT pore formation takes place. Indications are that the pore forms quickly on reperfusion. Measurements in isolated cardiomyocytes of mitochondrial inner membrane potential [2] and Ca2+ accumulation capacity [32] reveal that both are lost within 1–2 min after reoxygenation in non-recovering cells, consistent with PT pore formation within this time. Since anti-R1 therapeutics can only be delivered to organs on reperfusion, a key question to be resolved is whether mtCsA compounds can establish intra-mitochondrial concentrations within the effective range sufficiently quickly, and whether these concentrations can be maintained within (i.e. without exceeding) the effective range for a sufficient period of time. CsA itself seems kinetically adequate in this respect. In experimental animals, CsA reduces infarct size when introduced at the time of reperfusion [11]. In patients treated for acute myocardial infarction, CsA yields some protection against RI when administered intravenously immediately before coronary angioplasty [33]. Equally encouraging, conjugation to TPP+ has been used to deliver a wide range of lipophilic compounds to mitochondria in tissues and does so quickly, achieving maximal levels in the heart within 4 min of intravenous injection and maintaining these for >1 h [34].

**AUTHOR CONTRIBUTION**

Chemical syntheses were carried out by Henry Dube, Michela Simone and David Selwood. Biological preparations and assays were conducted by Sylvanie Malouire, Michela Capano and Martin Crompton. The project was directed by David Selwood (chemical aspects) and Martin Crompton (biological aspects). All authors contributed to writing the paper.

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