Targeting Acute Pancreatitis by Small Molecule Inhibitors of Cyclophilin D

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Introduction

Mitochondrial dysfunction is central to the pathogenesis of various diseases including acute pancreatitis (AP). Mitochondrial dysfunction is the result of a sudden increase in permeability of the inner mitochondrial membrane (IMM), via persistent opening of a multi-protein channel known as the mitochondrial permeability transition pore (MPTP). This allows uncontrolled proton flow across the IMM and unregulated flux of water, ions and solutes up to 1.5 kDa into and out of the mitochondrial matrix. A resultant loss of mitochondrial membrane potential ($\Delta\Psi_m$), essential for ATP production, coupled with disruption of calcium homeostasis, activates the necrotic cell death pathway. Cyclophilin D (CypD), a mitochondrial matrix protein with a peptidyl prolyl isomerase (PPIase) activity, promotes formation of the MPTP. We aim to develop small molecule specific inhibitors of CypD as a possible treatment for AP and other conditions where the MPTP plays a role.

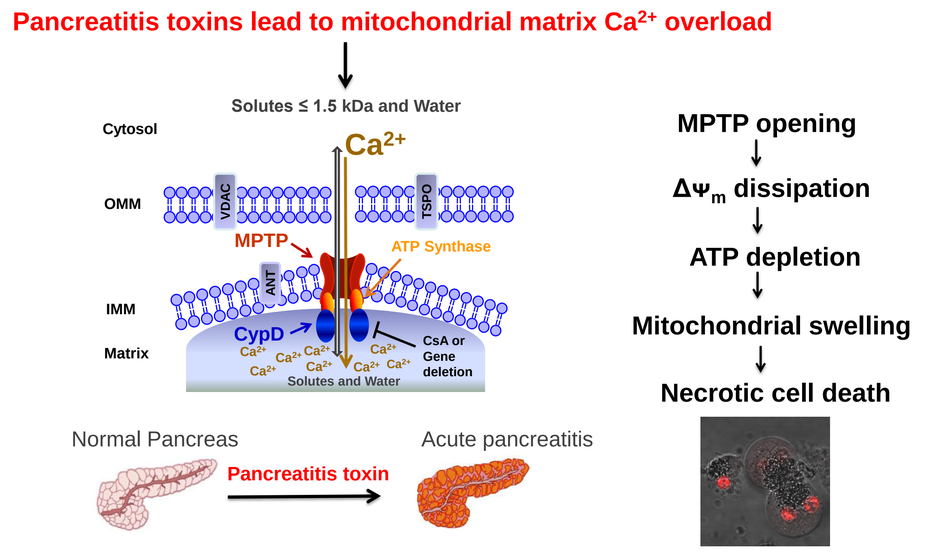


Figure 1. The MPTP plays a critical role in the development of AP. When MPTP opening is inhibited by genetic (Ppif$^{-/-}$, Ppif encodes CypD) or pharmacological means, $\Delta\Psi_m$ is preserved and ATP production sustained. This maintains the integrity of the pancreatic acinar cells that clear calcium more effectively and prevents the development of AP. OMM, Outer Mitochondrial Membrane; TSPO, Mitochondrial Translocator Protein; ANT, Adenine Nucleotide Translocase; VDAC, Voltage Dependent Anion Channel. Red in the image of pancreatic acinar cells is propidium iodide, marker of necrotic cell death.

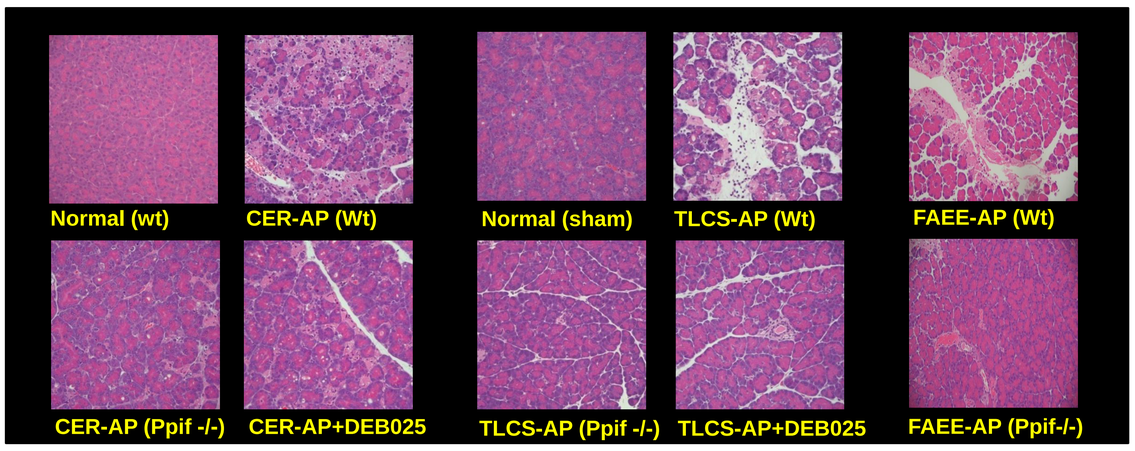


Figure 2. Target validation, Ppif gene encodes CypD. Normal pancreatic histology contrasted with cerulein (CER), taurolithocholic acid-3-sulfate (TLCS) and fatty acid ethyl ester (FAEE) induced AP in Wt, Ppif$^{-/-}$ or Wt treated with D-MeAla^3-EtVal^4-cyclosporin (Alisporivir, DEB025; 10mg/kg i.p. with third injection of CER or one hour after induction of TLCS-AP), showing extensive oedema, necrosis and inflammatory cell infiltration in Wt but not in Ppif$^{-/-}$ or Wt with DEB025. ***Mukherjee et al. Gut 2015***

Results, *in vitro*

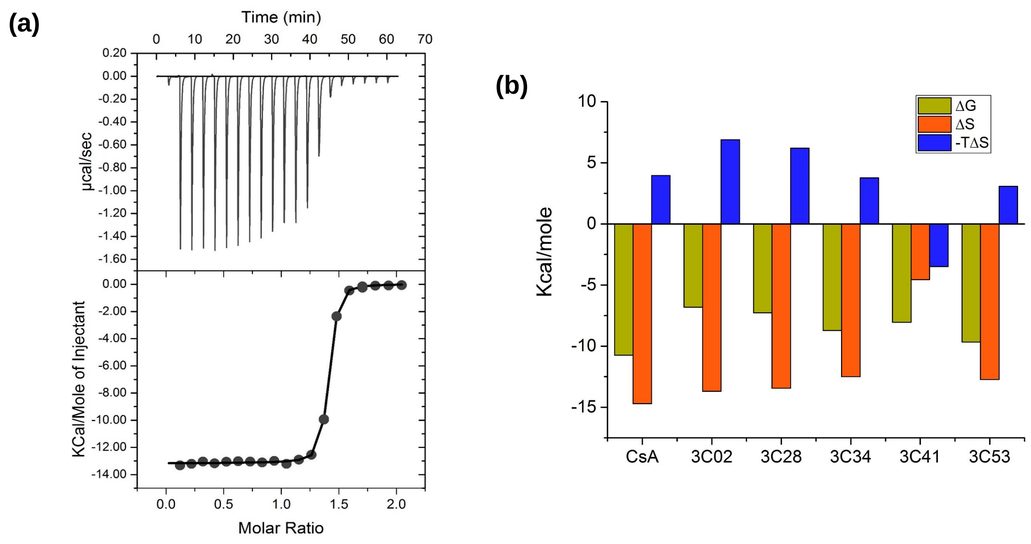


Figure 3. Isothermal titration calorimetry profile and thermodynamics for the binding of small molecule inhibitors to CypD. (a)The top panel shows the raw calorimetric data obtained upon titration of CypD with each inhibitor and the bottom panel shows the plot of the integrated heat signal as a function of molar ratio of ligand to protein. The ITC titration experiments for the interactions between the inhibitors and CypD were performed in duplicates, with 50-100 µM CypD in the cell and 1000-2000 µM compounds in the syringe, with 1% DMSO present in all the solutions. Equilibrium dissociation constant (Kd) for 3C28, 3C34, 3C41 and 3C53 were 4.9, 0.5, 1.1 and 0.06 µM, respectively. (b) Thermodynamic signatures for the binding of different compounds to CypD.

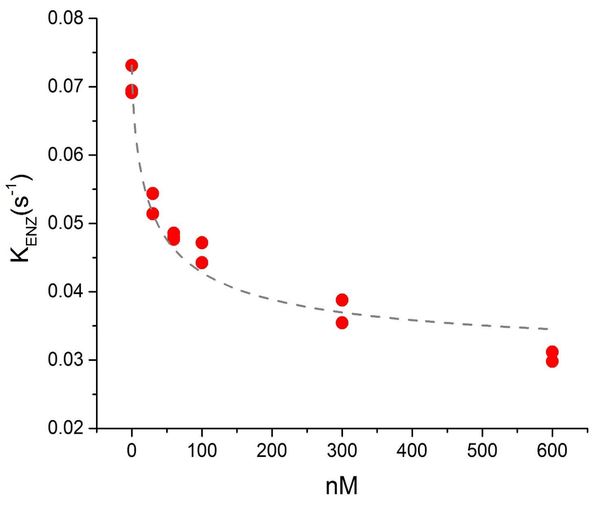
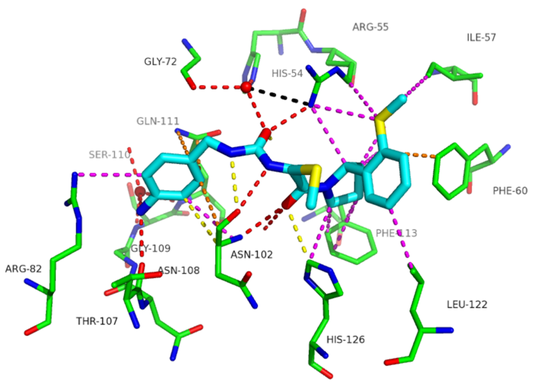


Figure 4. 3C53 inhibits enzymatic activity of CypD. Assay buffer (35 mM HEPES pH 7.8) is cooled to 10 °C in a quartz cuvette. A blank spectrum was obtained and then 3 µl enzyme (2 nM final concentration) and 3 µl substrate (N-SUCCINYL-ALA-ALA-PRO-PHE-P-NITROANILIDE 20 mg/ml in 0.5 M LiCl in trifluoroethanol, 60 µM final concentration) was added and the absorbance is measured at 330 nm over 5 min. A first order rate was fitted to the absorbance data to obtain a rate constant (first 10 to 15 s eliminated due to mixing). The catalytic rate was calculated from the enzymatic rate minus the background rate. Inhibitory constant (Ki) values for 3C28, 3C34, 3C41 and 3C53 were 9.1, 0.1, 0.95 and 0.032 µM, respectively.



	Non-covalent interaction
·····	Hydrogen bond
·····	Cation-dipole
·····	Hydrogen bond-π
·····	π- π
·····	Van der Waals

Figure 5. Crystal structure of 3C34 bound to CypD. 3C34 is rendered as sticks (carbon – cyan, nitrogen – blue, oxygen – red, sulphur – yellow). Residues involved in non-covalent interactions are rendered as thin sticks (carbon – green, nitrogen – blue, oxygen – red). Crystallised waters rendered as red spheres. Non-covalent contacts are shown as dotted lines with the colour code given in the key. CypD was concentrated to 25 mg/mL in the 50 mM Na/K Phos pH 6.8, 100 mM NaCl buffer. Co-crystallisations were conducted with the addition of the target compound at 1 mM final concentration with an equal volume of the reservoir solution, 4 M potassium formate, 0.1 M Bis-Tris propane pH 9 and 2% w/v PEG 2000mme. Diffraction data were collected at the PROXIMA1 beam line, SOLEIL Synchrotron.

Results, *ex vivo*

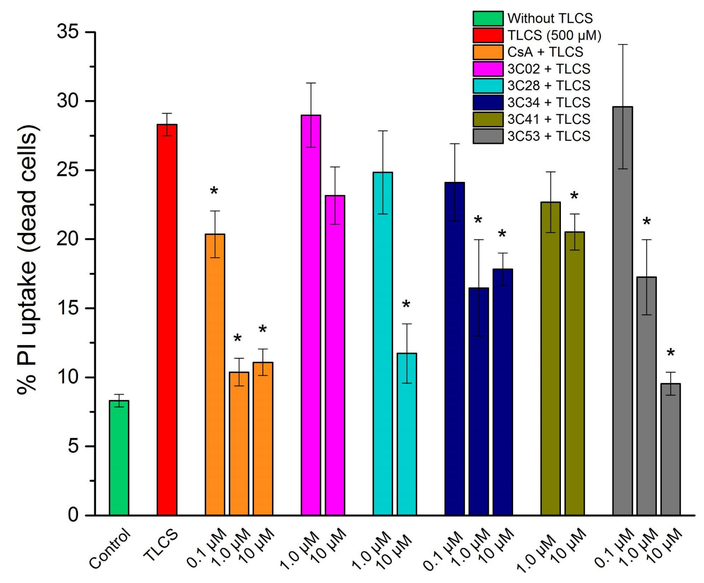


Figure 6. Effect of inhibitors on necrotic cell death of pancreatic acinar cells in the presence of TLCS. To assess the necrotic cell death, freshly isolated acinar cells from mouse pancreas were treated with TLCS (500 µM) and were incubated at room temperature with gentle shaking for 30 minutes in the absence or presence of a CypD inhibitor. Cells were washed with sodium HEPES buffer and loaded with propidium iodide (PI; 1 µM; excitation 543 nm, emission 610-690 nm) and imaged under confocal microscope. The uptake of PI is indicative of the necrotic cell death due to rupture of plasma membrane. For each group, 12 randomly selected fields - from 3 wells of a 96 well plate - of view under confocal microscope were taken of each mouse isolate, and the total number of cells displaying PI uptake were counted per field to give a percentage ratio for each field, averaged across fields, and converted to a mean and standard error of mean for a minimum of three mice per experimental group. A significant protection of cells from early plasma membrane rupture was observed at 1.0 µM of 3C34 or 3C53 and at 10 µM of 3C28, 3C34, 3C41 or 3C53 (n = 3, *p < 0.05, TLCS vs TLCS plus inhibitor). ***Emma & Awais et al. J Med Chem 2016***

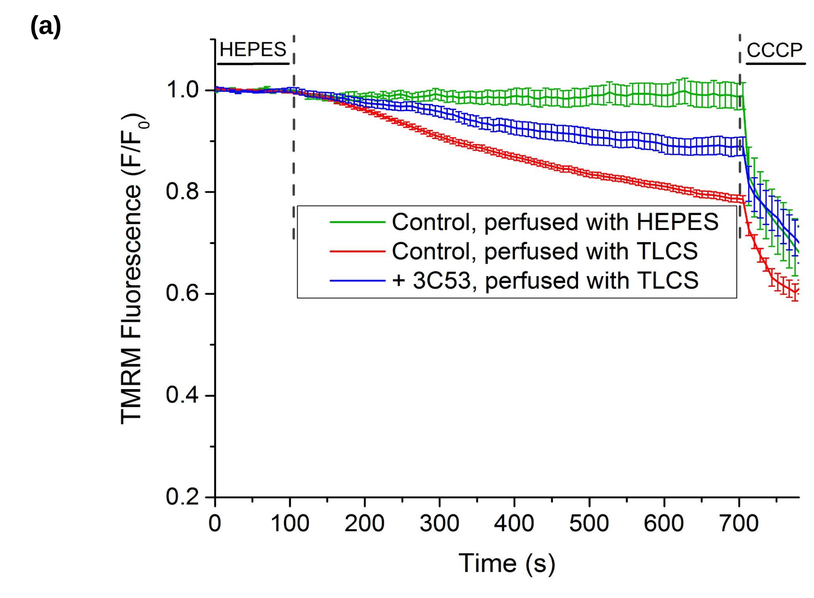


Figure 7. Real-time imaging of $\Delta\psi_m$ of murine pancreatic acinar cells to evaluate the ability of 3C53 to maintain $\Delta\psi_m$ in the presence of TLCS. **(a)** Freshly isolated acinar cells were loaded with 50 nM tetramethyl rhodamine methyl ester (TMRM, λ_{ex}: 543 nm, λ_{em}: >560 nm) in the presence or absence of an inhibitor (10 µM) for 30 minutes at RT and imaged under Zeiss Confocal 710. After assessing baseline fluorescence levels with HEPES buffer for 100 s, cells were perfused with TLCS (500 µM) for 600 s, to induce depolarisation of mitochondria, followed by addition of protonophore carbonyl cyanide m-chloro phenyl hydrazone (CCCP, 10 µM), which was used as a positive control. **(b)** Representative images of pancreatic acinar cells with or without 3C53 at different times perfused with TLCS.

Conclusion: We have shown that 3C28, 3C34, 3C41 and 3C53 bind with and modulate the PPIase activity of CypD, preserve $\Delta\psi_m$ and inhibit necrosis in freshly isolated pancreatic acinar cells, at K_d/K_i below 100 nM for 3C53. Further medicinal chemistry optimisation of our small molecule is underway. Promising candidates will be tested on the mouse *in vivo* models of AP.

Acknowledgement: Supported by UK National Institute for Health Research Biomedical Research Unit Funding Scheme.