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Small-molecule inhibitors of cyclophilins block opening of the mitochondrial permeability transition pore and protect mice from hepatic ischemia–reperfusion injury

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Abstract

Background & Aims: Hepatic ischemia–reperfusion injury is a complication of liver surgery that involves mitochondrial dysfunction resulting from mitochondrial permeability transition pore (mPTP) opening. Cyclophilin D (PPIF or CypD) is a peptidyl-prolyl cis-trans isomerase that regulates mPTP opening in the inner mitochondrial membrane. We investigated whether and how recently created small-molecule inhibitors of CypD prevent opening of the mPTP in hepatocytes and the resulting effects in cell models and livers of mice undergoing ischemia–reperfusion injury.

Methods: We measured the activity of 9 small-molecule inhibitors of Cyps in an assay of CypD activity. The effects of the small-molecule CypD inhibitors or vehicle on mPTP opening were assessed by measuring mitochondrial swelling and calcium retention in isolated liver mitochondria from C57BL/6J (wild-type) and *Ppif*^{−/−} (CypD knock-out) mice, and in primary mouse and human hepatocytes by fluorescence microscopy. We induced ischemia–reperfusion injury in livers of mice given a small-molecule CypD inhibitor or vehicle before and during reperfusion, and collected samples of blood and liver for histologic analysis.

Results: The compounds inhibited peptidyl-prolyl isomerase activity (IC₅₀ values, 0.2 to 16.2 μM) and, as a result, calcium-induced mitochondrial swelling, by preventing mPTP opening (IC₅₀ values, 1.4 to 132 μM) in a concentration-dependent manner. The most potent inhibitor (C31) bound CypD with high affinity and inhibited swelling in mitochondria from livers of wild-type and *Ppif*^{−/−} mice (indicating an additional, CypD-independent effect on mPTP opening) and in primary human and mouse hepatocytes. Administration of C31 in mice with ischemia–reperfusion injury before and during reperfusion restored hepatic calcium retention capacity and oxidative phosphorylation parameters and reduced liver damage compared with vehicle.

KEY WORDS: drug, mitochondrial swelling, PPIase activity, mouse model.

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Conflict of interest disclosures: Inserm Transfert is the owner of patent EP 09306294.1 covering the family of cyclophilin inhibitors described, for which Abdelhakim Ahmed-Belkacem, Jean-François Guichou and Jean-Michel Pawlotsky are inventors. All other authors declare no competing financial interests.

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Authors contributions:

- Mathieu Panel: acquisition, analysis and interpretation of data; drafting of the manuscript; statistical analysis.
- Isaac Ruiz: contribution to the experimental design of murine ischemia-reperfusion experiments; acquisition, analysis and interpretation of data.
- Rozenn Brillet: acquisition, analysis and interpretation of data.

- 100 - Fouad Lafdil: contribution to the experimental design of murine ischemia-reperfusion
101 experiments; revision of the manuscript.
- 102 - Fatima Teixeira-Clerc: contribution to the experimental design of murine ischemia-
103 reperfusion experiments; revision of the manuscript.
- 104 - Cong Trung Nguyen: acquisition, analysis and interpretation of histological data.
- 105 - Julien Calderaro: acquisition, analysis and interpretation of histological data.
- 106 - Muriel Gelin: acquisition, analysis and interpretation of chemical data.
- 107 - Fred Allemand: acquisition, analysis and interpretation of chemical data.
- 108 - Jean-François Guichou: compound design and synthesis; generation of structural data;
109 revision of the manuscript.
- 110 - Bijan Ghaleh: study supervision; analysis and interpretation of data; drafting of the
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- 112 - Abdelhakim Ahmed-Belkacem: study supervision; acquisition, analysis and
113 interpretation of data; drafting of the manuscript.
- 114 - Didier Morin: study supervision; analysis and interpretation of data; drafting of the
115 manuscript.
- 116 - Jean-Michel Pawlotsky: study supervision; analysis and interpretation of data; critical
117 revision of the manuscript for important intellectual content.

118

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Methods: We measured the activity of 9 small-molecule inhibitors of CyPs in an assay of CypD activity. The effects of the small-molecule CypD inhibitors or vehicle on mPTP opening were assessed by measuring mitochondrial swelling and calcium retention in isolated liver mitochondria from C57BL/6J (wild-type) and *Ppif*^{−/−} (CypD knock-out) mice, and in primary mouse and human hepatocytes by fluorescence microscopy. We induced ischemia–reperfusion injury in livers of mice given a small-molecule CypD inhibitor or vehicle before and during reperfusion, and collected samples of blood and liver for histologic analysis.

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Conclusions: Recently created small-molecule inhibitors of CypD reduced calcium-induced swelling in mitochondria from mouse and human liver tissues. Administration of these compounds to mice during ischemia–reperfusion restored hepatic calcium retention capacity and oxidative phosphorylation parameters and reduced liver damage. These compounds might be developed to protect patients from ischemia–reperfusion injury after liver surgery or for other hepatic or non-hepatic disorders related to abnormal mPTP opening.

KEY WORDS: drug, mitochondrial swelling, PPIase activity, mouse model.

Introduction

Hepatic ischemia-reperfusion injury is a major complication of liver surgery, including liver resection, liver transplantation, and trauma surgery¹. Ischemia results from the interruption of the blood flow that perturbs the cellular metabolism as a result of oxygen shortage. Reperfusion restores the blood flow, oxygen delivery and tissue pH, thereby exacerbating cellular damage initiated during hypoxia or anoxia^{2, 3}. Hepatic ischemia-reperfusion injury is an important cause of liver dysfunction or functional failure post-liver surgery, which affects perioperative morbidity, mortality and recovery^{2, 3}. There are two types of ischemia: the most frequent form, warm ischemia, is observed when vascular occlusion occurs during hepatic resection surgery or during exposure to low-flow incidences such as trauma, hemorrhagic shock, cardiac arrest or hepatic sinusoidal obstruction syndrome; cold ischemia is observed exclusively during orthotopic liver transplantation, when the graft is subjected to hypothermic preservation before warm reperfusion³. Their mechanisms and main target cells differ. Hepatocytes are a major target of warm ischemia-reperfusion injury through anoxia, nutrition depletion and cytosolic acidosis³. In particular, sodium, chloride and calcium homeostasis are significantly altered.

Mitochondrial dysfunction plays a major role in hepatic ischemia-reperfusion injury³. Indeed, ischemia-reperfusion triggers the mitochondrial permeability transition, characterized by an increase in the permeability of the inner mitochondrial membrane mediated by the opening of a channel, called the “mitochondrial permeability transition pore” (mPTP)⁴. Once mitochondrial permeability transition initiates, solutes with a molecular mass of up to 1.5 kDa diffuse across the mitochondrial inner membrane, inducing mitochondrial depolarization, uncoupling and swelling, which in turn induce ATP depletion and necrotic (and, to a lesser extent, apoptotic) cell death³. During hepatic ischemia-reperfusion, mPTP opening is triggered by calcium-mediated mitochondrial reactive oxygen species formation. The same phenomenon

has been reported to play an important role during ischemia-reperfusion affecting other organs (including heart and brain)⁵⁻⁸, neurodegenerative diseases⁹ and drug-induced liver injury¹⁰.

Therapies avoiding the consequences of hepatic ischemia-reperfusion injury remain limited. Given the key role of mPTP opening in ischemia-reperfusion injury, pharmacological inhibition of mPTP opening is a promising target for new therapies. However, the molecular structure and functioning of mPTP remain largely unknown. Several proteins have been suggested to be involved in the structure of mPTP, including ATP synthase, the adenine nucleotide translocase, a phosphate carrier, and cyclophilin D (CypD)¹¹. Cyclophilins are peptidyl prolyl *cis/trans* isomerases (PPIases) that catalyze the interconversion of the two energetically preferred conformers (*cis* and *trans*) of the planar peptide bond preceding an internal proline residue. Seventeen human cyclophilins have been identified, but the function of most of them is unknown^{12,13}. CypD is located within the mitochondrial matrix where it acts as a key component and regulator of the mPTP; mPTP formation appears to be catalyzed or stabilized by CypD through lowering of its calcium threshold¹⁴. Thus, CypD represents an attractive target for mPTP opening inhibition and cellular protection in the context of hepatic ischemia-reperfusion injury.

Cyclosporine A (CsA) and its derivatives (together with sanglifehrin A) are known macromolecular CypD ligands. They have been shown to efficiently desensitize mPTP opening in various cellular and *in vivo* models¹⁵⁻¹⁸. However, CsA is a potent immunosuppressor and its non-immunosuppressive derivatives suffer from many disadvantages, including their size, complex multistep synthesis, cell toxicity and lack of chemical plasticity. Thus, potent cyclophilin inhibitors unrelated to CsA or sanglifehrin A and lacking the disadvantages of non-immunosuppressive macromolecular CsA derivatives are needed.

By means of a fragment-based drug discovery approach based on X-ray crystallography and nuclear magnetic resonance, we generated a new family of nonpeptidic small-molecule cyclophilin inhibitors (SMCypIs) unrelated to CsA or sanglifehrin A, with potent inhibitory

activity against CypA, CypB and CypD¹⁹. These compounds lack cellular toxicity and immunosuppressive activity and bear druggable pharmacological properties¹⁹. In this study, we assessed the ability of the new SMCypIs to inhibit liver mitochondria mPTP opening through CypD inhibition, studied their mechanisms of inhibition, and evaluated *in vivo* their protective properties in the context of experimental hepatic ischemia-reperfusion injury.

Materials and Methods

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). SMCypIs were synthesized as previously described¹⁹. Primary human hepatocytes were obtained from Biopredict International (Saint-Grégoire, France).

Animals

Male C57BL/6J mice (8 to 10-week-old) were purchased from Janvier (Le Genest-St-Isle, France). *Ppif*^{−/−} mice (i.e., CypD knock-out mice) were obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were co-housed in an air-conditioned room with a 12-h light-dark cycle and received standard rodent chow and drinking water *ad libitum*. All animal procedures in this study were in strict accordance with the Directives of the European Parliament (2010/63/EU-848 EEC) and approved by the Animal Ethics Committee ANSES/ENVA/Université Paris-Est Créteil (approval numbers 09/12/14-02 and 11/10/16-01).

Molecular modeling and docking of C31 into CypD

Molecular modeling and docking experiments were performed by means of the @TOME-2 server²⁰. In each structural model, the boundaries of the active site were deduced from the vicinity of co-crystallized ligands (compounds C30, C27, and C24 selected as templates, with PDB accession numbers 4J5C, 4J5B and 4J5E, respectively) using @TOME-2 comparative option. They were used to guide docking in automatically computed models. The ligand files were generated with MarvinSketch 6.2.2 for SMILES and Frog2 server for mol2²¹. The Figure was generated by means of Pymol.

CypD PPIase enzyme assay

Inhibition of CypD PPIase activity was measured at 20°C by means of the standard chymotrypsin coupled assay. The assay buffer (25 mM HEPES, 100 mM NaCl, pH 7.8) and purified CypD (1900 nM stock solution) were pre-cooled at 4°C in the presence of serial dilutions of the SMCypIs. Five µL of 50 mg/mL chymotrypsin in 1 mM HCl was added. The reaction was initiated by adding 20 µL of 3.2 mM peptide substrate (N-succinyl-Ala-Ala-Cis-Pro-Phe-p-nitroanilide). P-nitroanilide absorbance was measured at 390 nm for 1 min. CsA was used as a positive control of PPIase inhibition. The percent inhibition of CypD PPIase activity was calculated from the slopes and the IC_{50s} were determined from percent inhibition curves using Sigmaplot software.

Isolation of liver mitochondria

Mouse livers were scissor-minced and homogenized on ice in a buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 4 mM EGTA, pH 7.4 at 4°C) using a Potter-

Elvehjem glass homogenizer in a final volume of 10 mL. The homogenate was centrifuged at 1000 *g* for 5 min at 4°C. The supernatant was centrifuged at 10,000 *g* for 10 min at 4°C. The mitochondrial pellet was resuspended in 600 µl of homogenization buffer without EGTA and protein concentration was determined.

Measurement of mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was measured with a Clark-type electrode fitted to a water-jacketed reaction chamber (Hansatech, Cergy, France). Mitochondria (1 mg protein/mL) were incubated at 30°C in a respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES and 5 mM KH₂PO₄ at pH 7.4. Respiration was initiated by the addition of glutamate/malate (5 mM each). After 1 min, ATP synthesis was induced by the addition of 1 mM ADP (state 3 respiration rate), and 1 µM carboxyatractyloside was added to measure the substrate-dependent respiration rate (state 4). The respiratory control ratio (state 3/state 4) was then calculated.

Mitochondrial swelling assays

Mitochondrial swelling was assessed by measuring the change in absorbance at 540 nm (A₅₄₀) using a Jasco V-530 spectrophotometer equipped with magnetic stirring and thermostatic control. Mitochondria (0.5 mg/mL) energized with pyruvate/malate (5 mM each) were incubated 30 sec in the respiration buffer before the induction of swelling with 10 mM phosphate, 100 µM atractyloside or 40 µM *tert*-butyl hydroperoxide in the presence of 50 µM of CaCl₂.

In de-energized conditions, mitochondria (0.5 mg/mL) were incubated 1 min at 30°C in a swelling buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES, 5 mM KH₂PO₄, 1

μM rotenone, and 1 μM antimycin at pH 7.4. Then, 50 μM or 100 μM CaCl₂ was added 1 min before swelling induction with 1 μM of A23187 or 10 μM phenylarsine oxide, respectively.

In both conditions, SMCypIs or CsA were introduced at the beginning of the incubation period.

Measurement of the calcium retention capacity of isolated mitochondria

Mitochondria were loaded with increasing concentrations of calcium until mPTP opening and fast calcium release occurred²². Liver mitochondria (1 mg/mL) energized with 5 mM glutamate/malate were incubated in the respiration buffer supplemented with 1 μM Calcium Green-5N fluorescent probe. The calcium concentration in the extra-mitochondrial medium was monitored by means of a Jasco FP-6300 spectrofluorimeter (Jasco, Bouguenais, France) at excitation and emission wavelengths of 506 and 532 nm, respectively. The calcium signal was calibrated by addition of known calcium amounts to the medium.

Isolation of primary mouse hepatocytes

Murine hepatocytes from C57BL6 animals were isolated by portal vein perfusion of collagenase²³. Freshly isolated hepatocytes were cultured in DMEM supplemented with 10% fetal calf serum, 10 U/mL penicillin, 10 μg/mL streptomycin, 10 μg/mL insulin, 5.5 μg/mL transferrin and 5 ng/mL sodium selenite. Four hours post-perfusion, the medium was removed and fresh medium, supplemented with 0.1 μM dexamethasone and 50 ng/mL epidermal growth factor, was added.

Measurement of mPTP opening in primary mouse and human hepatocytes

mPTP opening was assessed in primary mouse and human hepatocytes by inducing mitochondrial localization of calcein fluorescence²⁴. Cells were loaded with 2 mM CoCl₂ at 30°C in 1 mL of Tyrode's solution (NaCl 130 mM, KCl 5 mM, HEPES 10 mM, MgCl₂ 1 mM, CaCl₂ 1.8 mM, pH 7.4 at 37°C) for 30 min. After 10 min, the cells were supplemented with 1 μM calcein acetoxymethyl ester, washed free of calcein and CoCl₂, placed in a thermostated chamber and incubated in Tyrode's solution. After 5 min, 2 μM of calcium ionophore A23187 was added to induce mPTP opening²⁵. When specified, either C31 or CsA was added at the beginning of the incubation period.

Cells were imaged with an Olympus IX-81 motorized inverted microscope equipped with a mercury lamp as the light source for epifluorescence illumination, combined with a 12-bit cooled Hamamatsu ORCA-ER camera; 460-490 nm excitation and 510 nm emission filters were used for detection of calcein fluorescence. Images were acquired every min for 30 min after 30 ms of illumination per image using a digital epifluorescence imaging software (Cell M, Olympus, Rungis, France). Fluorescence from each cell in each region of interest ($\approx 80 \mu\text{m}^2$) was measured and background fluorescence was subtracted. Average fluorescence changes per field were calculated. Fluorescence was normalized to the maximal fluorescence value.

Ex vivo assessment of mPTP opening in mice treated with C31

The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (80 mg/kg). Increasing doses of C31 (10, 20, 50 and 150 mg/kg) or vehicle were randomly administered as a 3-min infusion through the jugular vein. Two min after the end of the infusion, the livers were excised and mitochondria were isolated to measure their capacity to retain calcium, as a marker of the ability of C31 to inhibit mPTP opening *in vivo*.

In vivo assessment of the mito- and hepatoprotective effect of SMCypIs in the context of hepatic ischemia-reperfusion

The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (80 mg/kg), intubated and mechanically ventilated. After section of the liver ligaments, hepatic normothermic ischemia of segments I to V was induced for 60 min by hilum clamping of the hepatic pedicle. Reperfusion occurred at removal of the clamp. One min before reperfusion, C31 or the vehicle was infused through the jugular vein and the infusion was maintained for the first 8 min of reperfusion.

Two sets of experiments were conducted. In the first set, livers were excised after 10 min of reperfusion and mitochondria were isolated for *ex vivo* experiments. In the second set, reperfusion was stopped after 60 min; 500 μ L of blood was collected to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, while livers were excised, formalin-fixed, paraffin-embedded and liver sections were stained with hematoxylin and eosin. “Hepatocyte clarification”, a histological feature of severe hepatocellular damage characterized by hepatocyte ballooning degeneration, was measured on whole sections of mice livers. Histological slides were scanned at an x20 magnification by means of an Aperio Slide Scanner (Leica Biosystems, Shanghai, China). Slides in “.svs” format were analyzed by means of Qupath open source software²⁶. Areas with hepatocyte ballooning were then manually annotated independently by two pathologists specialized in liver diseases.

Statistical analysis

Results are expressed as mean \pm SEM of at least 5 independent experiments. Statistical analysis was performed using one-way ANOVA followed by the Scheffé *post-hoc* test. Differences were considered significant for $p < 0.05$.

Results

SMCypIs bind CypD and inhibit its PPIase activity

We recently created a new family of small-molecule, nonpeptidic cyclophilin inhibitors (SMCypI)¹⁹. As shown in Supplementary Table 1 and Supplementary Figure 1, 9 members of the new SMCypI family, including compound C22, compounds C23 to C27 and compounds C29 to C31 (which all resulted from structure-guided chemical optimization of C22), inhibited CypD PPIase activity in an enzyme assay, with IC₅₀ values ranging from 0.2 to 16.2 μM. The most potent PPIase inhibitor was C31, with an IC₅₀ of 0.2±0.08 μM. Molecular modeling and docking experiments revealed that C31 binds both the catalytic site and the gatekeeper pocket of CypD (Figure 1A).

SMCypIs inhibit calcium-induced swelling as a result of the inhibition of CypD PPIase activity in isolated mouse liver mitochondria

As shown in Figure 1B, 1 μM CsA, used as a positive control of CypD inhibition, fully inhibited calcium-induced swelling of energized isolated mouse liver mitochondria. The 9 SMCypIs also inhibited mitochondrial swelling in a concentration-dependent manner (Supplementary Table 1). The most effective compound was C31, with an IC₅₀ of 1.4±0.2 μM (Figure 1B). SMCypI inhibition of calcium-induced mitochondrial swelling strongly correlated with SMCypI inhibition of CypD PPIase activity ($r=0.8535$; $p<0.004$; Figure 1C).

These results indicate that the new SMCypI family inhibits calcium-induced mitochondrial swelling as a result of CypD PPIase activity inhibition by binding to both the

PPIase catalytic site and the gatekeeper pocket of CypD. C31, the most potent SMCypI, was selected for subsequent experiments.

SMCypI inhibition of mitochondrial swelling is due to the inhibition of mPTP opening

To assess whether mitochondrial swelling inhibition by SMCypIs is related to the inhibition of mPTP opening, swelling was induced by different known mPTP opening inducers. As shown in Figures 2A and 2B, C31 inhibited mitochondrial swelling induced by both the pro-oxidant drug *tert*-butyl hydroperoxide and carboxyatractyloside, a ligand of adenine nucleotide translocase, in energized conditions.

Because mitochondrial parameters, such as the membrane potential or the ATP/ADP ratio, can modulate mPTP opening under energized conditions, thereby influencing the observed effect of the inhibitors, the inhibitory effect of C31 was analyzed under de-energized conditions, i.e., in the absence of substrate. Mitochondria were incubated in the presence of rotenone and antimycin to fully block the respiratory chain and supplemented with the calcium ionophore A23187 or the cross-linking thiol agent phenylarsine oxide in the presence of calcium. In these conditions (Figure 2C and 2D, respectively), mitochondrial swelling was slower than under energized conditions, but still inhibited by SMCypI C31.

These results indicate that SMCypI compound C31 inhibits mitochondrial swelling by inhibiting mPTP opening.

SMCypI compound C31 inhibits mPTP opening in both primary mouse and human hepatocytes

Primary mouse hepatocytes were loaded with calcein in the presence of CoCl₂ and mPTP opening was induced by the addition of 1μM A23187 (Figure 3A). As shown in Figure

3B, CsA inhibited the drop in calcein fluorescence associated with mPTP opening by only 55.4±0.8% at 30 min at the most effective concentration of 2 µM. C31 inhibited the drop in calcein fluorescence in a concentration-dependent manner, a result indicating that C31 inhibits mPTP opening in living mouse liver cells (Figure 3B). The inhibitory effect of C31 was stronger than that of 2 µM CsA, with full inhibition of mPTP opening obtained with 100 µM of C31.

The same experiments were performed with primary human hepatocytes (Figure 3C). CsA inhibited mPTP opening by approximately 50% at the most effective concentration of 0.2 µM. C31 inhibited mPTP opening in a concentration-dependent manner in primary human hepatocytes, with full inhibition achieved at the concentration of 100 µM (Figure 3C). This indicated that results obtained in mouse liver cells are representative of the human liver situation.

SMCypI compound C31 is more potent than CsA at increasing mitochondrial calcium retention capacity in isolated mouse mitochondria

The calcium retention capacity of isolated mouse mitochondria (i.e., the maximal calcium load achievable in the presence of a drug before mPTP opens) was measured, as previously described²². As shown in Figures 4A and 4B, 6±1 additions of 20 µM calcium, corresponding to 109±15 nmol/mg of mitochondrial proteins, were required to induce mPTP opening in the absence of CypD inhibitors. One µM of CsA increased the mitochondrial calcium retention capacity by 3-fold (337±47 nmol/mg of mitochondrial proteins). C31 at concentrations up to 100 µM also increased the mitochondrial calcium retention capacity. The maximum effect of C31 was markedly greater than that of CsA (550±37 nmol/mg of mitochondrial proteins; Figures 4A and 4B). C31 also more potently increased mitochondrial calcium retention capacity than CsA when the respiratory chain was fed with succinate in the

presence of rotenone, indicating that this effect is independent from activated respiratory complex I or II (data not shown).

Maximum mitochondrial calcium retention capacities of the same order were achieved in the presence of 10 μ M of C31 and 1 μ M of CsA, respectively. At these concentrations, that correspond to approximately 10 times the respective compound IC₅₀ values, CypD saturation is achieved. The combination of both C31 and CsA at the same concentrations did not result in any additive effect (Figure 4B), a result in keeping with them sharing CypD as a target.

Together, these results indicate that SMCypI compound C31 inhibits mPTP opening at least partly through CypD inhibition, and that this inhibition is more potent than that achieved by CsA.

The additional effectiveness of C31 as compared to CsA in inhibiting mPTP opening is unrelated to CypD inhibition

The mitochondrial calcium retention capacity in the presence of 100 μ M C31 was not modified by the addition of 1 μ M CsA (Figure 4B). This suggests that the greater capacity of C31 to inhibit mPTP opening as compared to CsA is unrelated to CypD inhibition. To verify this hypothesis, the effects of C31 and CsA were assessed on liver mitochondria isolated from *Ppif*^{-/-} mice, which have been knocked-out for CypD, in comparison with wild-type animals. As previously shown²⁷, the mitochondrial calcium retention capacity of *Ppif*^{-/-} mice was approximately 4-fold greater than that of wild-type animals (Figure 5A and 5B). CsA 1 μ M had no effect on *Ppif*^{-/-} mice mitochondrial calcium retention capacity. In contrast, C31 increased the calcium retention capacity by approximately 1.5-fold at concentrations >20 μ M in mitochondria isolated from *Ppif*^{-/-} mice livers (Figure 5A and 5B). The C31 concentrations at which this effect was observed were higher than those required to inhibit CypD PPIase activity and mitochondrial swelling and to enhance calcium retention capacity in wild-type mouse

mitochondria (see Figures 1 and 3 for comparison). In addition, C31 inhibited swelling induced by very high calcium concentrations in mitochondria isolated from *Ppif*^{-/-} mice (Figure 5C).

These results indicate that, in addition to blocking CypD, C31 interacts with another target at the mPTP level and that this interaction increases C31 blocking effectiveness on mPTP opening as compared to exclusive CypD inhibitors, such as CsA.

The additional effectiveness of C31 as compared to CsA in inhibiting mPTP opening is related to the chemical structure of its phenyl moiety

Comparison of the chemical structures of the SMCypIs (Supplementary Figure 1) suggested that the phenyl moiety of C31 is involved in this additional effect: indeed, SMCypI compound C34, that lacks the aromaticity of the cycle, lost the additional effect of C31 over CypD inhibition in isolated *Ppif*^{-/-} mouse liver mitochondria shown in Figure 5D.

The additional effectiveness of C31 as compared to CsA in inhibiting mPTP opening is unrelated to a ubiquinone-like effect or an interaction with the mitochondrial respiratory chain.

Ubiquinones have been shown to inhibit mPTP opening to a greater extent than CsA²⁸. The proximity between the urea and phenyl motifs of C31 would be compatible with a shared mPTP target with ubiquinones. As shown in Figure 5E, ubiquinone 0 (Ub₀), the most efficient ubiquinone, strongly increased the calcium retention capacity of liver mitochondria isolated from wild-type mice, but had no effect in liver mitochondria isolated from *Ppif*^{-/-} mice (Figure 5E). This result indicates that the additional, CypD-independent effect of C31 on mPTP opening is not ubiquinone-like.

Because it has been reported that inhibiting complex I with rotenone limits mPTP opening²⁹, we investigated whether C31 has an effect on respiratory chain functions. C31 did not alter substrate-dependent respiration rates or ADP-induced O₂ consumption, suggesting no C31-induced alteration of mitochondrial respiration (Supplementary Figure 2). No drop in the electron transport chain activity (lower $\Delta\Psi_m$ and slower calcium absorption) was detected in our experiments. Thus, the greater potency of C31 as compared to CsA in inhibiting mPTP opening is not related to an interaction with mitochondrial complex I or the respiratory chain.

SMCypI compound C31 inhibits mPTP opening in vivo in mice

Anesthetized mice were infused with increasing doses of C31 for 3 min and sacrificed 2 min later. Liver mitochondria were isolated and their calcium retention capacity was measured. Mitochondria isolated from C31-treated mouse livers exhibited higher calcium retention capacities than those from vehicle-treated mice, and this effect was dose-dependent. The effect of C31 was more potent than that of CsA (Figure 6A). This result, showing *in vivo* inhibition of mPTP opening by SMCypI compound C31, indicates that C31 reaches its mitochondrial target after infusion in living mice.

SMCypI compound C31 bears mito- and hepatoprotective properties during experimental liver ischemia-reperfusion in mice

Our final series of experiments aimed at demonstrating the protective effect of SMCypI compound C31 in an experimental murine model of hepatic ischemia-reperfusion injury. First, mice were subjected to 60 min of liver ischemia followed by 10 min of reperfusion. Ischemia-reperfusion reduced the calcium retention capacity of isolated mouse mitochondria by 59% (96.1±4.5 to 39.0±2.7 nmol/mg of protein; p <0.001). This reduction was associated with an

alteration of oxidative phosphorylation, as demonstrated by a 31% decrease of the rate of ADP-stimulated respiration ($p=0.0016$ vs Sham) and a 52% decrease of the respiratory control ratio ($p=0.0026$ vs Sham) (Figure 6B). Infusion of the most effective dose of C31 (150 mg/kg) 1 min before and during the first 8 min of reperfusion restored normal calcium retention capacity (84.9 ± 8.9 nmol/mg of protein). The protective effect of C31 also translated into a restoration of oxidative phosphorylation parameters (Figure 6B).

Secondly, the hepatoprotective effect of C31 was assessed in mice subjected to 60 min of liver ischemia, followed by 60 min of reperfusion. Blood samples were collected for ALT and AST level measurement and livers were excised for liver histology assessment (Figure 7A). C31 treatment significantly reduced ischemia-reperfusion-induced ALT elevation at the end of reperfusion (mean ALT levels: $2,297\pm 290$ versus $4,805\pm 1,088$ in C31-treated and vehicle-treated mice, respectively; $p=0.0375$) (Figure 7B). The effect of C31 on serum ALT levels was significantly greater than that of CsA, which was not significantly different from that of the vehicle (data not shown). C31 also substantially reduced AST elevation, but the difference did not reach significance (Supplementary Figure 3).

As shown in Figures 7C, 7D and 7E, C31 protected mouse livers from hepatic damages induced by ischemia-reperfusion. Indeed, 60 min after reperfusion, hepatic clarification areas that measure hepatocyte ballooning degeneration, a feature of acute liver injury (Figure 7D), covered a significantly smaller surface in C31-treated than in vehicle-treated mice ($30.4\pm 4.3\%$ versus $50.7\pm 3.2\%$ of liver section surface, respectively; $p=0.0012$). CsA also significantly reduced the surface of hepatic clarification in the model, to an extent similar to the effect of C31 (data not shown).

These findings indicate that SMCypI compound C31 exerts both mito- and hepatoprotective effects in mice exposed to ischemia-reperfusion.

Discussion

539

540 Hepatic ischemia-reperfusion injury is a major complication of liver surgery with
541 limited therapeutic options. Ischemia-reperfusion injury results in great part from hepatic
542 mitochondrial dysfunction related to abnormal mPTP opening. CypD is an important regulator
543 of the mPTP complex that plays a key role in its opening in several pathological conditions,
544 including hepatic ischemia-reperfusion^{11, 14}. Indeed, CypD translocation to the inner
545 mitochondrial membrane triggers mPTP opening and promotes cell death. Both effects can be
546 prevented by pharmacological CypD inhibitors or CypD knock-out^{27, 30-32}. Thus, CypD
547 represents an attractive target for inhibition of mPTP opening and hepatocellular protection in
548 the context of hepatic ischemia-reperfusion injury.

549 We designed a new family of SMCypIs made of two linked fragments binding the
550 cyclophilin catalytic and gatekeeper pockets, respectively¹⁹. Our aim was to develop CypA
551 inhibitors with antiviral activity against the hepatitis C virus. We showed that our SMCypIs
552 bind CypA and potently inhibit both CypA PPIase activity and the replication of hepatitis C
553 virus and related viruses in cell culture^{19, 33}. Because the different cyclophilins are structurally
554 very close (essentially differing by their cellular localizations and functions), we assessed
555 whether the new SMCypIs inhibit CypD activity. As shown here, their inhibitory activity was
556 potent and concentration-dependent. SMCypI compound C31 was the most potent CypD
557 inhibitor in our experiments. Its cytotoxic concentration 50% was >100 μ M in Huh7 cells,
558 suggesting a favorable therapeutic index¹⁹. We also showed that C31 binds both the catalytic
559 and gatekeeper pockets of Cyp D.

560 The ability of our SMCypIs to inhibit mPTP opening and its mitochondrial
561 consequences in a concentration-dependent manner correlated with their ability to block PPIase
562 activity. Inhibition of mPTP opening was observed in different models and conditions,
563 including energized and de-energized ones. This suggests that the SMCypIs act directly on
564 CypD and mPTP opening, downstream of the site of action of the inducers used in the

experiments. Importantly, this effect was observed in both human and mouse primary hepatocytes, a result validating the human relevance of our findings.

Although the CypD affinity of the SMCypIs was lower than that of CsA, C31 achieved more potent mPTP opening inhibition than CsA at its maximum soluble concentration in the medium. Because non-immunosuppressive derivatives of CsA, e.g., PKF220-384 or NIM811, have been shown to be equipotent to CsA in inhibiting mPTP opening³², these compounds are also likely to have a weaker effect on mPTP opening than C31 (not tested). Importantly, our findings suggest that the additional effect of C31 on mPTP opening as compared to CsA is independent from CypD inhibition. Indeed, first, the concentrations of CsA and C31 that fully inhibited CypD PPIase activity retained the same amount of calcium in mitochondria; secondly, CsA did not alter the maximal calcium retention capacity induced by C31; thirdly, C31 had an effect on calcium accumulation in liver mitochondria isolated from *Ppif*^{f/-} mice that do not express CypD.

The fact that CsA fully inhibited CypD PPIase activity in isolated mouse mitochondria (Supplementary Figure 3) suggests that the additional effect of C31 as compared to CsA is not related to its ability to inhibit PPIase activity. Because the structure and functioning of mPTP remain largely unknown, we could not identify the second target of C31 responsible for its greater potency as compared to CsA. We could however rule out an alteration of mitochondrial respiration²⁹ or a ubiquinone-like mechanism²⁸. Many potential components or regulators of the mPTP, such as the recently identified SPG7³⁴, could be involved, while many questions remain unanswered: is mPTP organized as a true physical channel? Is opening just a different state of the mitochondrial membrane with increased permeability? Which cellular components are actual constituents of the mPTP? Which ones only interact with it and/or regulate it? What are the mechanisms involved? Answering these questions was beyond the scope of our study, but we are confident that the new family of SMCypIs will be helpful in future mechanistic studies aimed at exploring these questions. Altogether, our result identify SMCypI C31 as a

particularly promising mPTP opening blocking agent, because its effect on mPTP opening related to CypD PPIase activity inhibition is enhanced by another complementary mechanism.

Key results in this study were obtained *in vivo* in murine models. We showed that C31 rapidly reaches its target after systemic administration. This is particularly important in the context of ischemia-reperfusion, because mPTP was reported to open within the first minutes of reperfusion after ischemia^{8, 35}. For this reason, proof-of-concept efficacy experiments were performed *in vivo* in a murine model of ischemia-reperfusion. The administration of C31 at reperfusion restored the mitochondrial function. In addition, ALT elevations and hepatic damage were significantly reduced in C31-treated mice, proving the hepatoprotective properties of the new SMCypI, as a result of mPTP opening inhibition.

In this study, liver damage was assessed by measuring “hepatocyte clarification”, i.e. hepatocyte ballooning degeneration, a feature of severe liver cell injury involving ATP depletion and a rise in intracellular calcium concentrations leading to a loss of cell volume control by plasma membranes and a disruption of the hepatocyte intermediate filament network. Hepatocyte ballooning degeneration has been observed in the early post-transplant period³⁶⁻³⁸³⁹, and described as an important morphological characteristic of ischemia-reperfusion injury measuring the degree of liver damage⁴⁰⁻⁴². As shown in Figure 7D in our model, hepatocyte ballooning degeneration was characterized by hepatocellular swelling, cytoplasm vacuolization and blebbing of the cell membrane. It was significantly improved by C31 administration in our murine model of ischemia-reperfusion injury. Because the half-life of C31 is short, requiring continuous infusion, it was not possible to determine how long the protective effects might last. Chemical, galenic and pharmacological optimization of the compounds is in progress and studies with more stable derivatives will be conducted to address this important question as part of future preclinical development.

In summary, we showed that our new family of small-molecule, non-peptidic cyclophilin inhibitors binds CypD and inhibits its PPIase activity, and that this effect is, at least

in part, responsible for their concentration-dependent inhibitory effect on calcium-induced swelling due to mPTP opening in both human and mouse hepatocytes. We also showed that the most potent SMCypI, compound C31, exerts an additional effect on mPTP opening, independent of its inhibitory effect on CypD, making it a promising pharmacological agent for hepatocellular protection in the context of diseases involving mitochondrial dysfunction related to abnormal mPTP opening. Finally, our study is the first to provide the *in vivo* proof-of-concept of mitochondrial and hepatocellular protection by SMCypI compound C31 in an experimental model of ischemia-reperfusion injury.

The new family of SMCypIs appears as a promising candidate for new drug development in the indication of hepatic protection in the context of warm ischemia-reperfusion after liver surgery. Whether similar protection can be obtained in the context of cold ischemia-reperfusion related to orthotopic liver transplantation remains to be assessed. Other applications in liver and non-hepatic diseases related to mPTP opening involving CypD deserve to be explored. They include chronic alcohol consumption, which enhances sensitivity to calcium-mediated mPTP opening and increases CypD expression⁴³, drug-induced liver injury, myocardial ischemia-reperfusion, brain injury and neurodegenerative disorders.

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Figure legends

Figure 1. (A) Molecular modeling and docking of SMCypI compound C31 bound to the catalytic site (left) and gatekeeper pocket (right) of CypD. **(B)** Concentration-dependent inhibitory effect of SMCypI C31 on isolated mouse liver mitochondrial swelling. Swelling was induced by 50 μ M calcium in the absence of inhibitors, in the presence of 1 μ M CsA (positive control of inhibition) or in the presence of increasing (0.1 to 10 μ M) concentrations of C31. No calcium was added in the negative control. The x axis indicates times of measurement; the y axis shows dynamic changes in absorbance at 540 nm (A_{540}) reflecting changes in mitochondrial swelling. **(C)** Relationship between SMCypI inhibition of CypD PPIase activity in an enzyme assay and swelling of isolated mouse liver mitochondria.

Figure 2. Inhibitory effect of SMCypI C31 on mitochondrial swelling triggered by mPTP opening inducers in isolated mouse mitochondria incubated in the presence of Ca^{2+} . The x axis indicates times of measurement; the y axis shows dynamic changes in absorbance at 540 nm (A_{540}) reflecting changes in mitochondrial swelling. **(A)** mPTP opening induced by 40 μ M *tert*-butyl hydroperoxide (*t*-BH) in energized mitochondria. **(B)** mPTP opening induced by 100 μ M carboxyatractyloside (CAT) in energized mitochondria. **(C)** mPTP opening induced by 1 μ M A23187 (A23) in non-energized mitochondria. **(D)** mPTP opening induced by 10 μ M phenylarsine oxide (PAO) in non-energized mitochondria.

Figure 3. Inhibition of mPTP opening by CsA and SMCypI compound C31 in primary mouse and human hepatocytes. **(A)** Experimental procedure: hepatocytes were loaded with calcein and $CoCl_2$ and mPTP opening was induced by the addition of 1 μ M of the calcium ionophore A23187 (A23), in the absence (control) or in the presence of CsA or of increasing concentrations of C31. Images were collected at 1-min intervals. Fluorescence was normalized

to 100% of the maximal value. The results of 4 to 7 experiments were averaged. **(B)** Inhibition of mPTP opening by CsA and SMCypI compound C31 in primary mouse hepatocytes. Left curves: kinetics of calcein fluorescence over time; right bar graph: calcein fluorescence measured at 30 min ($\#p < 0.05$ vs A23 alone; $*p < 0.05$ vs control; $\dagger p < 0.05$ vs CsA) **(C)** Inhibition of mPTP opening by CsA and SMCypI compound C31 in primary human hepatocytes. Left curves: kinetics of calcein fluorescence over time; right bar graph: calcein fluorescence measured at 30 min ($\#p < 0.05$ vs A23 alone; $*p < 0.05$ vs control (Ctrl); $\dagger p < 0.05$ vs CsA).

Figure 4. Calcium retention capacity of isolated mouse liver mitochondria in the presence of SMCypI compound C31 and of CsA. **(A)** Representative experiment showing mitochondrial calcium retention capacity in the presence of 1 μ M CsA or increasing concentrations of C31. Each fluorescence peak corresponds to the addition of 20 μ M calcium. **(B)** Average calcium concentrations required for mPTP opening, expressed as a percentage of the control value (100% represents 109 ± 15 μ M, as indicated by the dashed line). $*p < 0.05$ vs value observed with 0.1 μ M C31; $\dagger p < 0.05$ vs 1 μ M CsA. CsA: cyclosporine A; AU: arbitrary unit; CRC: calcium retention capacity; Ctrl: control.

Figure 5. Investigation of the mechanisms underlying the more potent effect of SMCypI compound C31 on mitochondrial calcium retention capacity, as compared to CsA. **(A)** Mean \pm SEM calcium retention capacity of isolated liver mitochondria from *Ppif*^{-/-} (CypD knocked-out) and wild-type mice. CRC: calcium retention capacity; WT: wild-type; $\#p < 0.05$ vs wild-type control; $*p < 0.05$ vs *Ppif*^{-/-} and wild-type controls, respectively; $\dagger p < 0.05$ vs CsA. **(B)** Representative experiment showing the calcium concentrations required for mPTP opening in liver mitochondria isolated from *Ppif*^{-/-} mice in the absence of compounds (control) or in the presence of 1 μ M CsA or 100 μ M C31. Each fluorescence peak corresponds to the addition of

20 μ M calcium. AU: arbitrary units. **(C)** Concentration-dependent C31 inhibition of mitochondrial swelling induced by 500 μ M calcium in liver mitochondria isolated from *Ppif*^{-/-} mice. A₅₄₀: absorbance at 540 nm. **(D)** Effect of 100 μ M C31 and C34 (a C31 derivative lacking the aromaticity of its phenyl moiety) on mitochondrial calcium retention capacity in isolated liver mitochondria from *Ppif*^{-/-} mice. Each fluorescence peak corresponds to the addition of 20 μ M calcium. AU: arbitrary units. **(E)** Left: representative experiments showing the effect of 50 μ M C31 and 50 μ M ubiquinone 0 on mPTP opening in liver mitochondria isolated from wild-type (top) and *Ppif*^{-/-} (bottom) mice. Each fluorescence peak corresponds to the addition of 20 μ M calcium. Right: Mean \pm SEM calcium retention capacity (CRC) in the corresponding experiments. WT: wild-type; Ubo: ubiquinone 0; AU: arbitrary units; **p* < 0.05 vs control (Ctrl).

Figure 6. *In vivo* effect of C31 on mPTP opening and mitochondrial alterations related to liver ischemia-reperfusion. **(A)** Anesthetized mice were infused with vehicle (VEH), CsA or different doses of C31 for 3 min and were sacrificed 2 min later. Liver mitochondria were isolated and the calcium retention capacities (CRC) of these mitochondria are shown. **p* < 0.05 vs VEH; #*p* < 0.05 vs CsA. **(B)** The mice were subjected to 60 min of a 70% partial liver ischemia, followed by 10 min of reperfusion, and received either 150 mg/kg C31 or vehicle (VEH). At the end of the reperfusion period, mouse livers were excised and mitochondria were isolated to assess the CRC (left) and mitochondrial respiration parameters (right), including the ADP-stimulated respiration rate (state 3), the substrate-dependent respiration rate (state 4) and the respiratory control ratio (RCR: state 3/state 4). **p* < 0.05 vs sham.

Figure 7. *In vivo* hepatoprotective effect of C31 in the context of liver ischemia-reperfusion. **(A)** The mice were subjected to 60 min of a 70% partial liver ischemia, followed by 60 min of reperfusion; they received either 150 mg/kg C31 or vehicle (VEH). At the end of the reperfusion period, blood samples and mouse livers were collected for assessment of liver damage. **(B)**

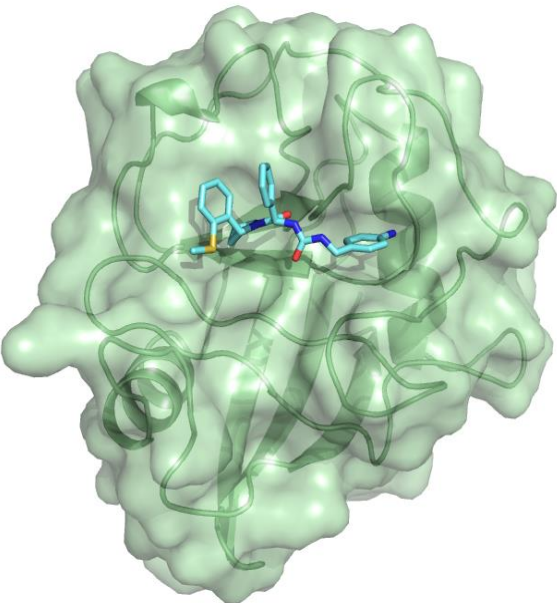
819 Mean±SEM ALT levels 60 min after reperfusion in VEH- and C31-treated animals; *p <0.05.
820 **(C)** Proportion of liver section surface occupied by hepatocyte clarification in VEH- and C31-
821 treated animals; *p <0.05. **(D)** Morphological alterations of hepatocyte clarification or hepatic
822 ballooning degeneration : A shows hepatocyte swelling and cytoplasmic clarification; B shows
823 cytoplasmic vacuolisation; C shows diffuse cell borders, an indirect feature of blebbing cell
824 membrane. **(E)** Representative hematoxylin-eosin-stained liver sections from VEH- and C31-
825 treated groups.

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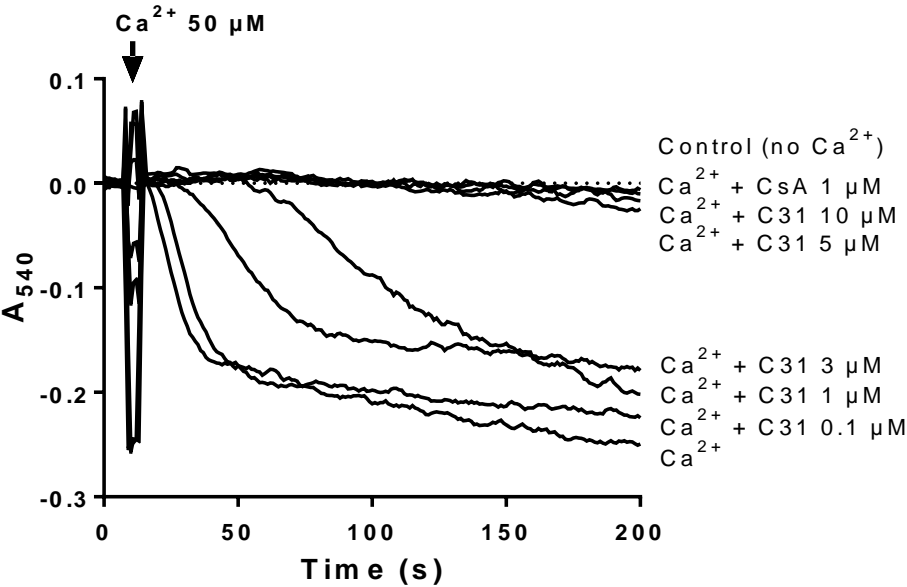
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Fig. 1

A



B



C

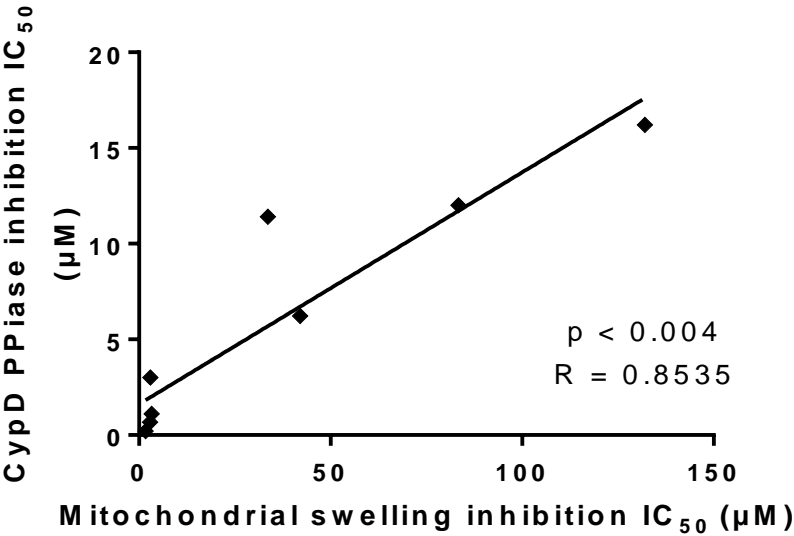
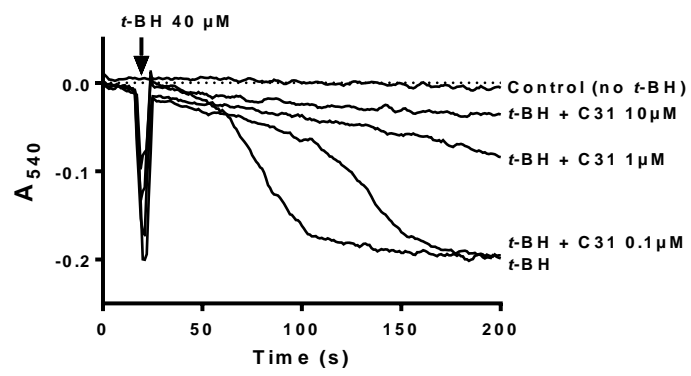
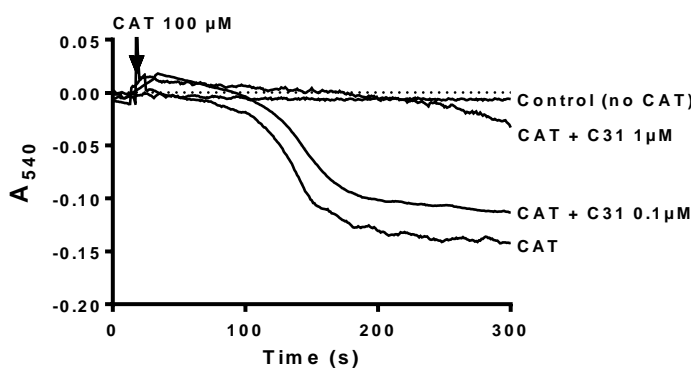


Fig. 2

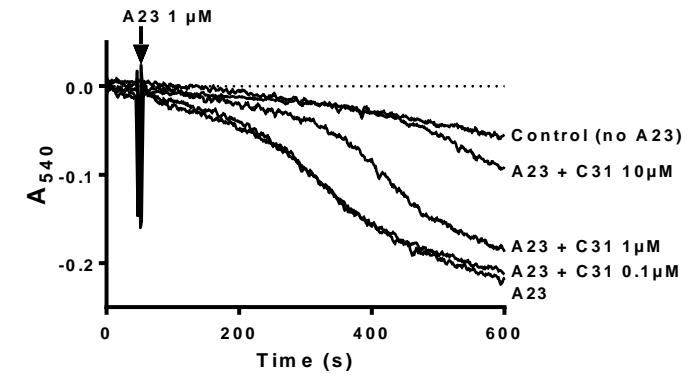
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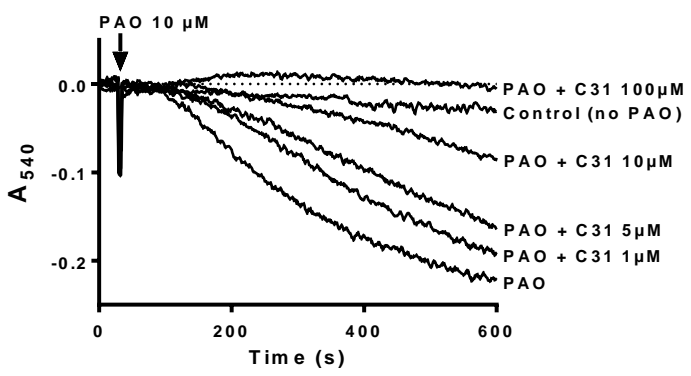


Fig. 3

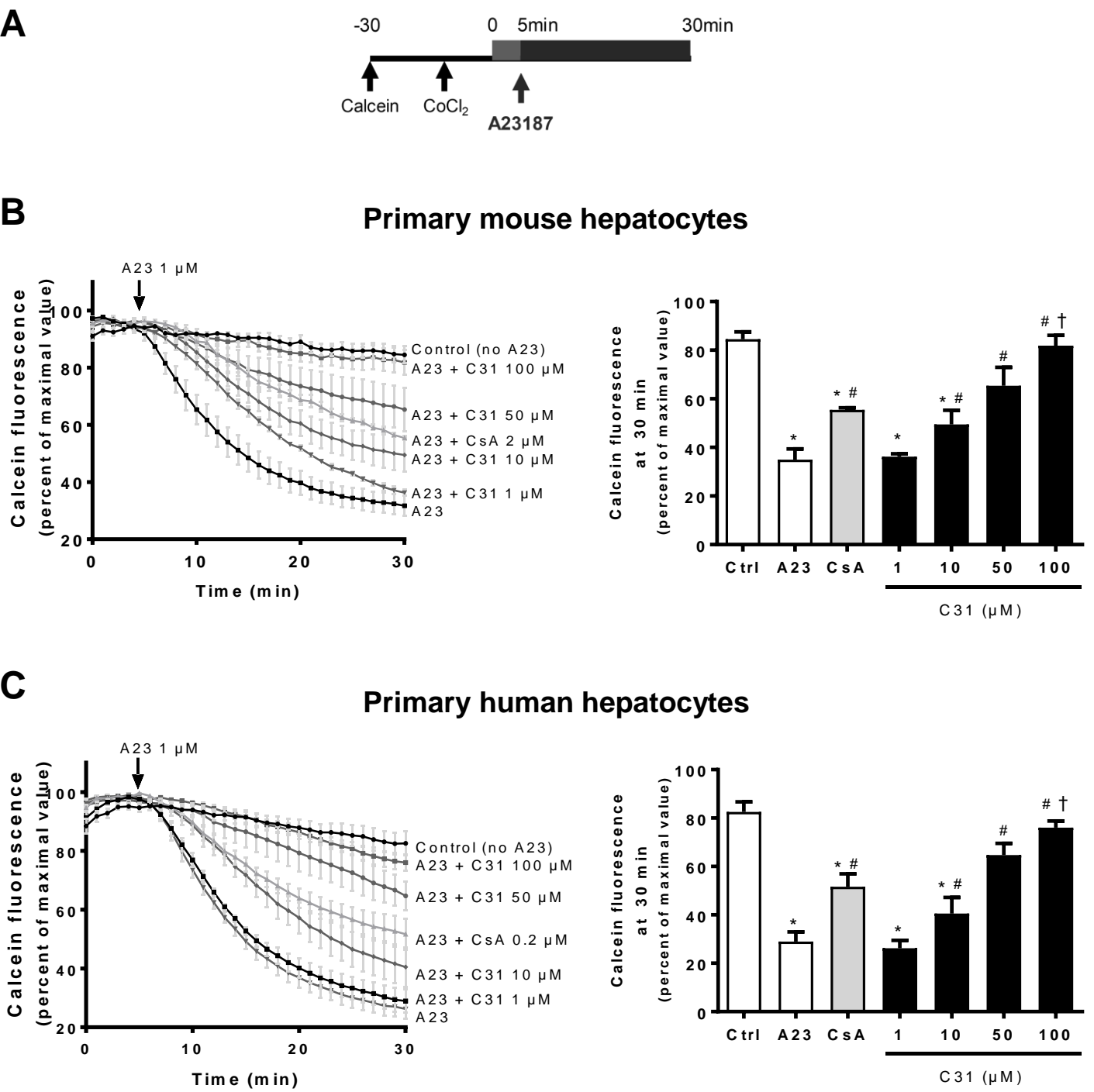


Fig. 4

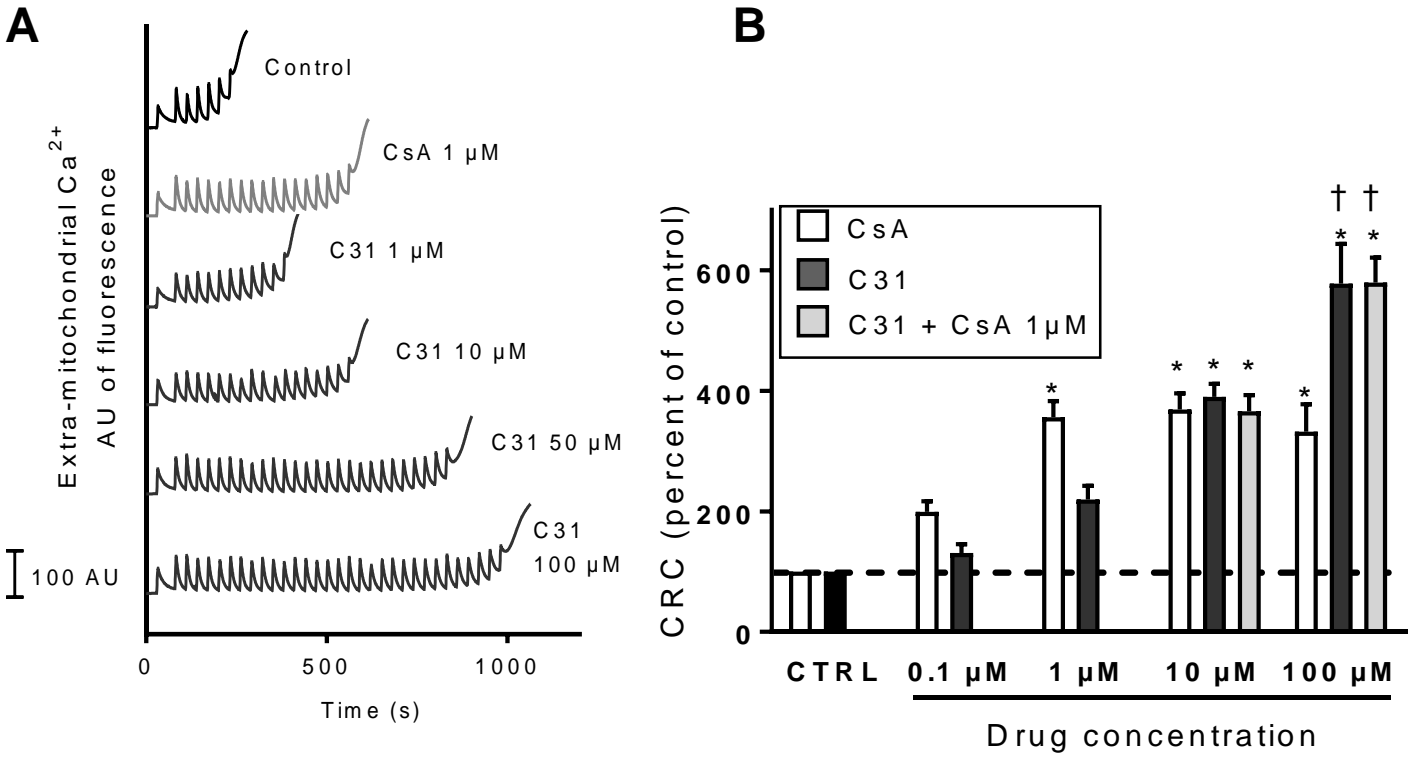


Fig. 5

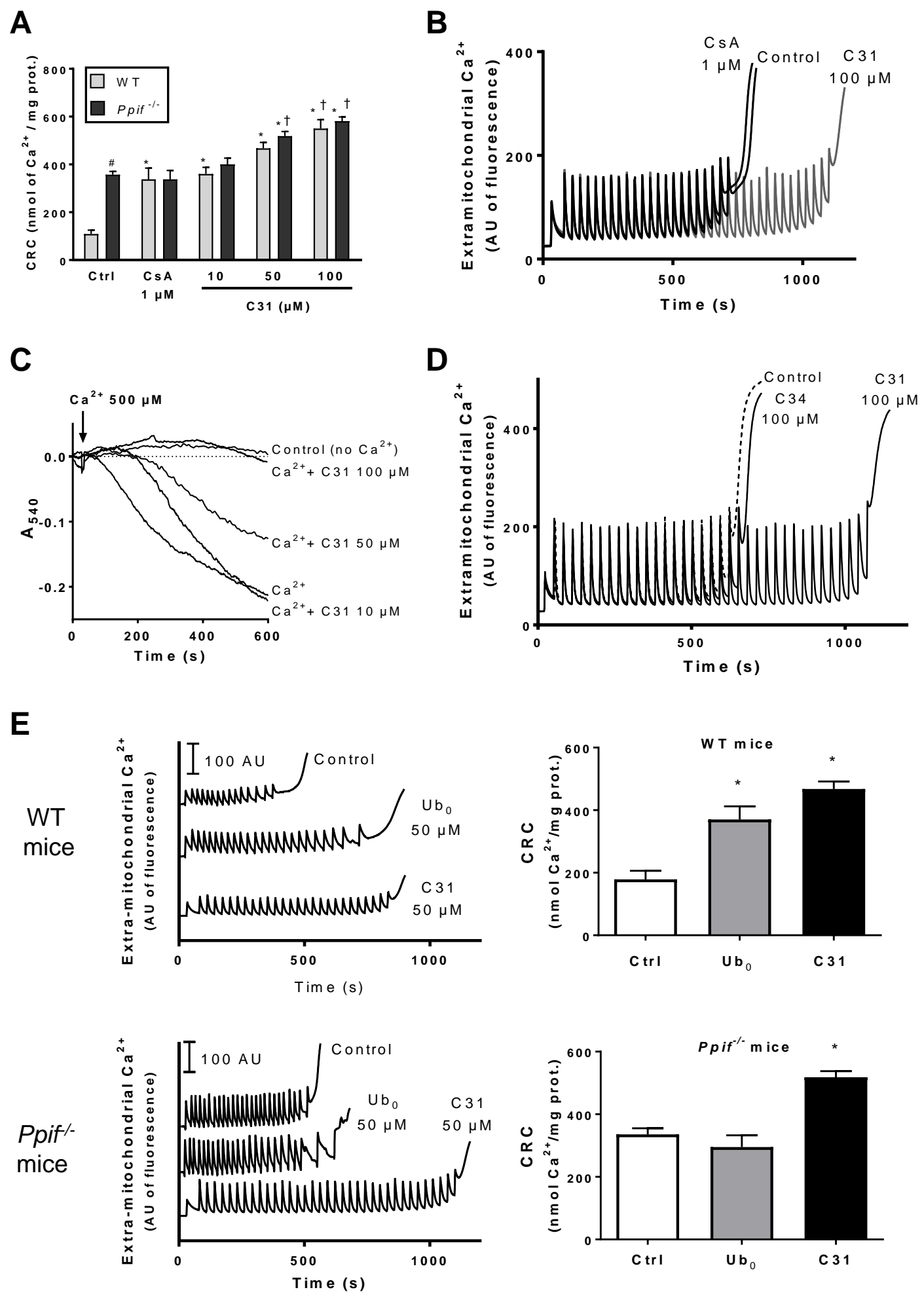


Fig. 6

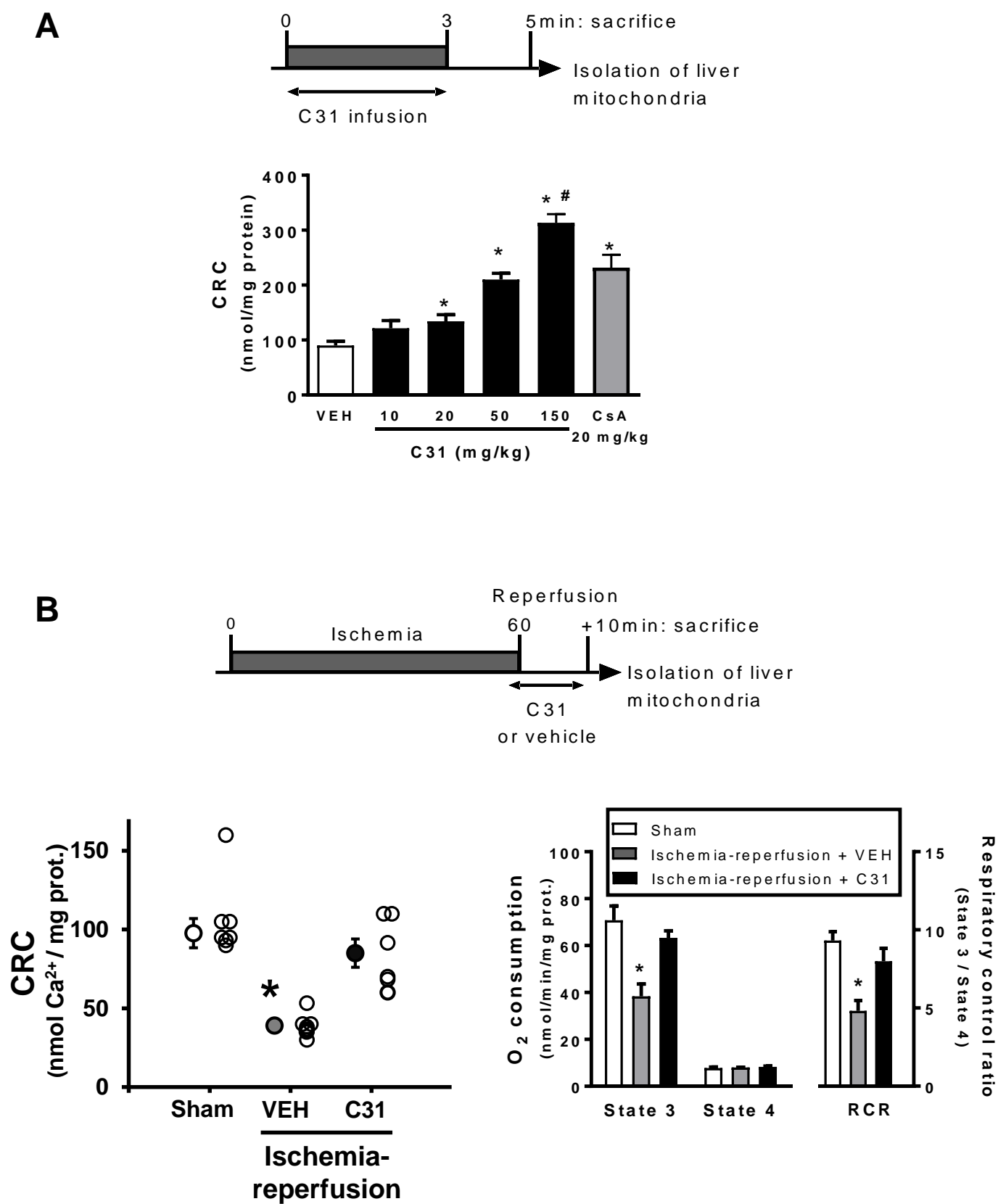


Fig. 7

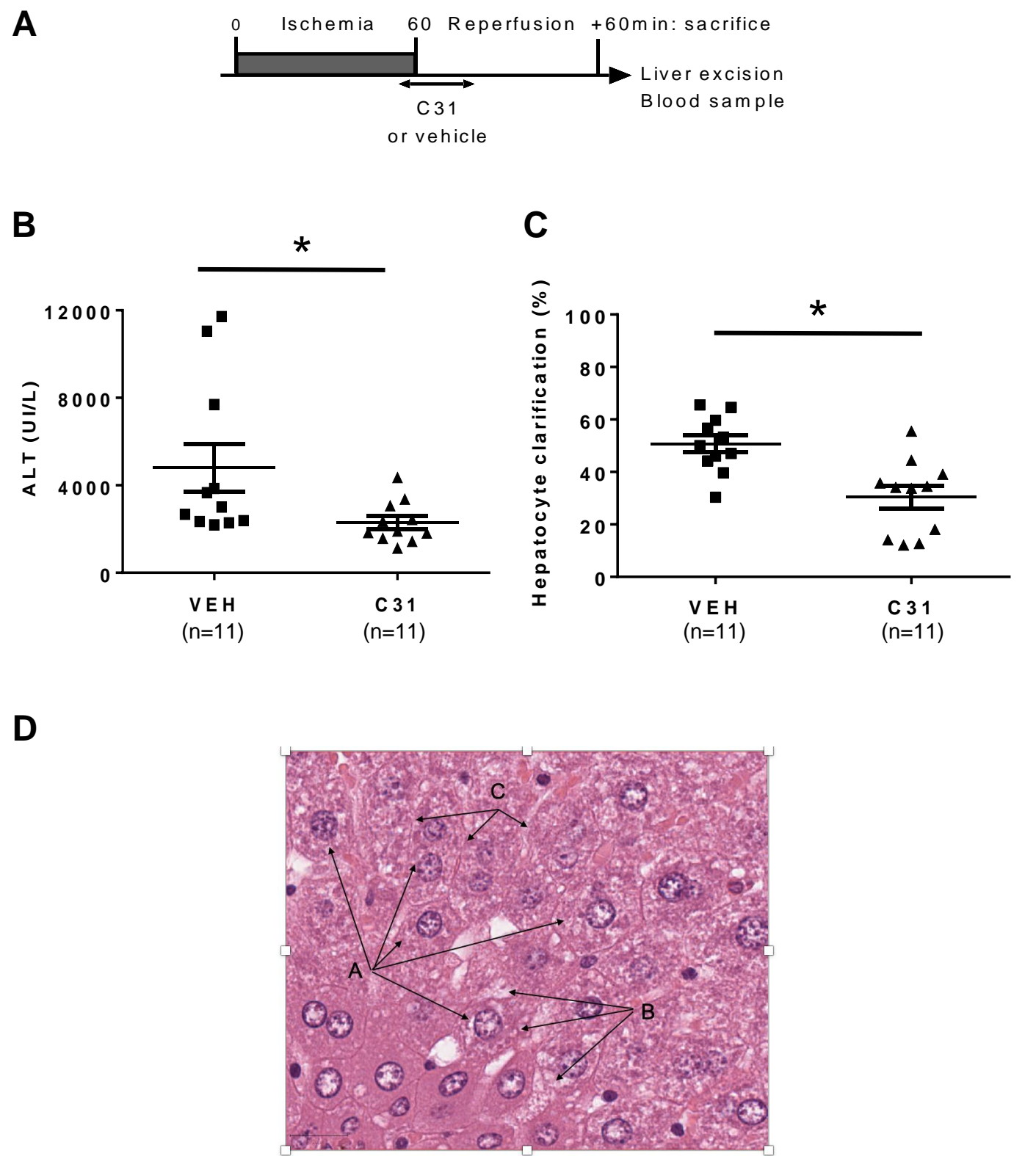


Fig. 7 (continued)

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