Novel mitochondrial transition pore inhibitor
$N$-methyl-$4$-isoleucine cyclosporin is a new therapeutic option in acute pancreatitis

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Key points

- Bile acids, ethanol and fatty acids affect pancreatic ductal fluid and bicarbonate secretion via mitochondrial damage, ATP depletion and calcium overload.
- Pancreatitis-inducing factors open the membrane transition pore (mPTP) channel via cyclophilin D activation in acinar cells, causing calcium overload and cell death; genetic or pharmacological inhibition of mPTP improves the outcome of acute pancreatitis in animal models.
- Here we show that genetic and pharmacological inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in pancreatic ductal cells.
- The results also show that the novel cyclosporin A derivative NIM811 protects mitochondrial function in acinar and ductal cells, and it preserves bicarbonate transport mechanisms in pancreatic ductal cells.
- We found that NIM811 is highly effective in different experimental pancreatitis models and has no side-effects. NIM811 is a highly suitable compound to be tested in clinical trials.

Abstract Mitochondrial dysfunction plays a crucial role in the development of acute pancreatitis (AP); however, no compound is currently available with clinically acceptable effectiveness and...
safety. In this study, we investigated the effects of a novel mitochondrial transition pore inhibitor, N-methyl-4-isoleucine cyclosporin (NIM811), in AP. Pancreatic ductal and acinar cells were isolated by enzymatic digestion from Bl/6 mice. In vitro measurements were performed by confocal microscopy and microfluorometry. Preventative effects of pharmacological [cyclosporin A (2 μM), NIM811 (2 μM)] or genetic (Ppif−/−/Cyp D KO) inhibition of the mitochondrial transition pore (mPTP) during the administration of either bile acids (BA) or ethanol + fatty acids (EtOH+FA) were examined. Toxicity of mPTP inhibition was investigated by detecting apoptosis and necrosis. In vivo effects of the most promising compound, NIM811 (5 or 10 mg kg−1 per os), were checked in three different AP models induced by either caerulein (10 × 50 μg kg−1), EtOH+FA (1.75 g kg−1 ethanol and 750 mg kg−1 palmic acid) or 4% taurocholic acid (2 ml kg−1). Both genetic and pharmacological inhibition of Cyp D significantly prevented the toxic effects of BA and EtOH+FA by restoring mitochondrial membrane potential (Δψ) and preventing the loss of mitochondrial mass. In vivo experiments revealed that per os administration of NIM811 has a protective effect in AP by reducing oedema, necrosis, leukocyte infiltration and serum amylase level in AP models. Administration of NIM811 had no toxic effects. The novel mitochondrial transition pore inhibitor NIM811 thus seems to be an exceptionally good candidate compound for clinical trials in AP.

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Introduction

Acute pancreatitis (AP) is among the most common gastrointestinal disorders requiring hospitalization in the United States (Fagenholz et al. 2007a; Peery et al. 2012). Although the disease is generally mild, the mortality rate in its severe form is still unacceptably high (Parniczky et al. 2016). In recent years, our understanding of the mechanisms that play a crucial role in the development of the disease has improved (Abu-El-Haija et al. 2018). Impaired autophagy, trypsinogen activation, excessive Ca2+ influx, calcineurin activation, mitochondrial dysfunction and inhibition of the cystic fibrosis transmembrane conductance regulator (CFTR) were shown to have considerable impact in the early phase of AP. Therefore, targeting one of these mechanisms may lead to the first specific therapy in AP.

Among the mechanisms noted above, one of the earliest events in AP is mitochondrial dysfunction (Sah & Saluja, 2011; Maleth et al. 2013; Abu-El-Haija et al. 2018; Biczo et al. 2018). It has been shown in acinar cells that bile acids (BAs) and ethanol and fatty acids (EtOH+FA) open the membrane transition pore (mPTP) channel via cyclophilin D (Cyp D) activation, keeping the channel continuously opened and thus resulting in mitochondrial depolarization, lower ATP synthesis and cell necrosis (Shalbueva et al. 2013; Mukherjee et al. 2016; Abu-El-Haija et al. 2018). Although it remains unknown how the pancreatitis-inducing factors noted above modify mPTP channel activity in pancreatic ductal epithelial cells (PDECs), it still seems to be one of the most promising drug targets and calls for further investigation.

Until now, cyclosporin A (CYA) is the only licensed compound used experimentally to inhibit mPTP (via Cyp D) (Javed et al. 2018); however, its clinical usefulness is highly questionable for several reasons. A pilot study found that CYA could reduce the size and damage of myocardial infarction, but larger studies showed no beneficial effects (Piot et al. 2008; Cung et al. 2015; Javed et al. 2018). Even efforts to decrease its immunosuppressive activity have not been successful. Moreover, the CYA derivative Debio025 (Alispoovirin, Debiopharm, Lausanne, Switzerland) has been found to be effective against the hepatitis C virus (HCV), but it had serious side-effects. Surprisingly, some of the patients developed pancreatitis, resulting in a clinical hold on the global Debio025 trial programme (Zeuzem et al. 2015; Stanciu et al. 2019). Another derivative, TRO40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-o, TROPHOS, Roche, Indianapolis, IN, USA), was not beneficial in a phase 2 trial of cardiac preservation following acute myocardial infarction, suggesting that this compound has low or no effectivity (Atar et al. 2015). Indeed it has recently been shown that TRO40303 does not even bind to Cyp D directly (Sileikyte & Forte, 2016; Javed et al. 2018). With regard to AP, both Debio025 and TRO40303 have been shown to be beneficial in animal models, but neither of them has reached ‘proof of concept’ clinical trials in AP, probably due to the clinical failures noted above. New compounds are therefore crucially needed.

A novel CYA derivative, N-methyl-4-isoleucine cyclosporin (NIM811), was found to be highly beneficial in different experimental and clinical studies. NIM811 was effective in animal models of CNS injury (Readnower et al. 2011), allergic encephalomyelitis (Huang et al. 2017), ischaemic-reperfusion injury after surgical intervention (Garbaisz et al. 2014), hepatitis C (Arai et al. 2014),
liver transplantation (Rehman et al. 2011) and pulmonary injury during liver transplantation (Liu et al. 2012). Importantly, none of the studies reported side-effects. NIM811 had no severe or serious adverse effects in a phase 2 clinical trial on HCV-infected patients, suggesting that it has no toxic immunosuppressant activity either (Lawitz et al. 2011).

In this study, we show in several in vitro and in vivo experiments that either pharmacological or genetic inhibition of Cyp D restores mitochondrial function not only in acinar cells, but also in ductal cells, highlighting the general importance of mPTP in AP. Moreover, we provide evidence that NIM811 is highly effective in different experimental pancreatitis models and that it has no side-effects.

**Materials and methods**

**Ethical approval**

The animal experiments were performed in compliance with European Union Directive 2010/63/EU and Hungarian Government Decree 40/2013 (II.14.). Experiments were approved by local ethics committees for investigations involving animals at the University of Szeged (XII/4988/2015). In our study all animals were killed via 200 mg kg$^{-1}$ pentobarbital i.P. (Bimed MTC, Cambridge, Canada).

**Animals**

Seventy wild type (WT) and Cyp D knockout (Cyp D KO, (B6;129-Ppiftm1Maf/J) mice were used. Cyp D KO mice were generated by targeted disruption of the Ppif gene (which encodes the Cyp D that is a component of the mPTP) (Baines et al. 2005). Cyp D KO animals were provided for by the Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary. WT and Cyp D-deficient littermate mice (of C57Bl/6J background, either sex, aged between 20 and 45 days) were housed in a room maintained at 20–22°C on a 12 h light–dark cycle with food and water available ad libitum. To ensure a homologous genetic background, mice were backcrossed with C57Bl6/J mice for at least eight generations.

**Solutions and chemicals**

Chemicals were obtained from Sigma-Aldrich (Budapest, Hungary), unless otherwise stated: 2.7-bis-(2-carboxyethyl)-5-(and-6-) carboxyfluorescein-acetoxy-methylester (BCECF-AM) and tetramethylrhodamine-methylester (TMRM) were purchased from ThermoFisher Scientific (Waltham, MA, USA); NIM811 was purchased from MedChem Express Europe (Sollentuna, Sweden). CYA, caerulein (CER), NIM811, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and fluorescence dyes were diluted in DMSO. Table 1 describes the constitution of solutions that we used during the study. In this study 500 µM chenodeoxycholic acid (BA) or 100 mM ethanol (EtOH) + 200 µM palmitoleic acid (FA) were used during the fluorescence, confocal microscopy and immunostaining measurements, to evaluate the effect of bile acids or the alcohol and fatty acid induced damage on the mitochondrial and cell function during the genetic or pharmacological inhibition of the mPTP in pancreatic ducts or acinar cells. CCCP at 100 µM was used in the mitochondrial measurements as a positive control for mitochondrial damage.

CYA (2 µM) and NIM811 (2 µM) were used to pharmacologically inhibit mPTP. Prior to the fluorescence and confocal microscopy, and immunostaining, the cells (duct and acinar cells as well) from the CYA- or NIM811-treated groups were pretreated for 25–30 min with the compounds (CYA or NIM811).

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**Table 1. Solutions used in the present study**

| Solution                  | Hepes (Standard) (mM) | HCO₃⁻ (Standard) (mM) | NH₄Cl⁻ HCO₃⁻ (mM) | 1× TBS (mM) | HBSS (Standard) (mM) |
|---------------------------|-----------------------|-----------------------|-------------------|-------------|----------------------|
| NaCl                      | 140                   | 115                   | 95                | 150         | 0.137                |
| KCl                       | 5                     | 5                     | 5                 | --          | 5.4                  |
| CaCl₂                     | 1                     | 1                     | 1                 | --          | 0.3                  |
| MgCl₂                     | 1                     | 1                     | 1                 | --          | --                   |
| Glucose                   | 10                    | 10                    | 10                | --          | 6                    |
| Hepes                     | 10                    | --                    | --                | --          | --                   |
| NaHCO₃⁻                   | --                    | 25                    | 25                | --          | 4.2                  |
| NH₄Cl⁻                    | --                    | --                    | 20                | --          | --                   |
| Trisma base               | --                    | --                    | --                | 50          | --                   |
| Na₂HPO₄                   | --                    | --                    | --                | --          | 0.25                 |
| KH₂PO₄                    | --                    | --                    | --                | --          | 0.44                 |
| MgSO₄                     | --                    | --                    | --                | --          | 1.03                 |
Figure 1. Genetic inhibition of Cyp D reduces the severity of bile acid- or ethanol and fatty acid-induced damage in PDECs

A, mitochondrial membrane potential measurements revealed that genetic inhibition of mPTP significantly reduces the mitochondrial membrane potential loss compared to WT controls during the administration of bile acid (500 µM CDC) or ethanol (100 mM) and fatty acid (200 µM FA) treatment (WT control vs. WT BA **P < 0.001, WT BA vs. Cyp D KO BA **P < 0.002, WT control vs. Cyp D KO BA p = 0.712, WT control vs. WT EtOH + FA P < 0.01, WT EtOH + FA vs. KO EtOH + FA *P < 0.05, WT control vs. Cyp D KO EtOH + FA p = 0.145), n = 4–6 experiments
Methods

Genotypes of Cyp D deficient mice were identified by PCR (typical PCR, analyses from tail genomic DNA). The PCR-mix contained: Taq DNA pol 5 U and 10 μl of Taq Buffer (Abgene, Portsmouth, NH, USA), MgCl₂ 1.5 mM, dNTP 2.5 mM, F-null2/LoxP1f /CyPuP2 primers (20–20 μM), dH₂O and template DNA sample. The total reaction mix volume was 25 μl. The wild type allele was detected using LoxP1f, 5′-AACATTCTCACTGAGTTGCTCTG-3′, as a forward primer and F-null2, 5′-GCTTTGTATATCCAGCAGCCA-3′, as a reverse primer. For genotyping of the mutant Cyp D deficient allele, F-null2, 5′-TTCTCACACGTGATAGGGGCTCTG-3′ was used as a forward primer with the reverse primer for WT (Table 2). DNA was denatured at 95°C for 2 min, followed by 30 cycles of amplification: 94°C for 30 s, 60°C for 30 s, 72°C for 45 s and a final primer extension step at 72°C for 7 min. Bands of 270 and 470 bp were amplified for WT and Cyp D KO mice, respectively.

Pancreatic ducts and acinar cells were isolated by microdissection and enzymatic digestion as described previously (Argent et al. 1986; Gout et al. 2013).

The mitochondrial membrane potential (Ψ) was determined by using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft, Budaörs, Hungary). BA or EtOH were used to induce mitochondrial damage. Isolated pancreatic ducts or acinar cells were incubated in standard Hepes solution and loaded with TMRM (100 nmol l⁻¹).

To monitor apoptotic and necrotic cells in isolated pancreatic ducts or acinar cells an apoptosis/necrosis kit was used (ab176750, Abcam, Cambridge, MA, USA). To determine live, necrotic or apoptotic cells, CytoCalcein Green DCS1 fluorescence dies (ab176750, Abcam) were used. Samples were incubated in the mixture of the above stated fluorochrome dyes at room temperature for 30–35 min (after 25 min of treatment with BA/EtOH + FA/CYA/NIM811) in the dark prior to the confocal microscopy measurements. For CYA- or NIM811-treated ducts or acinar cells, incubation with these compounds were performed before staining with the fluorescence dyes. Stainings were analysed using a Zeiss LSM 880 confocal laser scanning microscope. Live, necrotic or apoptotic cells were counted and summarized as a percentage of each sample, and data were then averaged and statistical analysis was performed.

Microfluorometry was used to measure pancreatic ductal HCO₃⁻ secretion as described earlier (Hegyi et al. 2013; Hegyi et al. 2004) by using BCECF-AM (1.5 mmol l⁻¹).

Functionally active mitochondria were detected with immunofluorescent staining (TOM20 mitochondrial marker EPR15581-39, Abcam). To determine mitochondrial localization in isolated pancreatic ductal or acinar cells we labelled the mitochondria by the using TOM20 primary antibody (Abcam, EPR15581-39). TOM20 is the central unit of the receptor TOM complex in the mitochondrial outer membrane and its role is to recognize and translocate cytosolically synthetized mitochondrial preproteins (Schatz et al. 1996; Pfanner, 1998; Rapaport, 2002). Isolated pancreatic ducts were frozen in cryomold at 20°C. The cryosections (thickness 7 μm) of the isolated pancreatic ducts from WT and Cyp D KO mice were cut via a Leica Cryostat. Sections were fixed in 4% paraformaldehyde. Washing periods were administered with 1× Tris-buffered saline (TBS) solution. Antigen retrieval was performed with 10 mM sodium citrate solution at pH 6 at 95°C for 15 min. Blocking was obtained for 1 h with 1% goat serum in 5% bovine serum albumin (BSA)-TBS solution. These sections were then incubated with TOM20 rabbit monoclonal antibody (dilution 1:400, Abcam) overnight at 4°C. The following day the samples were incubated with goat anti-rabbit secondary antibody (Alexa fluor 488, Thermo Fisher) for 2 h in the dark in room temperature. Nuclei were counterstained with Hoechst 33342 (Thermo Fisher). Immunofluorescence staining of the isolated pancreatic acinar cells was performed immediately after the isolation procedure with the same conditions as stated above (except: cells were fixed in 2% paraformaldehyde and dilution of the primary antibody was 1:200). Both ducal

| Table 2. Oligonucleotide primers used in genotyping |
|---------------------------------|---------------------------------|
| Primer | Sequence |
| F-null2 | TTCTCACAGTGCATAGGGCTCTG |
| LoxP1f | AAACCTTCTCACTGAGTTGCTCTG |
| CyPuP2 | GCTTGTATATCCAGCAGCCA |

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Figure 2. CYA reduces the severity of bile acid- or ethanol and fatty acid-induced pancreatic ductal damage

A, treatment with 2 µM CYA reduced the drop of mitochondrial membrane potential loss which accrued due to the BA or EtOH + FA treatment (WT vs. CYA). In WT ducts BA or EtOH + FA treatment resulted in significantly reduced mitochondrial membrane potential (WT control vs. WT BA ∗P < 0.05, WT control vs. WT EtOH + FA ∗P < 0.05), while between WT control groups compared to CYA-treated BA or EtOH + FA there was no significant
and acinar cell samples were mounted with Fluoromount and then analysed using a Zeiss LSM 880 confocal laser scanning microscope. To quantify TOM20 positively stained area, five or six representative images from each group were taken with the Zeiss LSM 880 microscope. Image J software was used to convert images to grey scale (16 bit), and threshold function was used to select the positively stained area. The fluorescence signal was calculated by the software [arbitrary scale from 0-negative (white) to 255-maximal staining (black)] (Venglovcev et al. 2018). Fluorescence intensity of the images was then normalized to the total ductal or acinar area of the samples, which were measured in arbitrary units. Fluorescence intensity was given as a percentage, normalized to the total ductal or acinar total area.

AP was induced by CER (10 × 50 μg kg⁻¹), 4% sodium taurocholate (TAU, 2 ml kg⁻¹, 4%) (Niederau et al. 1985; Ding et al. 2003; Perides et al. 2010; Pallagi & Balla et al. 2014) or alcohol and fatty acid (i.p. injection of 1.75 g kg⁻¹ ethanol and 750 mg kg⁻¹ palmitic acid, EtOH + FA) as described previously (Huang, 2014; Maleth et al. 2016). All control groups received physiological saline in the same amount as the CER, EtOH + FA or the TAU solutions respectively. Pre-treatment of the animals by NIM811 was performed and mice were gavaged orally once 1 h prior to the induction of AP (concentrations of NIM811 were 10 or 5 mg kg⁻¹). The dose of NIM811 was chosen according to a previous study in which NIM811 was effective against mitochondrial damage in liver transplantation (Rehman et al. 2011). Oral gavage treatment were performed by the use of plastic feeding tubes (20 gauge × 38 mm, Instech Laboratories, Plymouth Meeting, PA, USA). NIM811 were solubilized in a vehicle which contained 8.3% polyoxy 40 hydrogenated castor oil and 8.3% ethanol (Rehman et al. 2011).

NIM811 was used as a post-AP treatment as well. NIM811 was administered 12 h after the induction of AP in the TAU- or EtOH + FA-induced experimental pancreatitis models. Concerning the CER-induced AP, NIM811 was administered after the third injection of CER. The method for retrograde intraductal infusion of TAU has been described by Perides et al. (2010). The surgery was performed on anaesthetized mice (with ketamine–xylazine, dosage: 87.5 mg kg⁻¹ ketamine/12.5 mg kg⁻¹ xylazine). At the end of the procedure the mice were placed on a heating pad for 40 min and received buprenorphine i.p. (0.075 mg kg⁻¹) immediately to reduce pain. Following these mice were replaced into their cages for 24 h. They had free access to food and water. Twenty-four hours after the TAU- or EtOH + FA-induced AP the mice were killed via i.p. 200 mg kg⁻¹ pentobarbital (Bimeda MTC, Cambridge, Canada). During the CER-induced AP mice were killed with i.p. 200 mg kg⁻¹ pentobarbital (Bimeda MTC) 2 h after the last injections of CER. Mice were exsanguinated through cardiac puncture and the pancreas was removed. Blood from the cardiac puncture was placed on ice, then centrifuged with at 2500 g for 15 min at 4°C. Blood serum was collected from the pellet and stored at −20°C until use. Pancreas samples were placed into 8% neutral formaldehyde solution and stored at −4°C until the haematoxylin–eosin staining was performed. A colorimetric kit was used to measure serum amylase activity (Diagnosticum, Budapest, Hungary). Absorbance of the samples was detected at 405 nm with the use of a FLUOstar OPTIMA (BMG Labtech, Budapest, Hungary) microplate reader. Formaldehyde-fixed pancreas samples were embedded in paraffin and were cut into 3 μm thick sections and stained for haematoxylin–eosin by using a standard laboratory method. To quantify oedema, necrosis and leukocyte infiltration grades a semiquantitative scoring system was used according to Kui et al. (2015).

In vitro pancreatic ductal fluid secretion (luminal swelling) assays were developed by Fernández-Salazar et al. (2004) performed by videomicroscopy as described by Baláz et al. (2018). Briefly, stimulation of pancreatic ductal fluid secretion was induced by 5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX), and quantification were performed using ImageJ software (Baláz et al. 2018). In vivo fluid secretion measurements were performed on anaesthetized (i.p. 87.5 mg kg⁻¹ ketamine/12.5 mg kg⁻¹ xylazine) mice after CER- or EtOH + FA-induced AP before the animals were killed. Animals were placed on warm pads (37°C) to maintain body temperature. Briefly, the abdomen was opened and...
Figure 3. NIM811 protects mitochondrial and cell function in PDECs

A, NIM811-treated ducts revealed a significantly consolidated loss of mitochondrial membrane potential during the BA (WT BA vs. NIM811 BA *P < 0.05) or EtOH + FA (WT EtOH + FA vs. NIM811 EtOH + FA *P < 0.05) treatment. In NIM811-treated ducts the percentage of fluorescence intensity was significantly higher compared to non-NIM811-treated ducts during BA or EtOH + FA administration. B, in CCCP-treated ducts we found no significant difference in the amount of TOM20 staining in NIM811-treated or untreated groups. NIM811 itself did not alter the level of TOM20 staining compared to the WT control samples. C, NIM811 decreased the numbers of

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apoptotic and necrotic cells during bile acid or ethanol and fatty acid treatment (WT BA vs. NIM811 BA \( \ast P < 0.05 \), WT EtOH + FA vs. NIM811 \( \ast P < 0.05 \)). During the administration of CCCP the apoptosis and necrosis grades were not significantly different in the comparative groups. D–F, NIM811 treatment did not decrease the HCO\(_3\)\(^-\) secretion grade (control), while during the administration of BA or EtOH + FA it had a protective effect against the reduction of HCO\(_3\)\(^-\) secretory levels (E, F) (WT BA vs. NIM811 BA \( \ast P < 0.05 \), WT EtOH + FA vs. NIM811 EtOH + FA \( \ast P < 0.05 \)). Regarding recovery levels from alkali load during EtOH and FA treatment, differences were not significant in WT EtOH + FA- compared to the NIM811 and EtOH + FA-treated groups (E). [Colour figure can be viewed at wileyonlinelibrary.com]

Cannulation of the lumen of the common biliopancreatic duct was performed with a 30-gauge needle (Maléth et al. 2016). The proximal end of the common duct was closed by a microvessel clip (Braun-Aesculap, Tuttingen, Germany) to prevent contamination with bile, and the pancreatic juice was collected in a PE-10 tube for 15 min.

**In vivo** secretion was induced by i.p. administration of 0.75 CU kg\(^{-1}\) secretin (Maléth et al. 2016).

**Statistical analysis**

All data are expressed as means ± SEM. Data were compared by either one- or two-way ANOVA or

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![In vitro pancreatic ductal fluid secretion](image1)

**Figure 4.** Pancreatic ductal fluid secretion is not altered by NIM811 or CYA treatment. 
**A**, in vitro fluid secretion was stimulated by 5 \( \mu \)M forskolin and 100 \( \mu \)M IBMX (stimulation). **B** and **C**, BA or EtOH + PA treatment inhibited luminal swelling. **D**, relative luminal volume changes during forskolin and IBMX stimulation. Means ± SEM; \( n = 5–10 \) ducts per group. **E** and **F**, in vivo fluid secretion measurements were performed after CER- or EtOH + FA-induced AP. These experiments confirmed that pancreatic ductal fluid secretion is not affected by NIM811 or CYA. \( \ast P < 0.05 \) WT PS vs. WT EtOH + FA, \( \ast \ast P < 0.05 \) WT PS vs. WT CER \( n = 4–7 \) animals per group. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 5. NIM811 treatment protects mitochondrial function in pancreatic acinar cells

A. mitochondrial membrane potential measurements revealed a significant difference between WT untreated and NIM811-treated acinar cell response due to bile acid or ethanol and fatty acid treatment (WT BA vs. NIM811 BA *P < 0.05; WT EtOH + FA vs. NIM811 EtOH + FA *P < 0.05). A significant difference was detected between the NIM811-treated acinar cells and the groups which were not treated with NIM811 during BA or EtOH + FA.
Kruskal–Wallis tests followed by the Holm–Sidak method as appropriate (Sigma Plot). The effects were considered significant at $p < 0.05$.

**Results**

**Genetic inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in PDECs**

First, we measured the effects of the most relevant pancreatitis-inducing factors on mitochondria in primary intact ducts isolated from Ppif$^{-/-}$ and WT mice. Experiments with TMRM and TOM20 revealed that genetic inhibition of mPTP decreased both the loss of $\Delta \psi$ (Fig. 1A) and mitochondrial mass (Fig. 1B) caused by 500 $\mu$M chenodeoxycholic acid (CDC; BA) or co-administration of 100 mM ethanol and 200 $\mu$M palmitoleic acid (EtOH + FA). Co-staining the pancreatic ducts with CytoCalcein Violet, Apopxin Deep Red and Nuclear Green showed that genetic inhibition of mPTP also decreased the extent of necrosis and apoptosis during the administration of BA or EtOH + FA (Fig. 1C), suggesting that genetic inhibition of Cyp D has a protective effect on PDECs. Next, we investigated how the genetically preserved mitochondrial function affects the cellular function of PDECs (Fig. 1D). We used the NH$_4$Cl pulse technique, which is uniquely suited to characterizing both HCO$_3^-$ influx and efflux mechanisms. Our experiments demonstrated that the inhibitory effects of BA and EtOH + FA on Cl$^-$/HCO$_3^-$ exchangers (HCO$_3^-$ efflux) and on Na$^+$/HCO$_3^-$ co-transporters (HCO$_3^-$ influx) were totally blocked in Ppif$^{-/-}$ vs. WT mice, suggesting that inhibition of mPTP can preserve ductal function and thus has therapeutic benefits (Fig. 1D–F).

**Pharmacological inhibition of mPTP by CYA effectively prevents mitochondrial damage evoked by pancreatitis-inducing factors in PDECs**

Both BA and EtOH + FA significantly decreased the $\psi$ of PDECs (Fig. 2A). Importantly, 2 $\mu$m CYA effectively blocked the toxic effects of the BA- and EtOH + FA-preserving function of mitochondria during the presence of pancreatitis-inducing factors. As regards the quantity of mitochondria, CYA effectively inhibited loss, as observed during the genetic inhibition of mPTP (Fig. 2B). CYA at 2 $\mu$m decreased the extent of necrosis and apoptosis during the administration of BA or EtOH + FA in PDECs (Fig. 2C). Finally, we provided strong evidence of the beneficial effects of CYA on mPTP noted above, mitochondrial mass and cell death, resulting in preserved HCO$_3^-$ efflux and influx mechanisms during BA or EtOH + FA administration (Fig. 2D–F).

**NIM811 treatment protects mitochondrial function and preserves bicarbonate transport mechanisms in PDECs**

Next, we investigated the effects of the novel CYA derivative NIM811 on mitochondrial function and of bicarbonate secretion on isolated pancreatic ducts. According to our data, NIM811 reduces the BA- or EtOH + FA-induced damage to mitochondrial function and morphology in isolated pancreatic ducts (Fig. 3A, B). Experiments using CytoCalcein Violet, Apopxin Deep Red and Nuclear Green showed that NIM811 alone has no toxic effects on PDECs. Furthermore, it strongly decreases BA- or EtOH-FA-evoked necrosis and apoptosis (Fig. 3C). NH$_4$Cl$^-$ experiments revealed that the inhibitory effects of BA and EtOH + FA on Cl$^-$/HCO$_3^-$ exchangers (HCO$_3^-$ efflux) and on Na$^+$/HCO$_3^-$ co-transporters (HCO$_3^-$ influx) were significantly reduced in the NIM811-treated groups compared to the controls, showing a protective effect of NIM811 on PDECs (Fig. 3D).

**NIM811 and CYA have no effects on pancreatic ductal fluid secretion**

Both in vivo and in vitro measurements revealed that NIM811 or CYA treatment did not prevent BA- or EtOH + FA-induced fluid secretory damage in isolated ducts (Fig. 4A–D and E, F).

**NIM811 treatment protects mitochondrial function in acinar cells**

*In vitro* measurements of freshly isolated pancreatic acinar cells showed that NIM811 treatment decreased the BA- and EtOH-FA-induced loss of $\psi$ as effectively as seen in PDECs (Fig. 3A). However, results obtained from TOM20 staining suggest that NIM811 has no effect on mitochondrial mass in acinar cells (Fig. 5B). Microfluorometric measurements demonstrated that NIM811 alone has no toxic effects on acinar cells and has no effect on BA- or EtOH-FA-induced apoptosis, but is protective against BA- or EtOH-FA-induced necrosis (Fig. 5C).
Figure 6. NIM811 reduces the severity of CER-induced AP

A, representative images of pancreas sections. B, serum amylase levels were elevated in the CER-treated groups and NIM811 treatment resulted in a reduced serum amylase levels during CER-induced AP compared to the WT CER group (***P < 0.01 WT PS vs. WT CER, **P < 0.02 WT CER vs. pre 10 mg kg⁻¹ NIM811 CER, *P < 0.05 WT CER vs. pre 5 mg kg⁻¹ NIM811 CER, p = 0.717 CER + pre 5 mg kg⁻¹ NIM811 vs. CER + pre 10 mg kg⁻¹ NIM811). A–E, for CER-induced pancreatitis both 5 mg kg⁻¹ body weight NIM811 (P < 0.05 WT CER vs. pre 5 mg kg⁻¹ NIM811 CER) and pre 10 mg kg⁻¹ NIM811 (P < 0.05 WT CER vs. pre 10 mg kg⁻¹ NIM811 CER) treatment reduced the CER-induced damage. F–K, post 5 mg kg⁻¹ NIM811 treatment significantly reduced serum amylase levels compared to WT CER (**P < 0.05, ***P < 0.001 WT PS vs. WT CER. H, post-insult administration of 10 mg kg⁻¹ NIM811 significantly reduced oedema and leukocyte infiltration levels compared to WT CER-treated groups (***P < 0.05, n = 8–10 animals per group, data are means ± SEM). [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 7. NIM811 reduces the severity of TAU induced AP in mice

A–K, in TAU-induced pancreatitis, serum amylase measurements revealed that retrograde infusion of TAU led to elevated serum amylase levels (**P < 0.01 WT PS vs. WT TAU (B), ***P < 0.001 WT PS vs. WT TAU (G)), but 5 or 10 mg kg\(^{-1}\) body weight NIM811 treatment significantly reduced the enzyme levels both before and after treatment (B: ***P < 0.02 WT TAU vs. pre 5 mg kg\(^{-1}\) NIM811 + TAU, **P < 0.02 WT TAU vs. pre 10 mg kg\(^{-1}\) NIM811 + TAU, ***P < 0.001 WT TAU vs. pre 5 mg kg\(^{-1}\) NIM811 + TAU, **P < 0.02 WT TAU vs. pre 10 mg kg\(^{-1}\) NIM811 + TAU).
NIM811 has therapeutic benefits in CER-, TAU- and EtOH-FA-induced AP

First, we confirmed that per os administration of either 5 or 10 mg kg\(^{-1}\) NIM811 alone has no toxic effect on the pancreas (Fig. 9). Second, we tested the compound in three different experimental AP models: the CER-, EtOH + FA- and TAU-induced models (Niederau et al. 1985; Perides et al. 2010; Huang, 2014). Importantly, pre-treatment with both 5 and 10 mg kg\(^{-1}\) NIM811 significantly reduced the elevation of serum amylase activity, as well as pancreatic oedema, necrosis and leucocyte infiltration in experimental AP models (Figs 6–8). We also confirmed that subsequent treatment with 5 or 10 mg kg\(^{-1}\) NIM811 has protective effects against pancreatic damage (Figs 6–8).

Discussion

AP is a multifactorial disease (Hegyi & Petersen, 2003; Sahin-Toth & Hegyi, 2017) involving several types of cell, including acinar and ductal cells. None of the therapeutic efforts targeting only one of them has been successful. Intravenous administration of secretin, which targeted ductal cells only, was found to be either slightly beneficial or neutral in AP (Lankisch et al. 1983; Renner et al. 1983; Keim et al. 1985). By contrast, neither gabexate mesilate nor trasylol, which effectively inhibit trypsin activity, had beneficial effects in AP (Imrie et al. 1978; Buchler et al. 1993). Therefore, we need to find common targets which can restore both acinar and ductal cell functions in AP.

Mitochondrial damage is one of the key pathophysiological events in the early phase of AP in both types of cell (Hegyi & Petersen, 2003; Maleth et al. 2013; Maleth & Hegyi, 2015). It decreases ATP production, causing an elevation of intracellular calcium concentration; moreover, it negatively influences ATP-dependent Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchangers, CFTR Cl\(^{-}\) channels in ductal cells and enzyme secretory processes in acinar cells (Maleth et al. 2011, 2013, 2015; Judak et al. 2014; Maleth & Hegyi, 2015; Mukherjee et al. 2016; Biczo et al. 2018; Katona et al. 2016). In addition, mitochondrial damage is the main factor in determining cell death pathway necrosis and apoptosis. Release of mitochondrial cytochrome c into the cytosol causes apoptosis, whereas mitochondrial depolarization leads to necrosis (Odinokova et al. 2008). Generally, the standard apoptotic pathway involves mitochondrial outer membrane permeabilization, which causes apoptotic factors such as cytochrome c to be released from the inner membrane to the cytosol (Tait & Green, 2010; Maleth & Hegyi, 2015). On the other hand, opening of the mPTP leads to loss of \(\psi\) ATP depletion, increased inner membrane permeability, mitochondrial swelling and necrotic cell death (Goldstein & Kroemer, 2007; Halestrap et al. 2009; Maleth & Hegyi, 2015). Uniquely, inhibition of mPTP could prevent both cell death mechanisms in PDECs, which is different from that seen in acinar cells, where only necrosis could have been prevented. Inhibition of mPTP thus seems to be highly beneficial in both cell types. In the last decade, it has been shown that genetic or pharmacological inhibition of mPTP reduces BA- or EtOH + FA-induced acinar cell damage as well as augmenting the severity of AP (Sah & Saluja, 2011; Gukovskaya et al. 2016; Mukherjee et al. 2016; Biczo et al. 2018). As regards ductal cells, we have shown earlier that both BA and EtOH + FA induce inhibition of HCO\(_3\)\(^{-}\) secretion via severe mitochondrial damage in PDECs (Maleth et al. 2011, 2016). Now, we have continued our experiments investigating the role of mPTP and its inhibition in this type of epithelial cell. First, we characterized the role of mPTP (both genetic and pharmacological CYA) inhibition in PDECs and found that its inhibition has a strong protective effect against the toxic effects of BA or EtOH + FA in ductal cells, suggesting that targeting mPTP may have general benefits. Although many mPTP inhibitors have been tested, none of them has been successful. CYA itself inhibits calcineurin, which leads to immunosuppressant activity and thus could negatively affect the treatment of patients due to hazardous infections. Clinical testing of non-immunosuppressive CYA derivatives Debio025 and TRO40303 was also stopped before reaching ‘proof of concept’ phase 2 clinical trials in AP because of its inconsistent behaviour in other trials (see Introduction). Recently, other new mPTP inhibitors have been introduced in experimental studies. Isoxazoles had inconsistent effects in myocardial infarction (Sileikyte & Forte, 2016). Benzamides resulted in impaired ATP generation (Sileikyte & Forte, 2016; Javed et al. 2018). Cinnamic anilides were shown to be effective in myocardial infarction (Fancelli et al. 2014); however, it has since been shown that it has an age-related toxicity (Fang et al. 2019). In contrast, NIM811 seemed...
Figure 8. NIM811 has a protective effect against EtOH + FA induced pancreatic damage

A–K, in EtOH + FA-induced pancreatitis, serum amylase measurements revealed that in pretreatment with 10 mg kg⁻¹ NIM811 significantly reduced serum amylase levels (B; **P < 0.002 WT EtOH + FA vs. pre 10 mg kg⁻¹ NIM811 + EtOH + FA; B and G: ***P < 0.002 WT PS vs. WT EtOH + FA), whereas with post-NIM811 treatment
serum amylase levels did not differ significantly compared to its ETOH + FA control (G). With pre 10 mg kg\(^{-1}\) NIM811 treatment leukocyte infiltration (**\(P < 0.001\) WT ETOH + FA vs. 10 mg kg\(^{-1}\) NIM811) and necrosis levels (**\(P < 0.001\) WT ETOH + FA vs. 10 mg kg\(^{-1}\) NIM811) were significantly reduced compared to ETOH + FA AP group (D–E). C–E: **\(P < 0.001\) WT PS vs. WT ETOH + FA. Oedema and leukocyte infiltration levels were significantly reduced in post 5 mg kg\(^{-1}\) NIM811-treated groups compared to WT ETOH + FA groups (H and K). *\(P < 0.05\) WT ETOH + FA vs. post 5 mg kg\(^{-1}\) NIM811). n = 4–7 animals per group; data are means ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]
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Additional information

Competing interest

The authors have no conflicts of interest to disclose.

Author contributions

PH had the original idea, initiated the study, obtained funding and supervised the experimental procedures. Most of the protocols were designed by ET, JM, JF, PP, ZR and PH. ET, NZ, AG and RE performed the experiments. Experiments were performed at the Laboratory of Cell Physiology, First Department of Medicine, University of Szeged, or Institute for Translational Medicine and First Department of Medicine, University of Pécs, Pécs, Hungary. ERB contributed to the quantification of the histological samples. LT and GH provided the Ppif(−/−) mice and were involved in data interpretation. ET, NZ and PH evaluated the statistical analysis. JF, JM, PP, ERB and VV provided conceptual advice on the experimental protocols (JF: isolation procedure for pancreatic acinar cells; JM: confocal microscopy and study design; ERB: histological quantification; PP and VV: fluorescence microscopy). ET and PH wrote the paper. JM, NZ, JF, AG, RE, PP, LT, GH, ERB, ZR and VV reviewed and contributed to the manuscript. All authors approved the final manuscript.

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Keywords

acute pancreatitis, cyclophilin D, mitochondrial transition pore, NIM811
**Translational perspective**

Acute pancreatitis (AP) is a severe disorder with high morbidity, mortality and no specific treatment. It is generally accepted that one of the earliest events in initiation of the disease is mitochondrial dysfunction and ATP depletion. It has been shown that the pancreatitis-inducing factors ethanol, fatty acids and bile acids open the membrane transition pore (mPTP) channel, and keep it continuously open, resulting in mitochondrial depolarization, lower ATP synthesis and cell necrosis both in pancreatic acinar and ductal cells. In this study, we provided strong evidence that one of the mPTP inhibitors, namely NIM811, is highly effective in different experimental pancreatitis models. Since NIM811 had no side-effects and passed the important phase 1 stage in the clinical trial process, phase 2 clinical trials are needed with the use of this novel and promising drug candidate.