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A Phenyl-Pyrrolidine Derivative Reveals a Dual Inhibition Mechanism of Myocardial Mitochondrial Permeability Transition Pore, Which Is Limited by Its Myocardial Distribution

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ABSTRACT

Mitochondrial permeability transition pore (mPTP) opening is a key event in cell death during myocardial ischemia reperfusion. Inhibition of its modulator cyclophilin D (CypD) by cyclosporine A (CsA) reduces ischemia-reperfusion injury. The use of cyclosporine A in this indication is debated; however, targeting mPTP remains a major goal to achieve. We investigated the protective effects of a new original small-molecule cyclophilin inhibitor C31, which was specifically designed to target CypD. CypD peptidyl-prolyl *cis-trans* isomerase (PPIase) activity was assessed by the standard chemotrypsin-coupled assay. The effects of C31 on mPTP opening were investigated in isolated mouse cardiac mitochondria by measuring mitochondrial swelling and calcium retention capacity (CRC) in rat H9C2 cardiomyoblasts and in adult mouse cardiomyocytes by fluorescence microscopy in isolated perfused mouse hearts and ex vivo after drug infusion in mice. C31 potently inhibited CypD PPIase activity and mitochondrial swelling. C31 was more effective at increasing mitochondrial CRC than CsA and was still able to increase CRC in *Ppif*$^{-/-}$ (CypD-inactivated) cardiac mitochondria. C31 delayed both mPTP opening and cell death in cardiomyocytes subjected to hypoxia reoxygenation. However, high concentrations of both drugs were necessary to reduce mPTP opening in isolated perfused hearts, and neither CsA nor C31 inhibited mPTP opening in heart after in vivo infusion, underlying the importance of myocardial drug distribution for cardioprotection. C31 is an original inhibitor of mPTP opening involving both CypD-dependent and -independent mechanisms. It constitutes a promising new cytoprotective agent. Optimization of its pharmacokinetic properties is now required prior to its use against cardiac ischemia-reperfusion injury.

SIGNIFICANCE STATEMENT

This study demonstrates that the new cyclophilin inhibitor C31 potently inhibits cardiac mitochondrial permeability transition pore (mPTP) opening in vitro and ex vivo. The dual mechanism of action of C31 allows the prevention of mPTP opening beyond cyclophilin D inhibition. Further development of the compound might bring promising drug candidates for cardioprotection. However, the lack of effect of both C31 and cyclosporine A after systemic administration demonstrates the difficulties of targeting myocardial mitochondria in vivo and should be taken into account in cardioprotective strategies.

Introduction

Mitochondrial permeability transition pore (mPTP) opening is considered a critical event in cell death during myocardial ischemia reperfusion. Indeed, opening of the mPTP in the 1st minute of reperfusion causes necrosis or apoptosis and participates in ischemia-reperfusion injury (Yellon and Hausenloy, 2007; Morin et al., 2009; Halestrap, 2010; Di Lisa et al., 2011; Hausenloy et al., 2016). Therefore, targeting mPTP opening represents an interesting pharmacological strategy to limit the damages induced by the reperfusion of an ischemic myocardium. Even though the molecular structure of mPTP remains debated, cyclophilin D (CypD), a mitochondrial peptidylprolyl *cis-trans* isomerase (PPIase), is widely described as an essential modulator of pore opening. CypD is located within the mitochondrial matrix and catalyzes or stabilizes the formation of the pore (Gutiérrez-Aguilar and Baines, 2015). Inhibition or genetic ablation of CypD strongly decreases the susceptibility to mPTP opening by lowering its sensitivity to Ca^{2+} overload. Therefore, CypD became an attractive target to develop cardioprotective strategies. Animal studies demonstrated that CypD inhibition

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Conflict of interests: Inserm Transfert is the owner of patent EP 09306294.1 covering the family of cyclophilin inhibitors, including the C31 compound, for which A.A.-B., J.-F.G. and J.-M.P. are inventors. All other authors declare no competing financial interests.

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ABBREVIATIONS: calcein-AM, calcein acetoxymethyl ester; CIRCUS Does Cyclosporine ImpRove Clinical oUtcome in ST Elevation Myocardial Infarction Patients CRC, calcium retention capacity; CsA, cyclosporine A; CypD, cyclophilin D; PPIase, peptidylprolyl *cis-trans* isomerase; mPTP, mitochondrial permeability transition pore.

with cyclosporine A (CsA) and hence desensitization of mPTP opening decreased infarct size (Trankle et al., 2016). Clinical translation of CypD inhibition by CsA administered at reperfusion in patients during acute myocardial infarction demonstrated decreased biomarker release and less adverse remodeling (Piot et al., 2008; Mewton et al., 2010). However, the phase III clinical trial "Does Cyclosporine ImpRove Clinical oUtcome in ST Elevation Myocardial Infarction Patients" (CIRCUS) did not confirm the beneficial effects of CsA observed previously (Cung et al., 2015). The failure to achieve the CIRCUS predefined efficacy endpoint has been extensively discussed, and differences between the two trials, which are unrelated to CsA per se, may account for the discrepancy of the results (Heusch, 2015; Chen-Scarabelli and Scarabelli, 2016; Monassier et al., 2016). mPTP opening can also occur in a CypD-independent manner, and this might explain at least in part the difference between clinical studies. Indeed, CypD is only a modulator of mPTP opening, and its inhibition desensitizes mPTP opening rather than blocking it (Bernardi et al., 2006; Halestrap and Richardson, 2015).

Hence, mPTP opening remains achievable in case of very strong stimuli. These findings stimulated several groups to develop chemical strategies to identify novel selective and nonpeptidic compounds able to inhibit mPTP opening. Our group used fragment-based drug discovery combined with a linking strategy and structure-based compound optimization to generate a new family of nonpeptidic, small-molecule cyclophilin inhibitors unrelated to CsA or Sanglifehrin A with strong PPIase inhibitory activity (Ahmed-Belkacem et al., 2016). These compounds target cyclophilins by interacting with the PPIase domain and anchoring in the adjacent gatekeeper pocket (Ahmed-Belkacem et al., 2016). They bind into the active site of cyclophilins competitively with cyclosporine A (Nevers et al., 2018). We previously described that the most active compound, C31 (1-(4-aminobenzyl)-3-(2-(2-(2-(methylthio)phenyl)pyrrolidin-1-yl)-2-oxo-1-phenylethyl)urea), is an effective mPTP inhibitor that exerts protective effects in the context of hepatic ischemia-reperfusion injury through an original mechanism involving CypD at low concentrations and another target at higher concentrations (Panel et al., 2019).

Here, we compared the effect of C31 on mPTP opening in mouse liver and cardiac mitochondria. We demonstrated that C31 inhibits mPTP opening in isolated cardiac mitochondria with a dual mechanism identical to that observed in the liver. This effect was correlated with cytoprotective effects in cardiomyocytes subjected to hypoxia reoxygenation. Perfusion of CsA and C31 in Langendorff-perfused heart model showed that high concentrations of both compounds are required to inhibit mPTP opening. Strikingly, neither C31 nor CsA reached cardiac mitochondria after in vivo administration, emphasizing the need to improve intramitochondrial delivery of compound C31 for further therapeutic development.

Materials and Methods

Ethics Statement. All animal procedures used in this study were in accordance with the Directives of the European Parliament (2010/63/EU-848 EEC) and were approved by the Animal Ethics Committee French Agency for Food, Environmental and Occupational Health & Safety (ANSES)/Ecole Nationale Vétérinaire d'Alfort/Université Paris-Est Créteil (approval number 09/12/14-02) and by the French Ministry of Higher Education and Research (Project Authorization for the use of Animals for Scientific Purposes APAFIS 13504, December 18, 2017).

Drugs and Cells. Unless specified, all reagents were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Calcein-AM (C3100MP) and calcium Green 5N (C3737) were obtained from Invitrogen (Cergy-Pontoise, France). C31 was synthesized as previously described (Ahmed-Belkacem et al., 2016).

Rat H9C2 cells were obtained from the American Tissue Culture Collection (LGC Standards S.a.r.l., Molsheim, France) and were cultured in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were used between passage 20 and 25 and were seeded in complete medium in 35-mm Petri dishes 24 hours before microscopy experiments.

Animals. Male C57BL/6J mice (8–10-week-old) and male Wistar rats (250–300 g) were purchased from Janvier (Le Genest-St-Isle, France). CypD knockout mice (*Ppif*$^{-/-}$ mice) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in an air-conditioned room with a 12-hour light/dark cycle and received standard rodent chow, drinking water ad libitum.

Isolation of Mitochondria and Cytosols. For swelling experiments and evaluation of mitochondrial Ca^{2+} retention capacity, left ventricle tissues from C57BL6/J wild-type mice, *Ppif*$^{-/-}$ mice, or male Wistar rats were removed after cervical dislocation (mice) and sodium pentobarbital (80 mg/kg) anesthesia (rats). They were immediately immersed in ice-cold 0.9% NaCl, scissor-minced, and homogenized using a Polytron homogenizer in a cold buffer (4°C, pH 7.4) containing mannitol (220 mM), sucrose (70 mM), HEPES (10 mM), and EGTA (2 mM). The samples were further homogenized for 10 consecutive times using a Potter homogenizer at 1500 rpm. The homogenates were then centrifuged at 1000*g* for 5 minutes at 4°C to remove tissue debris and nuclei. The supernatants were centrifuged for 10 minutes at 10,000*g*. The final mitochondrial pellets were resuspended in the homogenization buffer with only 0.01 mM of EGTA, and protein concentration was determined using the Advanced protein assay reagent (catalog number 57697; Sigma).

For evaluation of PPIase activity, C57BL/6J mice hearts and livers were used to prepare mitochondrial and cytosolic extracts. Mitochondria were purified on a Percoll gradient (Townsend et al., 2007) as follows. Briefly, the left ventricle tissues were added to 5 ml of homogenization buffer [mannitol (220 mM), sucrose (70 mM), HEPES (10 mM), and EGTA (2 mM), pH 7.4 at 4°C] supplemented with 0.25% bovine serum albumin. The tissues were sliced and homogenized with a Potter-Elvehjem glass homogenizer by a motor-driven Teflon pestle at 1500 rpm in a final volume of 5 ml. Homogenates were centrifuged at 1000*g* for 5 minutes at 4°C. Then supernatants were centrifuged at 10,000*g* for 10 minutes at 4°C to pellet mitochondrial fractions. The resulting supernatants were centrifuged at 100,000*g* for 60 minutes at 4°C to obtain cytosolic fractions.

Mitochondrial pellets were added to 500 μl of homogenization buffer supplemented with 20% Percoll. Homogenates were centrifuged at 15,000*g* for 10 minutes at 4°C in a final volume of 10 ml. Supernatants were carefully removed, and pellet was added to 10 ml of homogenization buffer (without Percoll) before centrifugation at 12,000*g* for 5 minutes at 4°C. The final mitochondrial pellets were resuspended in the homogenization buffer. Sample protein concentrations were determined using the Advanced protein assay reagent (catalog number 57697; Sigma).

Evaluation of PPIase Activity. PPIase activity was evaluated at 25°C using the standard chymotrypsin-coupled assay. Mitochondrial or cytosolic fractions (0.3 mg protein/ml) were incubated in the assay buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 4 mM EGTA, pH 7.4 at 4°C) in the presence of 5 μl of 50 mg/ml chymotrypsin (in 1 mM HCl). After a 20-second stabilization period, reaction was initiated by adding 20 μl of 3.2 mM peptide substrate (*N*-Succinyl-Ala-

Ala-Pro-Phe-p-nitroanilide). The absorbance of p-nitroanilide was followed at 390 nm for 1 minute. For the inhibition assays, 1 μM CsA (in DMSO) was added to the preparation in the assay buffer. PPIase activity was determined from the slopes of the curves.

Mitochondrial Swelling Assays. Mitochondrial swelling was assessed in energized rat cardiac mitochondria by measuring the change in absorbance at 540 nm (A_{540}) using a Jasco V-530 spectrophotometer (Jasco, Bouguenais, France) equipped with magnetic stirring and thermostatic control. Experiments were carried out at 30°C in a respiration buffer including 100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM KH_2PO_4 (pH 7.4 at 30°C). Mitochondria (0.5 mg/ml) were incubated in the presence of pyruvate/malate (5 mM each). After 30 seconds, swelling was induced by addition of 250 μM $CaCl_2$. The determination of the initial rate of swelling allowed calculation of the IC_{50} values as previously described (Elimadi et al., 1997).

Evaluation of the Ca^{2+} Retention Capacity of Isolated Mitochondria. Rat or mouse cardiac mitochondria were loaded with increasing concentrations of Ca^{2+} until the load reached a threshold at which mitochondria underwent a fast process of Ca^{2+}release, which was due to mPTP opening, as previously described (Obame et al., 2008). Mitochondria (0.8 mg/ml) energized with 5 mM glutamate/malate were incubated in the respiration buffer supplemented with 1 μM Calcium Green-5N fluorescent probe. The concentration of Ca^{2+} in the extramitochondrial medium was monitored by means of a Jasco FP-6300 spectrofluorimeter (Jasco) at excitation and emission wavelengths of 506 and 532 nm, respectively. The Ca^{2+} signal was calibrated by addition to the medium of known Ca^{2+} amounts.

Isolation of Primary Adult Mouse Cardiomyocytes. Ventricular cardiomyocytes were isolated from mice by an enzymatic technique. Mice were anesthetized with sodium pentobarbital (100 mg/kg), and hearts were removed. The heart was retrogradely perfused for 15 minutes at 37°C with a stock perfusion buffer bubbled with 95%O_2/5%CO_2 containing 133 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM HEPES, 30 mM taurine, 0.032 mM phenol red, 5.5 mM glucose, and 10 mM 2,3-butanedionemonoxime (pH 7.4) to wash out blood. After 2 minutes of perfusion, liberase TM (10 mg/100 ml; Roche Applied Science, Mannheim, Germany), trypsin EDTA (14 mg/100 ml), and 12.5 μM Ca^{2+} were added to the buffer, and the heart was perfused for approximately 8 to 9 minutes. The heart was placed into a beaker in the same buffer supplemented with 10% bovine serum albumin (pH 7.4) at 37°C to stop the digestion. Ventricles were then cut into small fragments, and cells were isolated by stirring the tissue and successive aspirations of the fragments through a 10-ml pipette. Cell suspension was filtered (250-μm nylon mesh) and decanted for 10 minutes. The pellet (containing the cells) was resuspended in 10 ml of the perfusion buffer including 5% bovine serum albumin and 12.5 μM of Ca^{2+}. The cellular suspension was decanted again for 10 minutes. The supernatant was eliminated, and the same procedure (resuspension and settling) was repeated with increasing concentrations of Ca^{2+} (62, 112, 212, 500 μM) up to 1 mM. Finally, cardiomyocytes were suspended in an M199 culture medium, seeded on 35-mm Petri dishes precoated with 10 μg/ml sterilized laminin, and incubated for 90 minutes at 37°C before being used.

Measurement of mPTP Opening in Rat Cardiomyoblastic H9C2 Cells in Normoxic Conditions. Direct assessment of mPTP opening in rat cardiomyoblastic H9C2 cell line was performed using the established loading procedure of the cells with calcein acetoxymethyl ester (calcein-AM) and $CoCl_2$, resulting in mitochondrial localization of calcein fluorescence (Petronilli et al., 1999). Cells were loaded with 2 mM $CoCl_2$ at 30°C in 1 ml of Tyrode's solution (in millimolars: NaCl 130; KCl 5; HEPES 10; $MgCl_2$ 1; $CaCl_2$ 1.8, pH = 7.4 at 37°C) for 30 minutes. After 10 minutes, cells were supplemented with 1 μM calcein-AM. This labeling protocol was slightly different from that used in our previous studies performed with adult cardiomyocytes (Petronilli et al., 2001; Obame et al., 2008), but it was necessary to obtain the best labeling of the cells. Cells were then washed free of calcein and $CoCl_2$ with the Tyrode's solution and placed in a thermostated chamber (Warner Instruments Inc, CT), which was mounted on the stage of an IX81 Olympus microscope (Olympus, Rungis, France). After 5 minutes of incubation in the Tyrode's medium, 50 nM of the Ca^{2+} ionophore A23187 was added to the cells to induce mPTP opening as previously described (Schaller et al., 2010). When specified, C31 or CsA was added to the cells at the beginning of the incubation period.

Measurement of mPTP Opening in Primary Adult Cardiomyocytes Subjected to Hypoxia Reoxygenation. Mouse cardiomyocytes were placed into a thermostated (37°C) chamber (Warner Instruments Inc), which was mounted on the stage of an IX81 Olympus microscope (Olympus), and were perfused with the Tyrode's solution at a rate of 0.5 ml/min. The chamber was connected to a gas bottle diffusing a constant stream of O_2 (21%), N_2 (74%), and CO_2 (5%) and maintaining an O_2 concentration of 21%. Oxygen in the perfusate was measured in the chamber using a fiber optic sensor system (Ocean Optics Inc., FL). Cardiomyocytes were paced to beat by field stimulation (5 milliseconds, 0.5 Hz).

To simulate ischemia, the perfusion was stopped, and cardiomyocytes were exposed for 45 minutes to a hypoxic medium maintaining an O_2 concentration of 1% to 2%. This medium was the Tyrode's solution (bubbled with 100% N_2) supplemented with 20 mM 2-deoxyglucose and subjected to a constant stream of N_2 (100%). At the end of the ischemic period, reoxygenation was induced by rapidly restoring the Tyrode's flow and 21% O_2 in the chamber.

In these cells, mPTP opening was also assessed by means of the calcein loading procedure as previously described (Petronilli et al., 2001; Obame et al., 2008). Briefly, before introduction in the thermostated chamber, cells were loaded with 1 μM calcein-AM at 37°C for 30 minutes supplemented with 1 mM $CoCl_2$ after 20 minutes of incubation. To determine cell death, cardiomyocytes were coloaded with 1.5 μM propidium iodide, which permeates only the damaged cells.

Data Acquisition and Analysis of Fluorescence Microscopy Experiments. Cells were imaged with an Olympus IX-81 motorized inverted microscope equipped with a mercury lamp as a source of light for epifluorescence illumination and with a 12-bit cooled Hamamatsu ORCA-ER camera (Hamamatsu, Hamamatsu city, Japan). For detection of calcein fluorescence, 460–490-nm excitation and 510-nm emission filters were used. In normoxic experiments, images were acquired every 5 minutes for 30 minutes after an illumination time of 25 milliseconds per image using a digital epifluorescence imaging software (Cell M; Olympus). In hypoxia-reoxygenation experiments, images were acquired every 5 minutes during hypoxia, every minute throughout the first 10 minutes of reoxygenation, and then every 5 minutes for the remaining duration of the experiment after an illumination time of 25 milliseconds (calcein) and 70 milliseconds (propidium iodide).

Fluorescence was integrated over a region of interest (≈80 μm^2) for each cell, and a fluorescence background corresponding to an area without cells was subtracted. Then the global response was analyzed by averaging the fluorescence changes obtained from all the cells (25–30) contained in a single field.

For calcein experiments, intensity values were normalized according to the initial fluorescence values after subtracting background. Moreover, we calculated the average time to mPTP opening (t_{mPTP50}) in each experiment by measuring the reoxygenation time necessary to reach 50% decrease in calcein fluorescence intensity for each cell with opened mPTP in the field. Propidium iodide fluorescence intensity minus background was normalized to 100% cell death.

Ex Vivo Assessment of mPTP Opening in Mice and in Isolated Perfused Hearts. The ability of C31 and CsA to interact with CypD in vivo was evaluated by measuring their ability to inhibit mPTP after mitochondrial isolation according to the following protocol. Mice were anesthetized with intraperitoneal injection of sodium

pentobarbital (80 mg/kg). The depth of anesthesia was monitored using the tail-pinching response and the pedal reflex. Increasing doses of C31 (10, 20, 50, and 150 mg/kg), CsA (20 mg/kg), or vehicle were administered at random as a 3-minute infusion through the jugular vein. Two minutes after the end of the infusion, hearts and livers were excised, and mitochondria were isolated to measure their capacity to retain Ca^{2+}.

The ability of C31 and CsA to interact with CypD was also evaluated in isolated perfused mouse hearts. Wild-type mouse hearts were retrogradely perfused through the aorta with a perfusion medium (133 mM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 10 mM HEPES, 30 mM taurine, 5.5 mM glucose, and 10 mM 2,3-butanedionemonoxime, pH 7.4 at 37°C) containing DMSO (0.1%), CsA (2–10 μM), or C31 (100 μM). Perfusion pressure was set at 120 mm Hg in nonrecirculating mode, and after a 20-minute perfusion, cardiac mitochondria were isolated, and Ca^{2+} retention capacity was evaluated. For each preparation, maximal CypD-dependent Ca^{2+} retention capacity achievable was further assessed in vitro by adding 1 μM CsA to the mitochondrial suspension.

Data and Statistical Analysis. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Statistical analysis was performed using GraphPad prism v.6. Results are expressed as mean ± S.E.M. Difference among groups was assessed by one-way ANOVA analysis and was followed by Tukey's multiple comparison test if ANOVA produced a significant value of F ($P < 0.05$). For isolated perfused heart experiments, two different tests were used: a nonparametric Kruskall-Wallis multiple comparison test followed by Dunnett's post hoc test to compare calcium retention capacity (CRC) achieved by drug administration and a two-way ANOVA with paired values (CRC and CRC + CsA) followed by Fisher's least significant difference test to analyze the effect of CsA addition in vitro. Statistical significance was defined as a value of $P < 0.05$.

Results

C31 Inhibits mPTP Opening in Isolated Cardiac Mitochondria. We tested whether C31 was able to inhibit mPTP opening in isolated cardiac mitochondria. Isolated rat cardiac mitochondria were energized with pyruvate plus malate and exposed to 250 μM Ca^{2+} in the presence of phosphate to trigger swelling. Swelling was fully inhibited by 2 μM CsA, a well known desensitizer of mPTP opening, confirming that the observed change in absorbance was due to mPTP opening. In these conditions, C31 inhibited mitochondrial swelling in a concentration-dependent manner (Fig. 1). The IC_{50} obtained for both CsA and C31 (IC_{50} = 0.044 ± 0.001 and 1.29 ± 0.35 μM, respectively) were comparable between cardiac and liver mitochondria recently reported for the latter (Panel et al., 2019). Thus, C31 inhibits mPTP opening in cardiac mitochondria to a similar extent as what we recently observed in liver mitochondria.

Next, we evaluated the effect of C31 on cardiac mitochondrial CRC (i.e., a sensitive assay to analyze mPTP inhibition or sensitization) (Fontaine et al., 1998). This technique is complementary to swelling experiments because it allows the determination of the maximal Ca^{2+} loading achievable in the presence of a drug before mPTP opening. Rat cardiac mitochondria required 143 ± 14 μM Ca^{2+} before mPTP opening. Fig. 2, A and B show that the maximal effect of CsA allowed mitochondria to retain twice (287 ± 29 μM, $P < 0.05$) the amount of Ca^{2+} as compared with control (DMSO)-treated

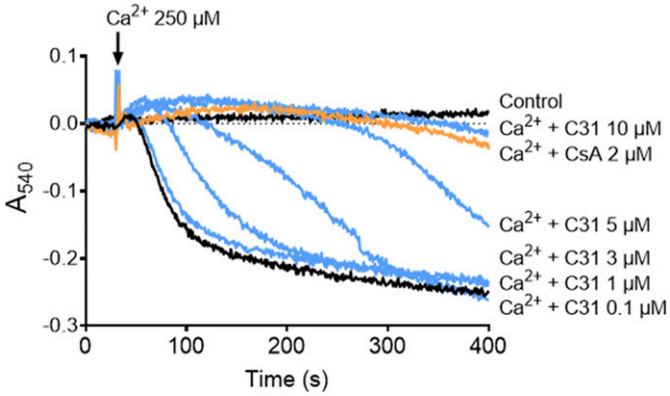


Fig. 1. C31 prevents calcium-induced swelling in isolated cardiac and liver mitochondria. Representative experiment showing the inhibition of swelling by increasing concentrations of C31 (0.1–10 μM) or CsA (2 μM) in energized rat cardiac mitochondria. Swelling was induced by addition of 250 μM Ca^{2+}. IC_{50} were further calculated from the slopes of the curves. A_{540}, absorbance at 540 nm.

mitochondria. Interestingly, C31 increased Ca^{2+} retention in a concentration-dependent manner and was significantly more effective than CsA with a significantly greater CRC (405 ± 48 μM at 100 μM).

C31 Exerts Additional mPTP-Inhibiting Properties Independently from CypD. To decipher the mechanism by which C31 exerts a stronger mPTP inhibition than CsA, CRC was assessed in cardiac mitochondria isolated from *Ppif*$^{-/-}$ mice. CRC under CsA was similar to control conditions in *Ppif*$^{-/-}$ mitochondria (164 ± 17 μM) and was comparable to that obtained in wild-type mitochondria treated with 1 μM CsA (148 ± 17 μM), indicating that CsA totally inhibited CypD in these mitochondria (Fig. 2, C and D). C31 (100 μM) exhibited significantly greater CRC. These results show that a CypD-independent mechanism participates in mPTP inhibition by C31 in addition to the CypD-dependent mechanism.

C31 Inhibits mPTP Opening in Cells and Delays Hypoxia Reoxygenation–Induced Cell Death. We then investigated the protective effect of C31 in whole cells. In the first step, rat cardiomyoblast H9C2 cells were loaded with 1 μM calcein in the presence of 1 mM $CoCl_2$ for 30 minutes at 37°C. Cells were treated with DMSO (0.1%), increasing concentrations of CsA (0.2, 2, and 5 μM) or C31 (20, 50, and 100 μM). Then mPTP opening was induced by exposing cells to the Ca^{2+} ionophore A23187 (50 nM), which resulted in a drop in calcein fluorescence (Fig. 3, B and C). Treatment with 2 μM CsA partially reduced the decrease in calcein fluorescence. This was the most effective concentration, and we did not observe any effect at 0.2 and 5 μM. This confirmed the studies indicating that CsA is only protective within a narrow concentration range (Halestrap et al., 2004). C31 inhibited calcein loss in a concentration-dependent manner and totally abrogated mPTP opening at 100 μM (Fig. 3, B and C).

Cytoprotective effect of C31 was then assessed in freshly isolated adult murine cardiomyocytes subjected to 45-minute hypoxia followed by 180-minute reoxygenation to mimic ischemia reperfusion. Freshly isolated adult cardiomyocytes were coloaded with calcein-AM, $CoCl_2$, and propidium iodide. They were electrically paced and imaged during the

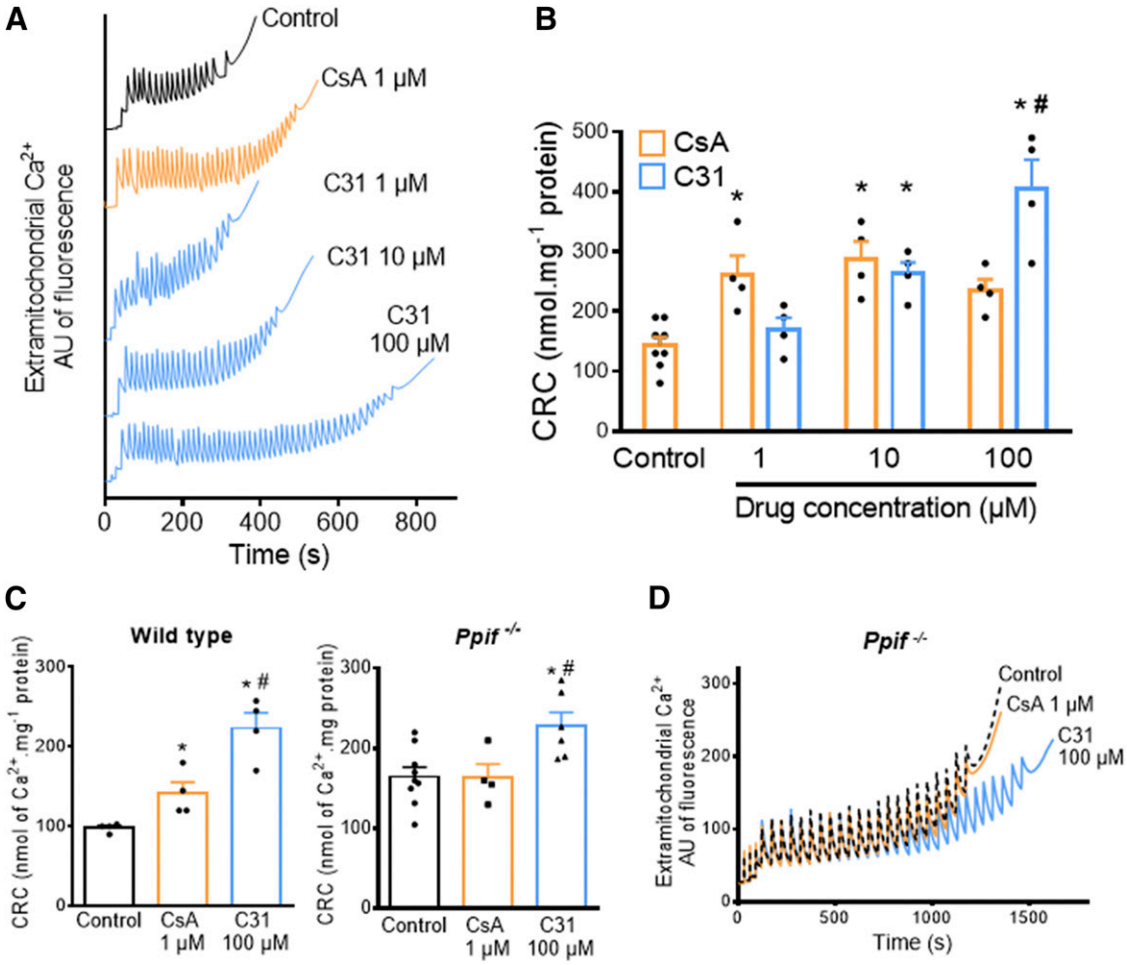


Fig. 2. C31 increases calcium retention capacity in cardiac mitochondria. Energized rat cardiac mitochondria were challenged with successive Ca^{2+} quanta (10 μM) until the load reached a threshold at which Ca^{2+} was released through mPTP. Then CRC was determined. Typical experiment (A) and bar graph (B) showing that treatment with increasing conc. of C31 allowed mitochondria to sustain higher Ca^{2+} amount than CsA. In the bar graph (B), each value is the mean ± S.E.M. of at least four independent experiments (different mitochondrial preparations) performed in duplicate. *$P < 0.05$ vs. control (no drug added); #$P < 0.05$ vs. CsA. (C and D) CRC was evaluated in cardiac mitochondria isolated from wild-type (C) or *Ppif*$^{-/-}$ mice (C and D) in the presence of CsA (1 μM) or C31 (100 μM). In the bar graph (C), each value represents the mean ± S.E.M. of at least four independent experiments (different mitochondrial preparations) performed in duplicate. Difference among groups was assessed by one-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$ vs. control (no drug added); #$P < 0.05$ vs. CsA. AU, arbitrary units

whole procedure to monitor mPTP opening and cell death. Hypoxic medium was supplemented with CsA (2 μM) or C31 (100 μM, 0.1% DMSO) 15 minutes before reoxygenation, and the compounds were further present in the perfusion medium during the first 10 minutes of reoxygenation (Fig. 4A). As shown in Fig. 4B, reoxygenation induced mPTP opening with a mean time for 50% mPTP opening (t_{mPTP50}) of 62 ± 13 minutes in control conditions. Surprisingly, in these cardiomyocytes, CsA was not able to delay mPTP opening (t_{mPTP50} = 55 ± 6 minutes) and cell death (Fig. 4, B and C), whereas CypD gene deletion delayed calcein loss and cell death in the same model (Panel et al., 2017). In accordance with the results obtained in H9C2 cells (Fig. 3), increasing CsA concentration to 5 or 10 μM did not afford more protection (unpublished data). In contrast, C31 delayed mPTP opening (t_{mPTP50} = 120 ± 8 minutes), and this was associated with a reduction in cell death (Fig. 4, B–D). These results demonstrate that C31 permeates cell membranes and protects cardiomyocytes from mPTP opening.

High CypD Inhibitor Concentrations Are Required to Inhibit mPTP Opening in Isolated Perfused Heart. The next part of this work was designed to study the ability of CypD inhibitors to reach CypD ex vivo using a mouse model of isolated perfused heart. In these experiments, wild-type mouse hearts were retrogradely perfused through the aorta with a perfusion medium containing DMSO (0.1%), CsA (2–10 μM), or C31 (100 μM). After a 20-minute perfusion, cardiac mitochondria were isolated, and CRC was evaluated in the presence or absence of 1 μM CsA added directly to the mitochondria in the medium, a CsA concentration totally inhibiting CypD (Fig. 2C). As shown in Fig. 5, control mitochondria retained 61.3 ± 5.5 μM Ca^{2+} before mPTP opening, and this retention doubled by adding 1 μM CsA (115.0 ± 2.9 μM) in the incubation medium. Interestingly, heart perfusion of 2 μM CsA only increased the Ca^{2+} retention capacity to 96.2 ± 6.2 μM (+56.9% vs. control value), which reflects a weak inhibition of CypD activity. CsA concentration had to be increased to 10 μM to obtain a full inhibition of

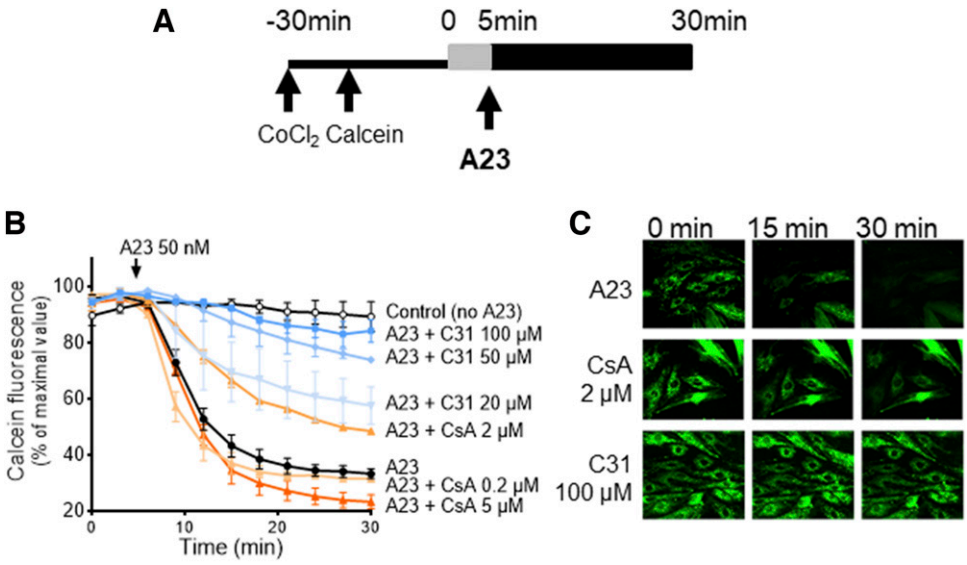
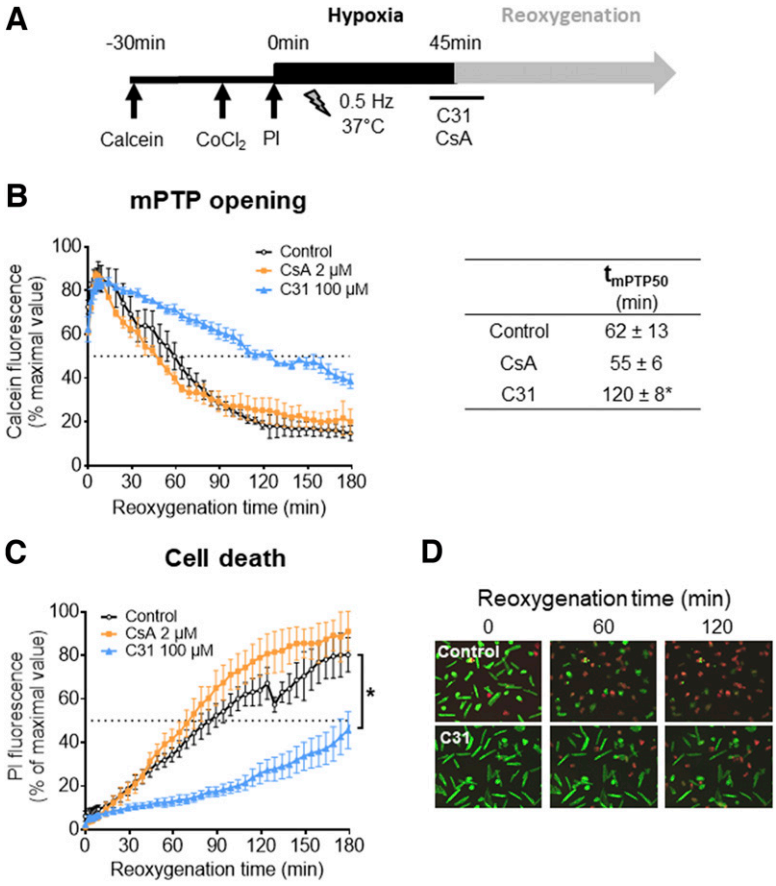


Fig. 3. C31 inhibits A23187-induced mPTP opening in H9C2 cells. (A) Experimental procedure: H9C2 cells were labeled with calcein-AM in the presence of $CoCl_2$ for 30 minutes and then placed in a thermostatic chamber for fluorescence imaging. After 5 minutes of incubation, A23187 (A23, 50 nM) was added to the medium to induce mPTP opening, and calcein fluorescence was monitored until 30 minutes. When used, C31 or CsA was incubated 20 minutes prior to A23187 addition. For each curve, three experiments were averaged. (B) Inhibition of mPTP opening by CsA and C31: kinetics of calcein fluorescence over reoxygenation time. (C) Typical experiment showing the change of calcein fluorescence in the absence (control) or in the presence CsA or C31.

CypD-dependent mPTP opening. In these conditions, perfusion with 100 µM C31 allowed retention of 100.8 ± 4.5 µM Ca^{2+}, inducing a partial inhibition of CypD. These data demonstrate that CypD inhibitors are able to reach cardiac mitochondria when they are directly perfused to the heart. Nevertheless, a total inhibition of CypD required high concentrations of CsA, and it was impossible to obtain this effect with C31, even at the highest concentration obtainable in solution (100 µM, solubility limitation).

C31 Reaches Liver but Not Cardiac Mitochondria in a Mouse Model In Vivo. Based on our previous results demonstrating that C31 inhibits mPTP opening in mouse liver after in vivo administration (Panel et al., 2019), we questioned whether C31 was also able to inhibit mPTP in cardiac



	t_{mPTP50} (min)
Control	62 ± 13
CsA	55 ± 6
C31	120 ± 8*

Fig. 4. C31 inhibits mPTP opening and cell death in mouse isolated cardiac myocytes during hypoxia reoxygenation. (A) Experimental procedure: mouse cardiomyocytes were colabeled with the association calcein-AM–$CoCl_2$ and propidium iodide (PI) to assess mPTP opening and cell death, respectively. Electrically paced cells were subjected to 45 minutes hypoxia (1% to 2% O_2, 20 mM 2-deoxyglucose) followed by reoxygenation (21% O_2, 5.5 mM glucose) and were monitored during the whole procedure. C31 (100 µM) or CsA (2 µM) was added 15 minutes before reoxygenation and maintained during the first 10 minutes of reoxygenation. (B) Inhibition of mPTP opening by CsA and C31. Left curves: kinetics of calcein fluorescence over reoxygenation time. Right table: avg. time for mPTP opening (t_{mPTP50}). Each curve represents the mean ± S.E.M. of five experiments performed with five different cell preparations. Difference between groups was assessed by one-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$ vs. control. (C) Inhibition of cell death by CsA and C31 during reoxygenation monitored by the appearance of PI fluorescence. Each curve represents the mean ± S.E.M. of five experiments performed with five different cell preparations. Difference between groups was assessed by one-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$ vs. control. (D) Typical experiment showing the change of calcein (green) and propidium iodide (red) fluorescence during reoxygenation time in the absence (control) or in the presence of C31.

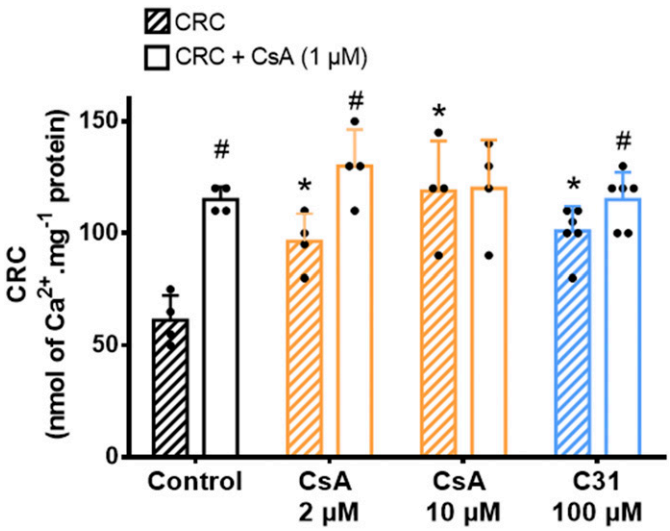


Fig. 5. Effect of C31 and CsA on mitochondrial calcium retention capacity after Langendorff perfusion of mouse hearts. Wild-type mouse hearts were cannulated and retrogradely perfused through the aorta. Perfusion pressure was set at 120 mm Hg to ensure coronary perfusion. Hearts received vehicle (control), C31 (100 μM), or CsA (2 and 10 μM) as a 20-minute perfusion. Then mitochondria were isolated, and CRC was evaluated in the absence (hatched bars) or in the presence of 1 μM CsA in the incubation medium as a control for maximal CypD-dependent mPTP inhibition (empty bars). CRC (hatched bars, without CsA) were compared by means of a nonparametric test Kruskall-Wallis multiple comparison followed by Dunnett's post hoc test. $^*P < 0.05$ vs. control value. To analyze the effect of CsA addition in the incubation medium, a two-way ANOVA with paired values (CRC and CRC + CsA) followed by Fisher's least significant difference test was used. $\#P < 0.05$ vs. respective CRC. Each CRC value is the mean ± S.E.M. of four to six independent experiments (four to six hearts).

mitochondria when administrated to living mice. C57BL/6J mice were infused with either vehicle, 20 mg.kg^{-1} CsA, or increasing concentrations of C31 ranging from 10 to 150 mg.kg^{-1} (i.e., the dose that was previously described as the most efficient in liver studies). Hearts and livers were excised 2 minutes after the end of infusion, and mitochondria were isolated. In C31-treated mice, CRC in cardiac mitochondria was not changed as compared with mice receiving vehicle, although liver mitochondria demonstrated increased CRC with both C31 and CsA (Fig. 6). The same lack of effect was observed when mice were treated with 20 mg.kg^{-1} CsA, as mitochondria exhibited CRC indistinguishable from vehicle. These results suggest that neither C31 nor CsA reached cardiac mitochondria in our experimental conditions.

Cytosolic Cyclophilins Are Not Responsible for the Absence of In Vivo Effect of CsA and C31. A possible factor hampering CypD inhibitors to reach cardiac mitochondrial matrix might rely on the presence of extramitochondrial cyclophilins, such as cyclophilin A, which is abundant in the cytosol (Wang and Heitman, 2005). We hypothesized that CsA or C31 could interact with these cytosolic cyclophilins, preventing their translocation to mitochondria. Thus, we evaluated PPIase activity of mitochondrial and cytosolic extracts of mouse liver and heart. Figure 7A shows that at the same protein concentration the rate of peptide isomerization was lower in mitochondria than in cytosols. This tends to indicate that the ratio of PPIase proteins over total protein is lower in mitochondria. Besides this difference, PPIase activity was similar in each compartment in both tissues (Fig. 7, B and C), indicating that a difference in PPIase activity cannot explain the discrepancy observed in vivo between the two organs.

Discussion

mPTP is thought to play a major role in myocardial ischemia-reperfusion injury. Indeed, the cellular conditions that prevail at reperfusion match those required to trigger mPTP opening. Ca^{2+} overload, oxidative stress, high phosphate concentrations, and adenine nucleotide depletion encountered by cardiomyocytes are known to induce translocation of CypD to membrane mPTP components, which will, in turn, favor opening of the pore. Inhibition of mPTP opening by targeting CypD results in decreased infarct size in numerous animal models and was translated to clinical studies. The phase

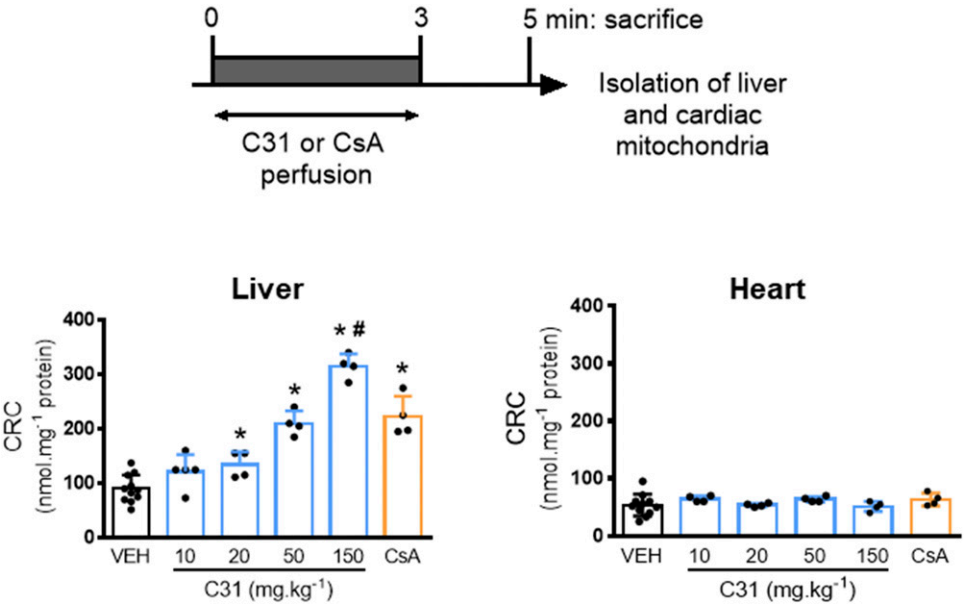


Fig. 6. Effect of in vivo administration of C31 on mitochondrial calcium retention capacity. Mice were infused with increasing doses of C31 or 20 mg/kg CsA for 3 minutes. Two minutes after the end of infusion, cardiac and liver mitochondria were isolated, and mitochondrial CRC was assessed. Each CRC value is the mean ± S.E.M. of at least four independent experiments (four animals). Difference among groups was assessed by one-way ANOVA followed by Tukey's multiple comparison test. $^*P < 0.05$ vs. vehicle (VEH); $\#P < 0.05$ vs. CsA.

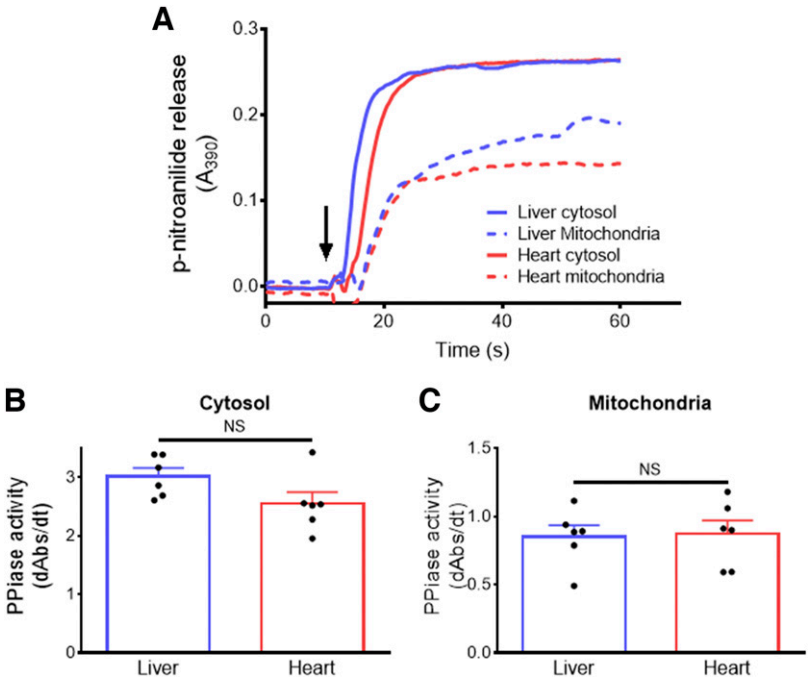


Fig. 7. Peptidylpropyl isomerase activity (PPIase) in cytosolic and mitochondrial fractions isolated from mouse heart and liver. (A) Typical curves showing PPIase activity monitored by the release of p-nitroanilide. Arrow: addition of *N*-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. (B and C) Each bar graph represents the mean ± S.E.M. of six independent experiments (six different mitochondrial or cytosolic preparations). Results are expressed as the maximal rate value of p-nitroanilide release. Unpaired student's *t* test; NS, not statistically significant.

III clinical trials CIRCUS (Cung et al., 2015) and CYCLosporinE A in Reperfused Acute Myocardial Infarction (CYCLE, Ottani et al., 2016) failed to confirm the beneficial effect of CsA previously observed (Piot et al., 2008). These results have been extensively commented on elsewhere, and several hypotheses have been proposed to explain CsA failure (Chen-Scarabelli and Scarabelli, 2016; Monassier et al., 2016). Nevertheless, CypD inhibition and, more broadly, mPTP blockade remain major goals to achieve and interesting drug targets.

The present study shows that C31, a small-molecule cyclophilin inhibitor derived from phenyl-pyrrolidine, inhibits mPTP opening in mouse cardiac mitochondria. Interestingly, high concentrations of C31 still increase Ca^{2+} retention capacity in mitochondria issued from *Ppif*$^{-/-}$ mice. This indicates that in addition to its CypD-inhibiting properties, C31 inhibits mPTP opening through another mechanism that remains to be elucidated. This compound is therefore of particular interest because it escapes the limitations imposed by the regulatory role of CypD in mPTP function. Indeed, mPTP opening can still occur in the absence of CypD (Baines et al., 2005) but also when the stress conditions imposed to the cell are strong, which limits this pharmacological strategy. Such stress conditions might have been encountered in our experimental conditions by isolated cardiomyocytes in the hypoxia-reoxygenation model. Indeed, C31 delayed mPTP opening and cell death, whereas CsA was totally ineffective on both phenomena. This suggests that C31 might be more effective in inducing cardioprotection than CsA.

The present study emphasizes a role for mitochondrial targeting and bioavailability of such inhibitors within the heart itself. Indeed, we observed that inhibition of mPTP required high CsA concentrations to fully inhibit CypD in a model of isolated perfused heart. This suggests that drug uptake by cardiomyocytes and mitochondrial delivery remains limited, even in the absence of other organs potentially extracting the compound from the blood. In the context of whole organism, only 5% of the cardiac output is distributed to the heart itself through coronary blood flow. In these conditions, only a low fraction of the administered compound reaches the myocardium and its mitochondrial compartment at the time of reperfusion (i.e., a fraction probably too low to permit protection of the cells). This is clearly demonstrated by our data showing that systemic administration of CsA and C31 inhibited mPTP opening in the liver but not in the heart. Therefore, systemic intravenous administration of mitochondrial protective agents might limit cardioprotection because of the massive uptake by the liver. This can explain, at least partly, the failure of the recent two large randomized clinical trials using CsA administered prior to percutaneous coronary intervention in patients (Davis et al., 2010; Cung et al., 2015). Thus, intracoronary administration of cardioprotective compounds at the time of percutaneous intervention might be more relevant to afford local protection within the area at risk.

Aside from myocardial drug distribution, other factors can influence mitochondrial targeting of the drugs. Proteins from the cyclophilin superfamily share the same catalytic site that is targeted by CsA and other cyclophilin inhibitors (Davis et al., 2010; Dunyak and Gestwicki, 2016). Therefore, exogenous inhibitors have to cross cell compartments filled with other cyclophilins than CypD before reaching mitochondrial matrix and thus might be entrapped outside mitochondria. This could limit mPTP inhibition and also participate in the lack of cardioprotection observed with CsA in recent clinical trials (Cung et al., 2015; Ottani et al., 2016). This is why we questioned whether proteins other than CypD exhibiting PPIase activity might hamper mitochondrial targeting. The present study showed that mitochondria exhibited lower PPIase activity than cytosol, reinforcing the idea of a possible entrapment by extramitochondrial cyclophilins. Thus, our data underline the urge to develop small inhibitors directly targeted to mitochondria. Such strategy has been mainly used

for antioxidant agents (Silva et al., 2016) and can be obtained, for instance, by coupling compound with lipophilic cations, such as triphenylphosphonium, which will accumulate in mitochondria in response to the membrane potential. A demonstration of the efficacy of this strategy was brought by Crompton's team who successfully coupled CsA to triphenylphosphonium cation. This resulted in CsA accumulation in mitochondria and a decrease in the concentration required to inhibit mPTP as well as a lower cellular toxicity (Malouitre et al., 2009; Dube et al., 2012). Another strategy consists of the use of nanoparticles filled with protective compound. Previous work using nanoparticle-mediated mitochondrial targeting of CsA demonstrated that the compound accumulates predominantly in the area at risk, enhancing cardioprotection (Ikeda et al., 2016).

In conclusion, our results demonstrated that C31 is a strong inhibitor of mPTP opening in the myocardium. C31 combines both CypD-dependent and CypD-independent inhibitory effects, suggesting that it might be more effective than CsA at inducing cardioprotection. Its low cardiac bioavailability limits its use in vivo, but optimization of this phenylpyrrolidine derivative aiming at increasing its metabolic stability and affinity might bring new interesting candidates to protect the heart against ischemia-reperfusion injury. This study also reveals that myocardial drug distribution may play a key role in the failure of mitochondrial agents to protect the myocardium, with distribution parameters being often overlooked.

Authorship Contributions

Participated in research design: Panel, Ahmed-Belkacem, Ruiz, Pawlotsky, Ghaleh, Morin.

Conducted experiments: Panel, Ahmed-Belkacem, Ruiz, Morin.

Contributed new reagents or analytic tools: Guichou.

Performed data analysis: Panel, Ahmed-Belkacem, Ruiz, Morin.

Wrote or contributed to the writing of the manuscript: Panel, Ahmed-Belkacem, Ruiz, Guichou, Pawlotsky, Ghaleh, Morin.

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