




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

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Edited by: Kim Barrett & Pawel Ferdek

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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

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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 μ M), NIM811 (2 μ M)] 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 \times 50 μ g kg⁻¹), EtOH+FA (1.75 g kg⁻¹ ethanol and 750 mg kg⁻¹ palmitic 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 ($\Delta\psi$) 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.

(Received 21 June 2019; accepted after revision 11 October 2019; first published online 21 October 2019)

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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; Biczó *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 (Alisporivir, 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),

Table 1. Solutions used in the present study

	Hepes (Standard) (mM)	HCO ₃ ⁻ (Standard) (mM)	NH ₄ Cl ⁻ HCO ₃ ⁻ (mM)	1× TBS (mM)	HBSS (Standard) (mM)
NaCl	140	115	95	150	0.137
KCl	5	5	5	–	5.4
CaCl ₂	1	1	1	–	0.3
MgCl ₂	1	1	1	–	–
Glucose	10	10	10	–	6
Hepes	10	–	–	–	–
NaHCO ₃ ⁻	–	25	25	–	4.2
NH ₄ Cl ⁻	–	–	20	–	–
Trisma base	–	–	–	50	–
Na ₂ HPO ₄	–	–	–	–	0.25
KH ₂ PO ₄	–	–	–	–	0.44
MgSO ₄	–	–	–	–	1.03

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. (Bimeda MTC, Cambridge, Canada).

Animals

Seventy wild type (WT) and Cyp D knockout (Cyp D KO, (B6;129-Ppif^{tm1Maf/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 C57Bl/6J 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-acetoxymethyl ester (BCECF-AM) and tetramethylrhodamine-methyl ester (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).

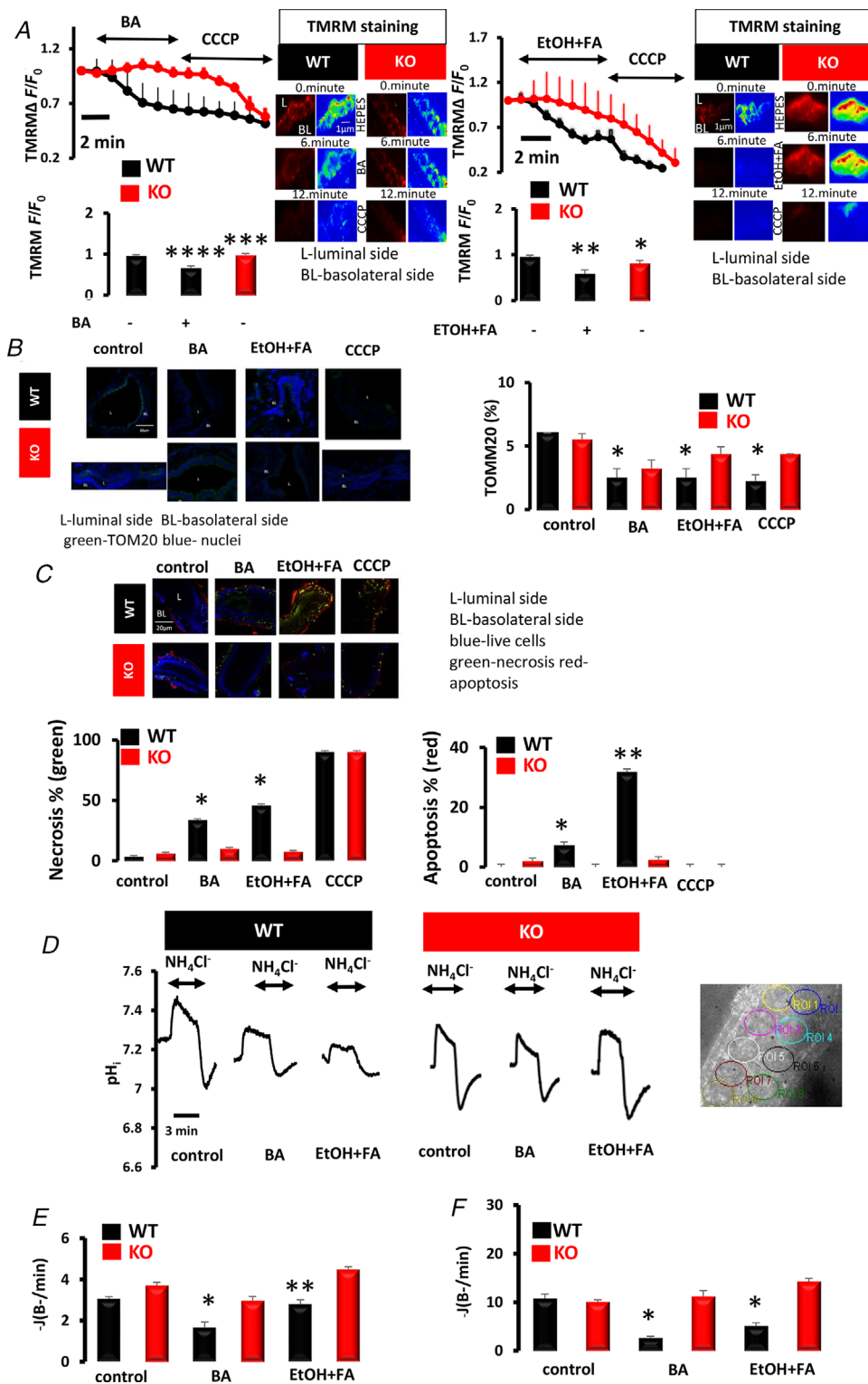


Figure 1. Genetic inhibition of Cyp D reduces the severity of bile acid- or ethanol and fatty acid-induced damage in PDECs

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 μ M CDC) or ethanol (100 mM) and fatty acid (200 μ M 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

Table 2. Oligonucleotide primers used in genotyping

Primer	Sequence
F-null2	TTCTACCAGTGCATAGGGCTCTG
LoxP1f	AAACTTCTCAGTCAGCTGTTGCCTCTG
CyPuP2	GCTTTGTTATCCCAGCTGGCG

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× 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'-AAACTTCTCAGTCAGCTGTTGCCTCTG-3', as a forward primer and F-null2, 5'-GCTTTGTTATC CCAGCTGGCGC-3', as a reverse primer. For genotyping of the mutant Cyp D deficient allele, F-null2, 5'-TTCTACCAGTGCATAGGGCTCTG-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 micro-dissection 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 + FA 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 determinate live, necrotic or apoptotic cells, CytoCalcein Violet 450 fluorescent, Apopxin Deep Red Indicator and Nuclear Green DCS1 fluorescence dyes (ab176750, Abcam) were used. Samples were incubated in the mixture of the above stated fluorescence 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 (Hegyí *et al.* 2013; Hegyí *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 synthesized 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 ductal

per group; data are means ± SEM. *B*, immunostaining revealed a significant decrease of the TOM20 stainings in BA-, EtOH + FA- or CCCP-treated WT ducts; results were compared to Cyp D KO stainings (**P* < 0.05). *C*, genetic inhibition of mPTP also decreased the necrosis and apoptosis levels during bile acid; ethanol and fatty acid or CCCP treatment (**P* < 0.05) *D*, representative traces from the pancreatic ductal HCO₃⁻ secretion measurements. *E* and *F*, the data revealed that levels of alkalosis recovery were significantly lower due to BA or EtOH + FA administration (**P* < 0.05) compared to the results from Cyp D KO ducts. Levels of alkalosis recovery were significantly lower in the WT ducts due to the treatment with BA or EtOH + FA (**P* < 0.05), while in Cyp D KO ducts these levels were significantly higher (**P* < 0.05). *n* = 5–7 experiments per group; data are means ± SEM. *y* axis=base flux [–J(B–/min)] was calculated from the 1pH/1t obtained by linear regression analysis of pHi (HCO₃⁻ secretion) measurements. [Colour figure can be viewed at wileyonlinelibrary.com]

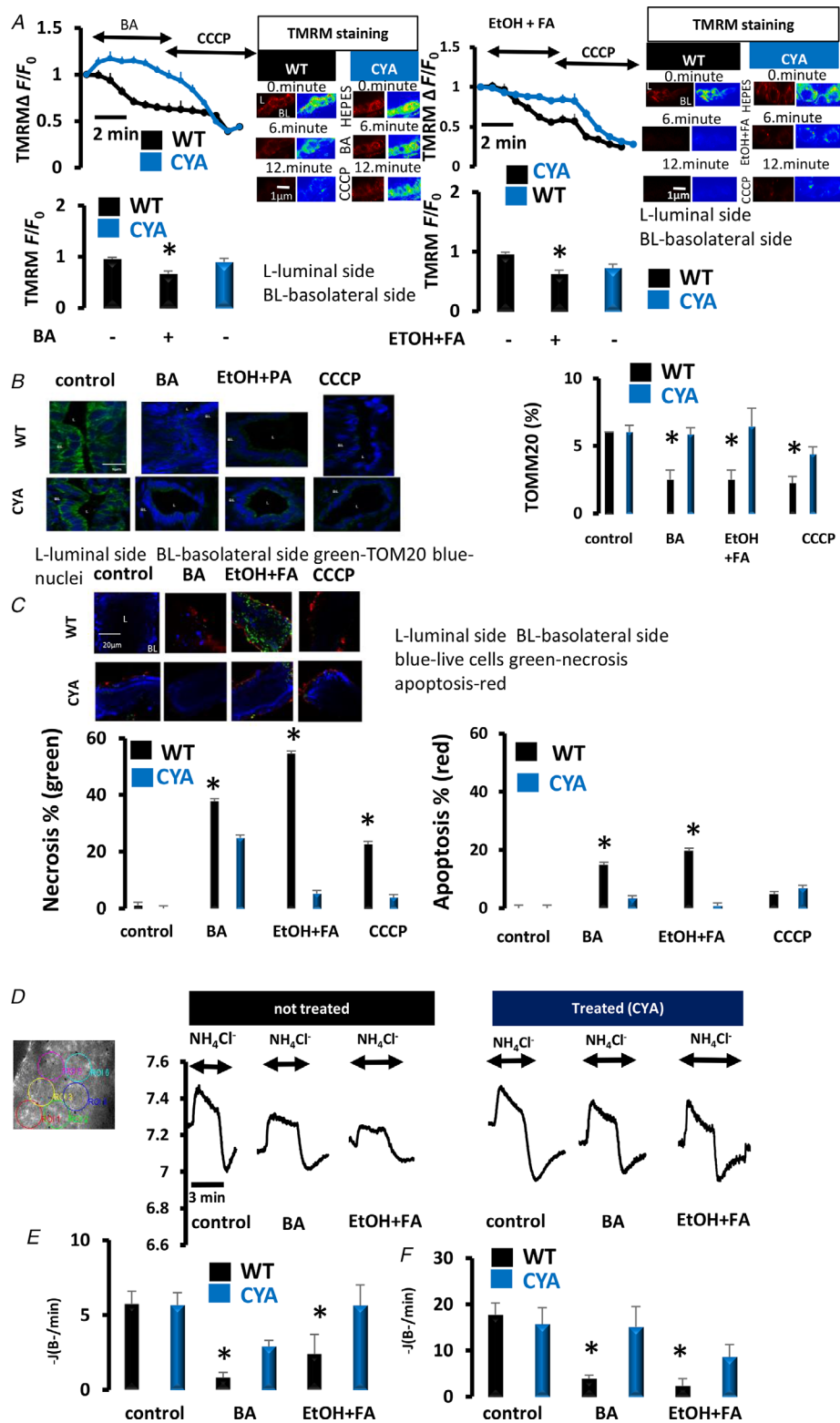


Figure 2. CYA reduces the severity of bile acid- or ethanol and fatty acid-induced pancreatic ductal damage

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 by 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)] (Venglovecz *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 ($10 \times 50 \mu\text{g kg}^{-1}$), 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 \times 38 mm, Instech Laboratories, Plymouth Meeting, PA, USA). NIM811 were solubilized in a vehicle which contained 8.3% polyoxyl 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 $3 \mu\text{m}$ 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 Balázs *et al.* (2018). Briefly, stimulation of pancreatic ductal fluid secretion was induced by $5 \mu\text{M}$ forskolin and $100 \mu\text{M}$ 3-isobutyl-1-methylxanthine (IBMX), and quantification were performed using ImageJ software (Balázs *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

decrease. *B*, TOM20 levels were significantly reduced in BA, EtOH + FA or CCCP control (not CYA treated) ducts, while in the CYA-treated groups the percentage of TOM20-stained area was significantly higher ($*P < 0.05$). Between the control groups (WT control or only CYA-treated samples) we found no significant alterations in the stainings. *C*, necrosis was much higher in BA- or EtOH-treated groups in WT ducts but not in CYA-treated groups. Apoptosis levels were significantly higher as well in the non-CYA-treated groups compared to the CYA-treated groups. Measurements of HCO_3^- secretion levels revealed a significant difference in WT and CYA-treated ducts during administration of BA ($P < 0.05$ WT BA vs. CYA BA) or EtOH + FA ($*P < 0.05$). *E* and *F*, in WT ducts the levels of base flux [$-\text{J}(\text{B}/\text{min})$] grades were significantly decreased due to BA (WT vs. WT BA $P < 0.05$) or EtOH + FA (WT vs. WT EtOH + FA $P < 0.05$) treatment. Recovery from alkalosis (*E*) and recovery from acidosis (*F*) values are presented \pm SEM. Comparison within CYA-treated groups revealed no significant difference (CYA control vs. CYA BA $p = 0.644$). *y* axis=base flux [$-\text{J}(\text{B}/\text{min})$] was calculated from the $1\text{pH}/1\text{t}$ obtained by linear regression analysis of pHi (HCO_3^- -secretion) measurements. [Colour figure can be viewed at wileyonlinelibrary.com]

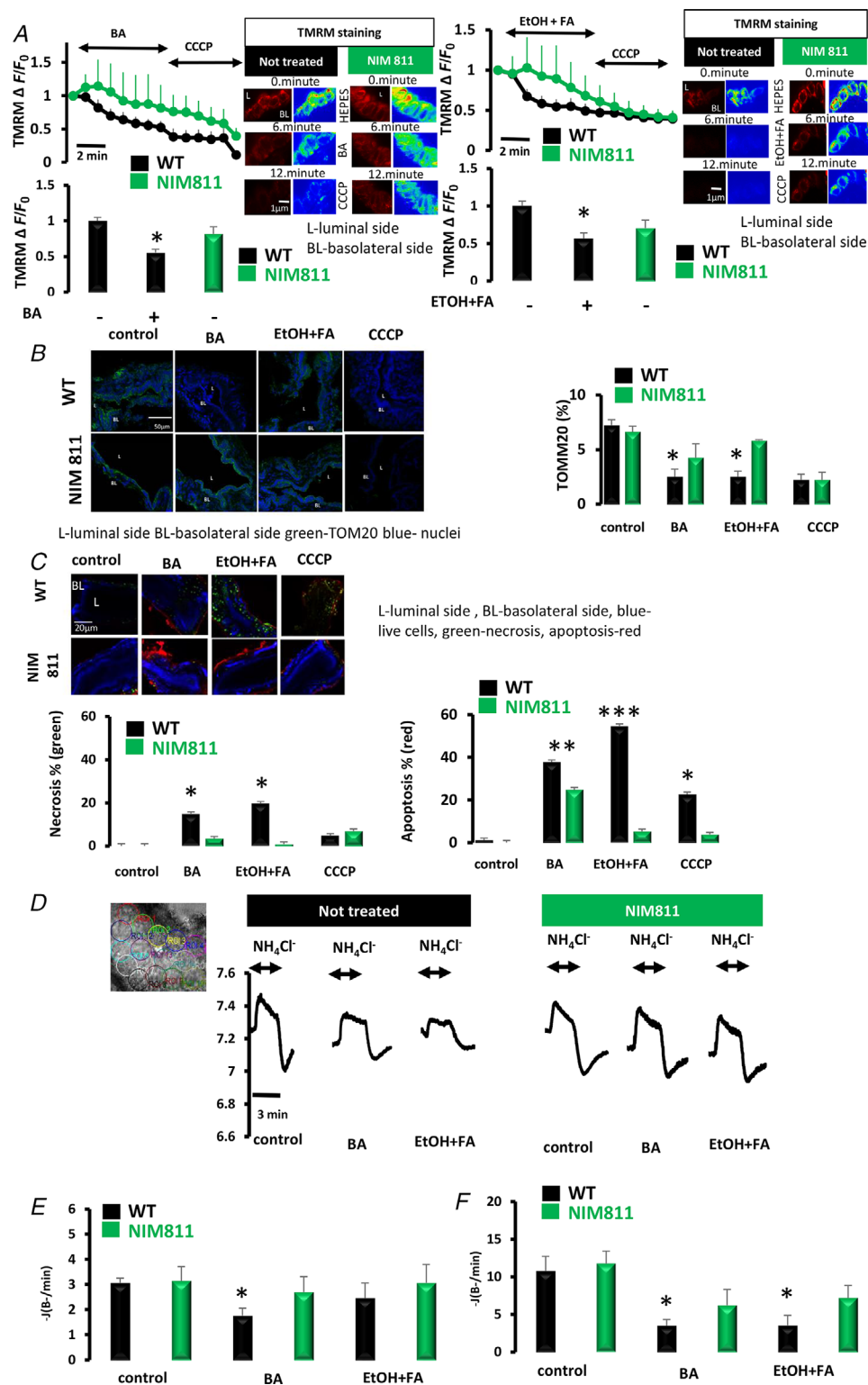


Figure 3. NIM811 protects mitochondrial and cell function in PDECs

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

apoptotic and necrotic cells during bile acid or ethanol and fatty acid treatment (WT BA vs. NIM811 BA * $P < 0.05$, WT EtOH + FA vs. NIM811 * $P < 0.05$). 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 * $P < 0.05$, WT EtOH + FA vs. NIM811 EtOH + FA * $P < 0.05$). 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, Tuttlingen, 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 \pm SEM. Data were compared by either one- or two-way ANOVA or

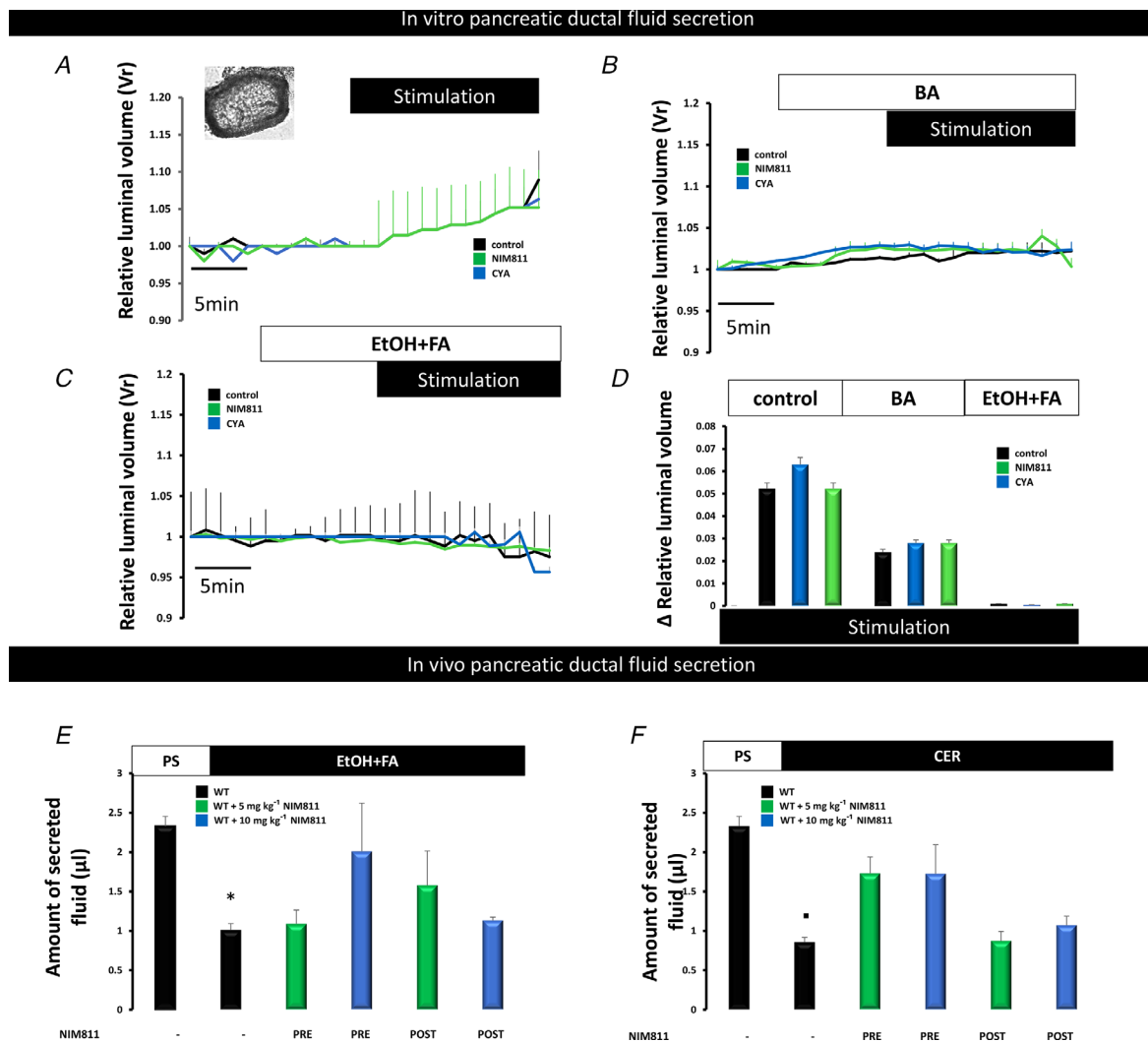


Figure 4. Pancreatic ductal fluid secretion is not altered by NIM811 or CYA treatment
 A, *in vitro* fluid secretion was stimulated by $5 \mu\text{M}$ forskolin and $100 \mu\text{M}$ IBMX (stimulation). B and C, BA or EtOH + PA treatment inhibited luminal swelling. D, relative luminal volume changes during forskolin and IBMX stimulation. Means \pm SEM; $n = 5\text{--}10$ 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. * $P < 0.05$ WT PS vs. WT EtOH + FA, $\square P < 0.05$ WT PS vs. WT CER $n = 4\text{--}7$ animals per group. [Colour figure can be viewed at wileyonlinelibrary.com]

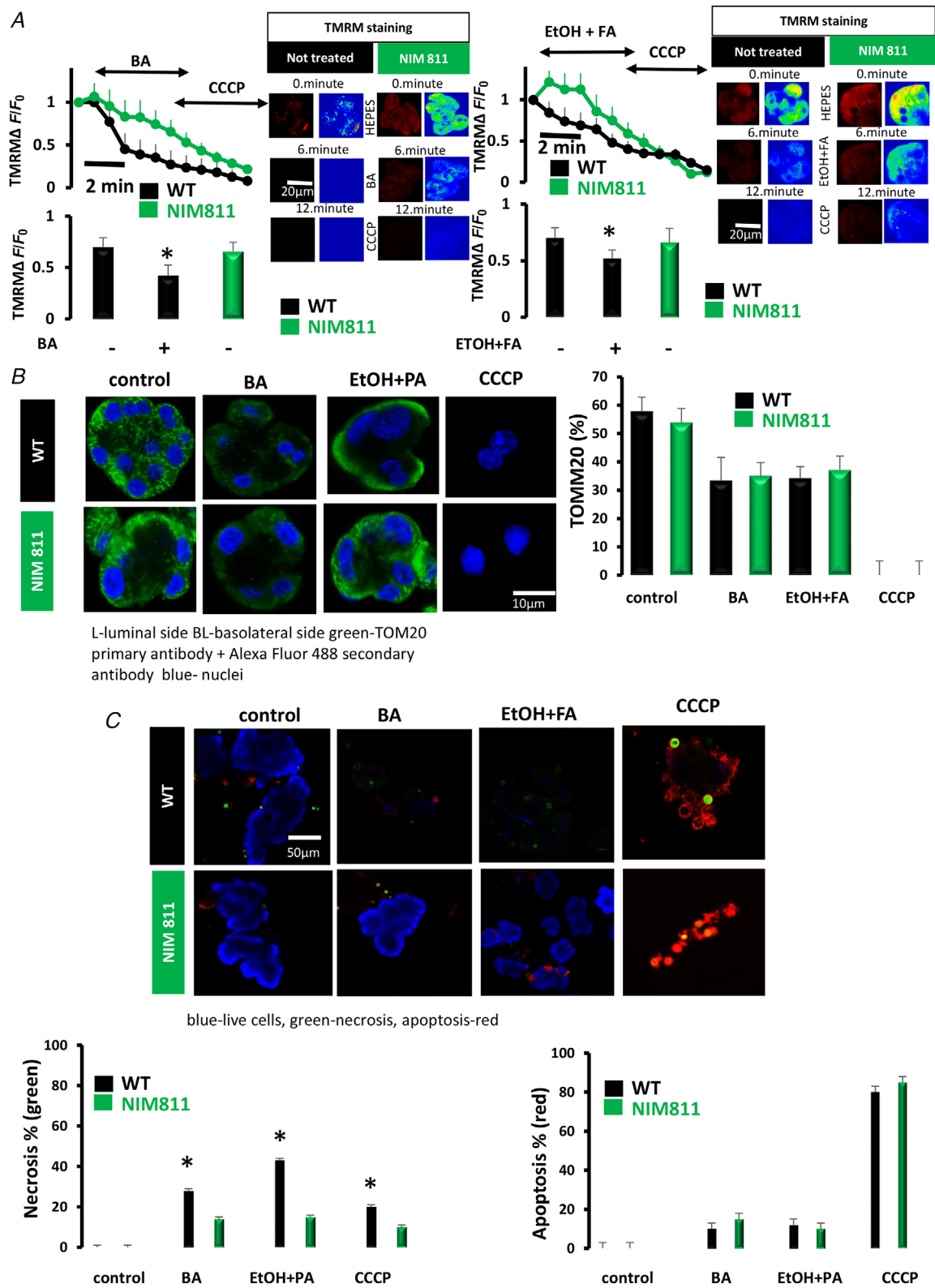


Figure 5. NIM811 treatment protects mitochondrial function in pancreatic acinar cells

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 μM chenodeoxycholic acid (CDC; BA) or co-administration of 100 mM ethanol and 200 μM palmitoleic acid (EtOH + FA). Co-staining the pancreatic ducts with CytoCalcein Violet, Apoptin 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_4Cl 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 $\text{Cl}^-/\text{HCO}_3^-$ exchangers (HCO_3^- efflux) and on $\text{Na}^+/\text{HCO}_3^-$ 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 ψ of PDECs (Fig. 2A). Importantly, 2 μM 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 μM 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, Apoptin 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_4Cl^- experiments revealed that the inhibitory effects of BA and EtOH + FA on $\text{Cl}^-/\text{HCO}_3^-$ exchangers (HCO_3^- efflux) and on $\text{Na}^+/\text{HCO}_3^-$ 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 ψ 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). Micro-fluorometric 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).

treatment. B, mitochondrial protein TOM20 levels did not show a difference in the NIM811-treated or untreated groups after BA, EtOH + FA or CCCP treatment ($P > 0.05$). C, a significant difference in necrosis levels was found between NIM811-treated and untreated groups in BA or EtOH + FA ($*P < 0.05$). However, no difference was found for the CCCP-treated groups. Apoptosis levels were not altered significantly by NIM811 during BA or EtOH + FA treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

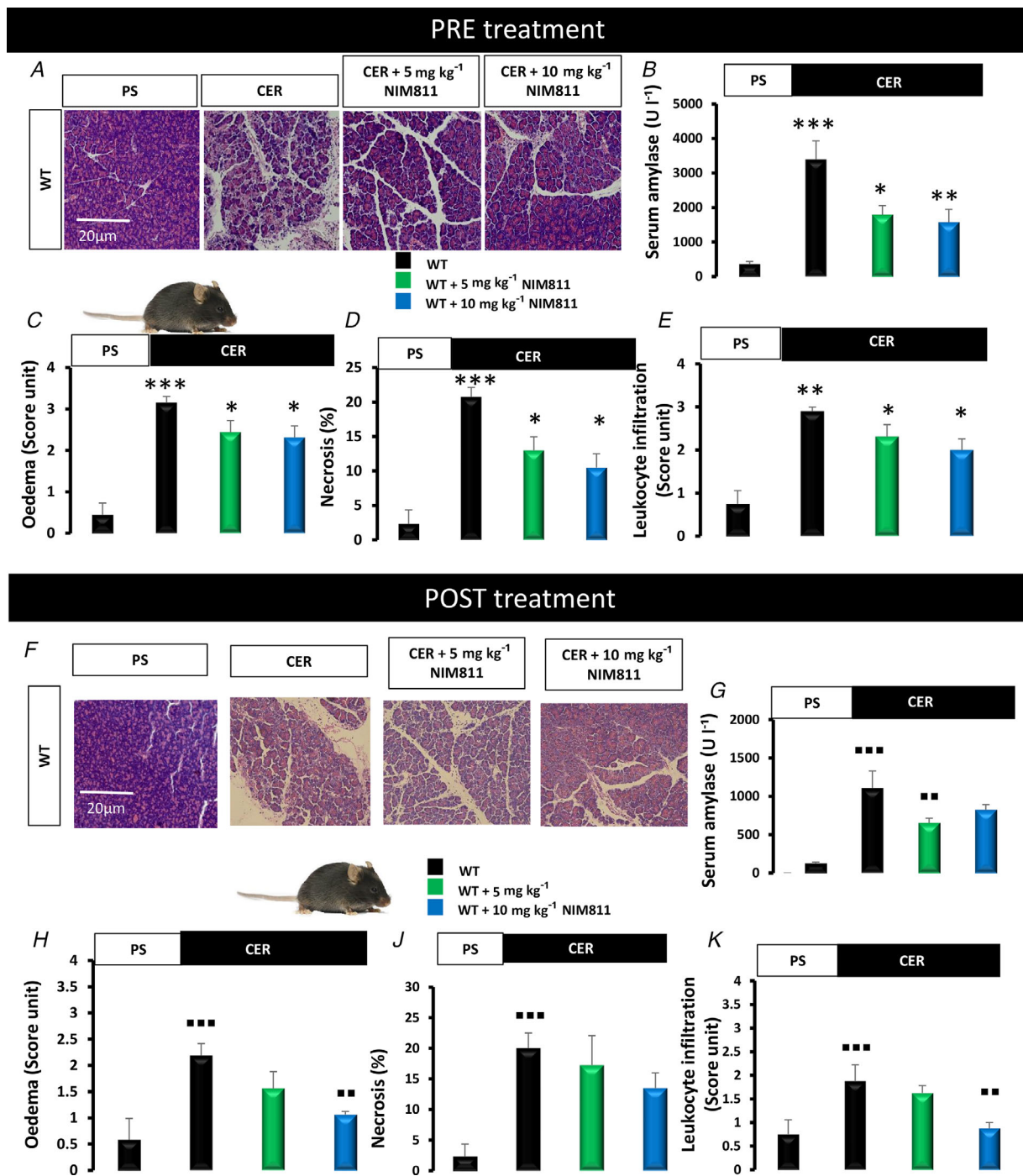


Figure 6. NIM811 reduces the severity of CER induced AP

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 and 10 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 \pm SEM). [Colour figure can be viewed at wileyonlinelibrary.com]

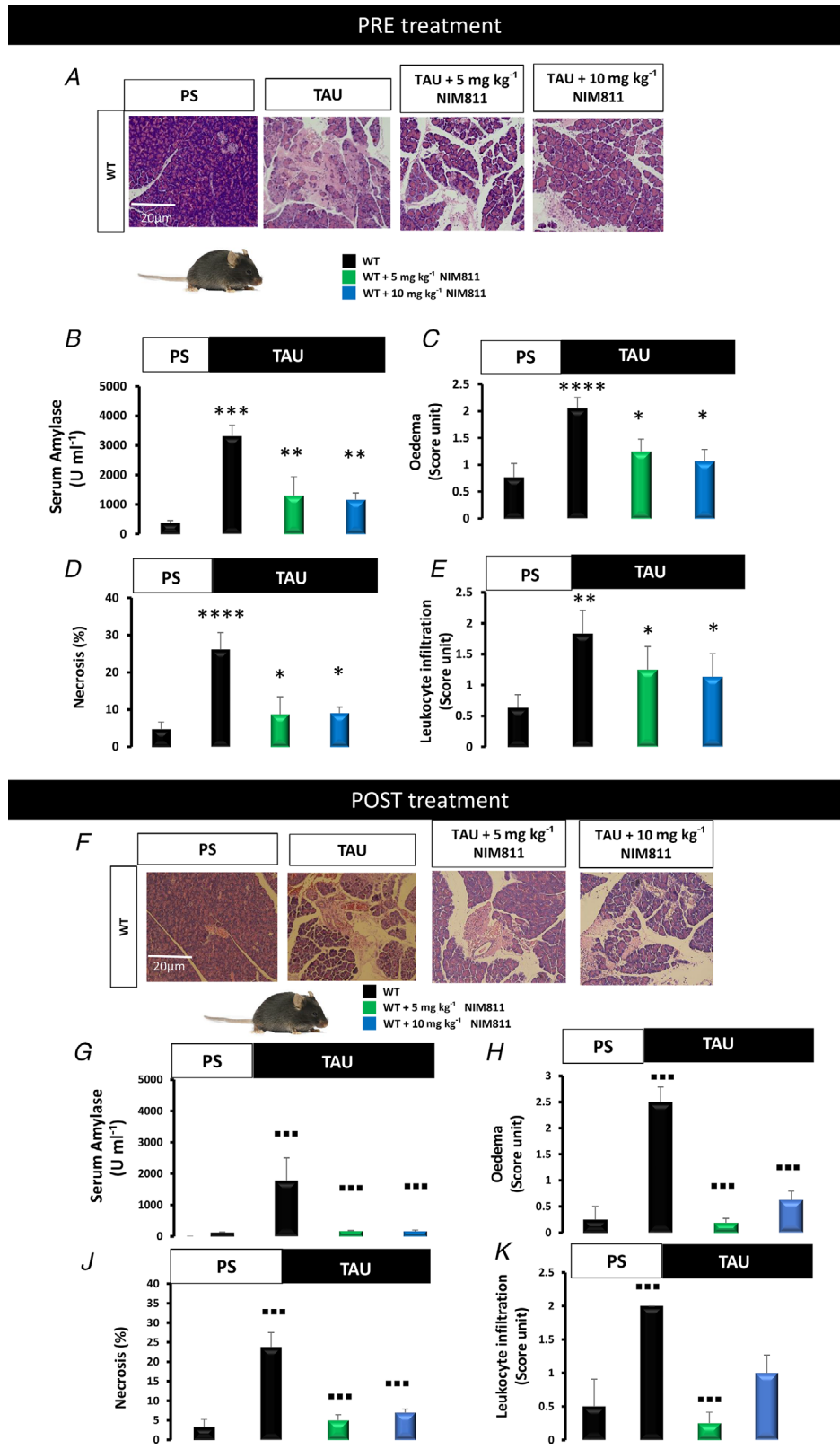


Figure 7. NIM811 reduces the severity of TAU induced AP in mice
 A–K, in TAU-induced pancreatitis, serum amylase measurements revealed that retrograde infusion of TAU led to elevated serum amylase levels [****P* < 0.01 WT PS vs. WT TAU (B), *****P* < 0.001 WT PS vs. WT TAU (G)], but 5 or 10 mg kg⁻¹ body 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. post 5 mg kg⁻¹ NIM811 TAU, $***P < 0.001$ WT TAU vs. post 10 mg kg⁻¹ NIM811 + TAU), and serum amylase levels were reduced compared to WT TAU-treated groups (B and G: $*P < 0.01$ WT TAU vs. WT 5 mg kg⁻¹ NIM811 TAU and $*P < 0.01$ WT TAU vs. WT 10 mg kg⁻¹ NIM811 TAU). During pre-NIM811 treatment oedema, necrosis and leukocyte infiltration scores were significantly decreased compared to the only TAU-treated groups (A, C, D, E: $P < 0.05$ WT TAU vs. pre 5 mg kg⁻¹ NIM811 TAU/10 mg kg⁻¹ NIM811 TAU). Post-insult administration of NIM811 decreased oedema, leukocyte infiltration and necrosis levels in the TAU group (G–K: $***P < 0.001$). $n = 4–6$ animals per group; data are means \pm SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

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 (Niederer *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 leukocyte 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⁻/HCO₃⁻ 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; Biczó *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 ψ ATP depletion, increased inner membrane permeability, mitochondrial swelling and necrotic cell death (Golstein & 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; Biczó *et al.* 2018). As regards ductal cells, we have shown earlier that both BA and EtOH + FA induce inhibition of HCO₃⁻ 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

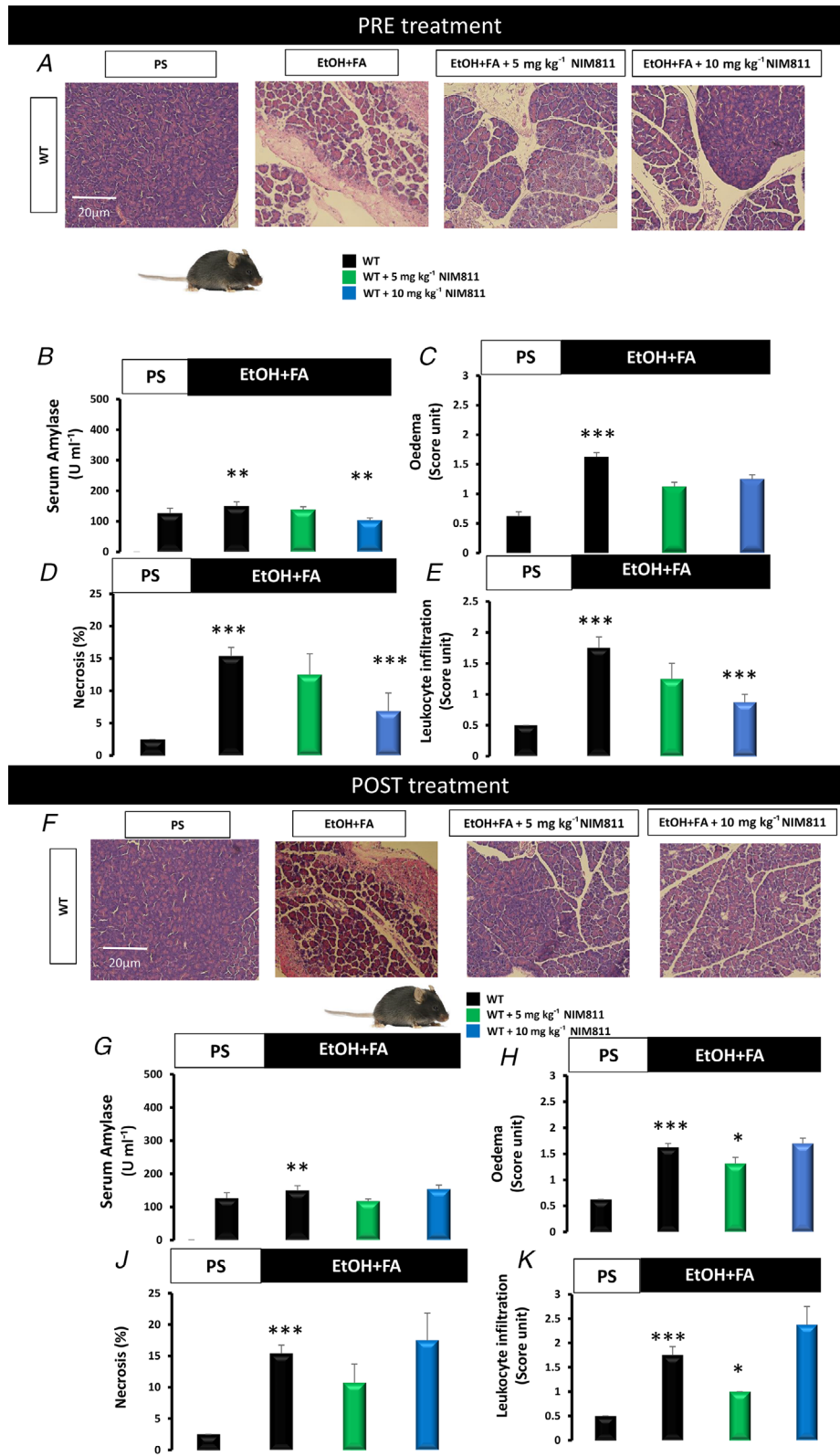


Figure 8. NIM811 has a protective effect against EtOH + FA induced pancreatic damage
 A–K, in EtOH + FA-induced pancreatitis, serum amylase measurements revealed that in pretreatment with 10 mg kg⁻¹ NIM811 significantly reduced serum amylase levels (B: ***P* < 0.002 WT EtOH + FA vs. pre 10 mg kg⁻¹ NIM811 + 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]

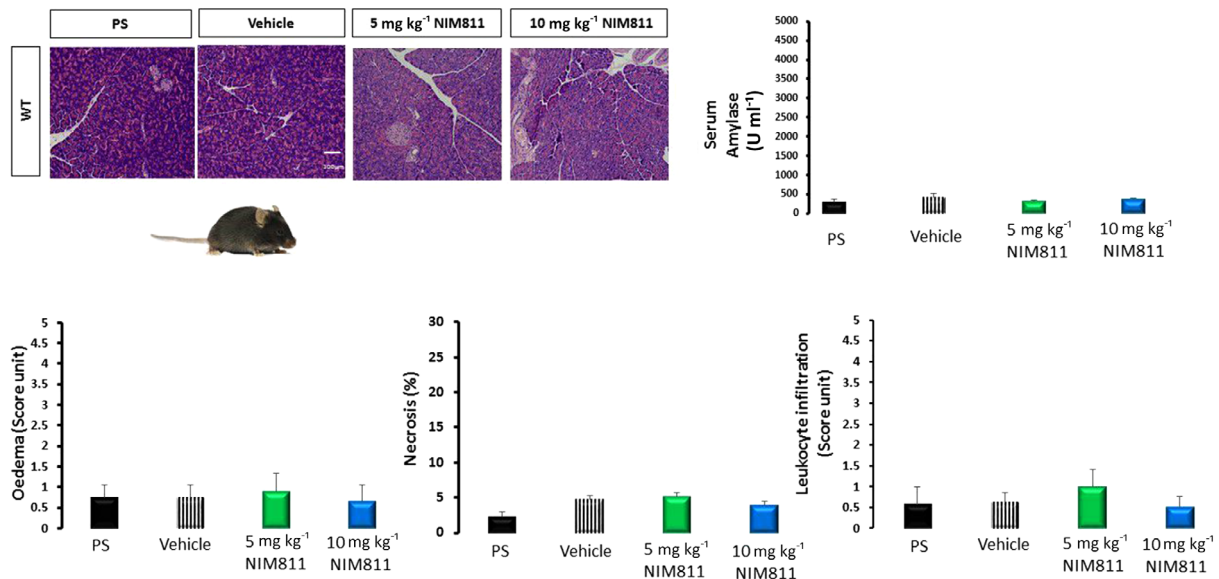


Figure 9. NIM811 itself does not induce pancreatic damage

No significant difference was found between the NIM811-treated - (8.3% polyoxyl 40 hydrogenated castor oil, 8.3% EtOH) vs. the control groups. *n* = 4–5 animals per group. [Colour figure can be viewed at wileyonlinelibrary.com]

to be a perfect choice. It has been shown to be protective in several diseases, and until now no toxic effects have been demonstrated. Therefore, we continued our study by testing the effects of NIM811 on both ductal and acinar cells *in vitro*. We found that NIM811 reduces the mitochondrial damage caused by BA or EtOH + FA. Importantly, NIM811 decreased apoptosis levels during BA or EtOH + FA treatment in ductal cells, but not in acinar cells, a result which could be due to the observation that ductal cells have more mitochondria than acinar cells (Maleth *et al.* 2013). Surprisingly, inhibition of mPTP protected pancreatic ductal bicarbonate but not fluid secretion during BA or EtOH + FA treatment. These data suggest that rescuing intracellular ATP levels and the activity of Na⁺/K⁺-ATPase do not result in overall protection alone and other fluid transport mechanisms such as aquaporins may remain diminished (Venglovecz *et al.* 2018). Per os administration of 5 or 10 mg kg⁻¹ NIM811 alone had no toxic effect, but significantly reduced the severity of AP. We found that NIM811 treatment was more beneficial in the TAU- than in the EtOH + FA-induced AP model. One explanation could be that besides the direct toxic effect of EtOH and FA, the non-oxidative metabolites of FA (fatty acid ethyl esters) have even higher toxicity on the mitochondria in both

acinar and ductal cells (Criddle *et al.* 2006; Petersen *et al.* 2009).

Taken together, mitochondrial function and bioenergetics play a crucial role in the development of AP; however, translation of these results to a patient benefit remains lacking (Maleth *et al.* 2013; Gukovskaya *et al.* 2016; Maleth & Hegyi, 2015; Mukherjee *et al.* 2016; Biczó *et al.* 2018). In this study, we have confirmed that the mPTP inhibitor NIM811 is a highly suitable compound to be tested in clinical trials. As a next step, phase 2 clinical trials are needed with the use of this novel and promising drug candidate.

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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, VV, 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.

Funding

This study was funded by a Momentum Grant from the Hungarian Academy of Sciences (LP2014-10/2014 to PH) as well as Economic Development and Innovation Operational Programme Grants and Project Grants from the National Research, Development and Innovation Office (GINOP-2.3.2-15-2016-00015, EFOP-3.6.2-16-2017-00006 and K116634 to PH, UNKP-19-3-SZTE-303 to ET, K109756 to VV, PD115974 to JM and K119938 to ZR).

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.