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

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KEYPOINTS

Bile acids, ethanol and fatty acids deteriorate pancreatic ductal fluid and bicarbonate secretion via mitochondrial damage, ATP depletion and calcium overload.

It is known that pancreatitis inducing factors open the membrane transition pore (mPTP) channel via cyclophilin D activation in acinar cells causing calcium overload and cell death and genetic or pharmacological inhibition of mPTP improves the outcome of acute pancreatitis in animal models.

In our study we show that genetic and pharmacological inhibition of mPTP protects mitochondrial homeostasis and cell function evoked by pancreatitis-inducing factors in pancreatic ductal cells.

Our results also reveal that the novel Cyclosporin A derivative NIM811 protects mitochondrial function in acinar and ductal cells, moreover it preserves bicarbonate transport mechanisms in pancreatic ductal cells.

We found that NIM811 is highly effective in different experimental pancreatitis models and that NIM811 has no side-effects. NIM811 is a highly suitable compound to be tested in clinical trials .

ABSTRACT

Background and aims

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.

Methods

Pancreatic ductal and acinar cells were isolated by enzymatic digestion from Bl/6 mice. In vitro measurements were performed by confocal microscopy and microfluorometry. Preventive effects of pharmacological (cylosporin A (2μM), NIM811 (2μM)) 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 *per os*), were checked in three different AP models induced by either caerulein (10x50μg/kg), ethanol+ fatty acid (1.75 g/kg ethanol and 750 mg/kg palmitic acid) or 4% taurocholic acid (2ml/kg).

Results

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.

Conclusion

The novel mitochondrial transition pore inhibitor NIM811 seems to be an exceptionally good candidate compound for clinical trials in AP.

KEYWORDS

Acute pancreatitis, mitochondrial transition pore, cyclophilin D, NIM811

INTRODUCTION

Acute pancreatitis (AP) is among the most common gastrointestinal disorders requiring hospitalization in the United States (Fangenholz *et al*,2007 ; Fagenholz *et al*, 2007 ; 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 Ca^{2+} influx, calcineurin activation, mitochondrial dysfunction and cystic fibrosis transmembrane conductance regulator (CFTR) inhibition were shown to have a great 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 and Saluja,2011; Maleth *et al*, 2013; Abu-El-Haija *et al*, 2018 ; Biczo and Vegh *et al* ,2018 ;) . It has been shown in acinar cells that bile acids (BA) 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 is still unknown how the pancreatitis-inducing

85 factors noted above modify mPTP channel activity in pancreatic ductal epithelial cells (PDEC),
86 it still seems to be one of the most promising drug targets and calls for further investigation.

87 Until now, cyclosporin A (CyA) is the only licenced compound used experimentally to
88 inhibit mPTP (via Cyp D) (Javed *et al*, 2018); however, its clinical usefulness is highly
89 questionable for several reasons. A pilot study found that CyA could reduce the size and
90 damage of myocardial infarction, but larger studies showed no beneficial effects (Piot *et*
91 *al*,2008; Cung *et al*,2015; Javed *et al*, 2018) . Even efforts to decrease its immunosuppressive
92 activity have not been successful. Moreover, CyA derivative Debio025 (Alispovirir,
93 Debiopharm) has been found effective against the hepatitis C virus (HCV), but it had serious
94 side-effects. Surprisingly, some of the patients developed pancreatitis, resulting in a clinical
95 hold on the global Debio025 trial programme (Zeuzem *et al*,2015; Stanciu *et al*, 2019). Another
96 derivative, TRO40303 (3,5-seco-4-nor-cholestan-5-one oxime-3-o, TROPHOS, Roche), was
97 not beneficial in a phase 2 trial of cardiac preservation following acute myocardial infarction,
98 suggesting that this compound has low or no effectivity (Atar *et al*, 2015) . Lately, it has turned
99 out that TRO40303 does not even bind to Cyp D directly (Sileikyte,2016 ; Javed *et al*, 2018;) .
100 With regard to AP, both Debio025 and TRO40303 have been shown to be beneficial in animal
101 models, but neither of them have reached "proof of concept" clinical trials in AP, most probably
102 due to the clinical failures noted above. All in all, new compounds are crucially needed.

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

112 In this study, we show in several in vitro and in vivo experiments that either
113 pharmacological or genetic inhibition of Cyp D restores mitochondrial function not only in
114 acinar cells, but also in ductal cells, highlighting the general importance of mPTP in AP.
115 Moreover, we provide evidence that NIM811 is highly effective in different experimental
116 pancreatitis models and that NIM811 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 euthanized by 200 mg/kg pentobarbital i.p. (Bimeda MTC, Cambridge, Canada).

Animals

A total of 70 wild type (WT) and cyclophilin D knockout (Cyp D KO, (B6;129-Ppiftm1Maf/J) mice were sacrificed. *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 us by the Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary. Wild type and Cyp D-deficient littermate mice (of C57Bl/6 J 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-acetoxymethylester (BCECF-AM) and Tetramethylrhodamine-methylester (TMRM) were purchased from Termofischer Scientific . NIM811 were purchased from MedChem Express Europe (Sweden). Cyclosporin A (CYA) , caerulein (CER) , NIM811, CCCP and fluorescence dies were diluted in dimethyl sulfoxide (DMSO) . Table 1 describes the constitution of solutions that we used during the study. In this study 500μM Chenodeoxycholic acid (bile acid,BA) or 100mM ethanol (EtOH) + 200μM palmitoleic acid (fatty acid, FA) was 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 pharmachological inhibition of the mPTP in pancreatic ducts or acinar cells. 100 μM of Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used in the mitochondrional measurements as a positive control for mitochondrial damage.

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

Abbrevations used in this study:

AP- acute pancreatitis , NIM811- N-metil-izoleucine cyclosporine , mPTP- mitochondrial transition pore , mitochondrial membrane potencial- ψ, CFTR- cystic fibrosis transmembrane conductance regulator, PDEC-pancreatic ductal epithelial cells , Cyclophylin D- Cyp D , CYA- cylosporin A , Hepatitis C virus-HCV, Debio025- Alispovirir, Tro40303-3,5-seco-4-nor-cholestan-5-one-oxime-3-o, PCR-polymerase chain reaction, TMRM-Tetramethylrhodamine Methyl Ester Perchlorate, TOM20- Mitochondrial import receptor subunit, FA- fatty acid (palmitoliec acid), FAEE- Fatty acid ethyl ester , ETOH-ethanol , BA- CDC- chenodeoxycholic acid , BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester), CER-caerulein , TAU- sodium taurocholate , TBS- Tris Buffered Solution, BSA- Bovine Serum Albumin, HBSS- Hank1s Stock Solution, CBD- Common Pancreatic Biliary Duct, CCCP- Carbonyl cyanide 3-chlorophenylhydrazone

Methods

Mouse genotyping

Genotypes of cyclophilin D deficient mice were identified by PCR (typical polymerase chain reaction , analyses from tail genomic DNA) . PCR-mix contained: Taq DNA pol 5 U and 10xTaq Buffer (Abgene, Portmouth, USA), $MgCl_2$ 1,5 mM, dNTP 2.5mM, F-null2/LoxP1f /CyPuP2 primers (20-20μM), dH_2O and template DNA sample. Total reactions mix volume was 25 μl.
The wild type allele was detected using LoxP1f, 5'-AAA CTT CTC AGT CAG CTG TTG CCT CTG-3' as a forward primer and F-null2, 5'- GCT TTG TTA TCC CAG CTG GCG C-3' as a reverse primer. For genotyping of the mutant cyclophilin D deficient allele, F-null2, 5'-TTC TCA CCA GTG CAT AGG GCT CTG –3' was used as a forward primer with the reverse primer for WT (Table 2.) . DNA was denatured at 95°C for 2 mins, followed by 30 cycles of amplification: 94°C for 30 secs, 60°C for 30 secs, 72°C for 45 secs and a final primer extension

step at 72°C for 7 mins. Bands of 270 and 470 base pairs were amplified for WT and CypDKO mice, respectively.

Pancreatic ducts and acinar cells were isolated by microdissection and enzymatic digestion as described earlier (Argent *et al*,1986 ; Gout *et al*, 2013) (Argent, Arkle et al. 1986, Gout, Pommier et al. 2013).

Mitochondrial membrane potential (Ψ) were determined by 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 (Tetramethylrhodamine Methyl Ester Perchlorate ,100 nmol/L).

In order to monitor apoptotic and necrotic cells in isolated pancreatic ducts or acinar cells an apoptosis/necrosis kit was used (ab176750, Abcam). To determinate live, necrotic or apoptotic cells, CytoCalcein Violet 450 fluorescent, Apopxin Deep Red Indicator and Nuclear Green DCS1 fluorecence dies (ab176750, Abcam) were used. Samples were incubated in the mixture of the above stated fluorescence dyes at room temperature for 30-35 mins (after 25 min treatment of with BA/ETOH+FA/CYA/NIM811) in dark prior to the confocal microscopy measuremets. In case of CYA or NIM811 treated ducts or acinar cells, the incubation with these compounds were performed before staining with the fluorescence dyes . Stainings were analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). Live, necrotic or apoptotic cells were counted and summarized in percentage of each sample, then data were summarized to average and statistical analysis was performed.

Microfluorometry was used to measure pancreatic ductal HCO_3^- secretion as described earlier (Hegyi *et al*, 2003, Hegyi *et al*, 2004) by using BCECF-AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester, 1.5 mmol/L).

Functionally active mitochondria were detected with immunofluorescent staining (TOM20 mitochondrial marker, (EPR15581-39, Abcam)). In order to determine mitochondrial localisation in isolated pancreatic ductal or acinar cells we labeled the mitochondria by the using of TOM20 primary antibody (Abcam, EPR15581-39). TOM20 is the central unit of the receptor TOM complex in the mitochondrial outer membrane and the role of it is to recognise and translocate cytosolically synthetized mitochondrial preproteins (Shatz *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 by Leica Cryostat. Sections were fixed in 4% paraformaldehyde . Washing periods were administered with 1xTBS solution. Antigen retrieval was performed with 10 mM Sodium –Citrate solution at the pH of 6 at 95 ˚C for 15 minutes. Blocking was obtained for 1h with 1% goat serum in 5% BSA-TBS solution. After these sections were incubated with TOM20 rabbit monoclonal antibody (dilution 1:400,Abcam) overnight incubation at 4˚C. The following day the samples were incubated with goat anti rabbit secondary antibody (Alexa fluor 488, Thermo Fisher, Rockford, IL, United States) for 2 hours at dark in room temperature. The nuclei were counterstained with Hoechst 33342 (Termofischer, Rockford,IL,United States) . Immunofluorescence staining of the isolated pancreatic acinar cells were performed freshly after the isolation procedure with the same conditions as stated above, (except two parameters ; cells were fixed in 2% paraformaldehyde and dilution fo the primary antibody was 1:200) as stated above. Both ductal and acinar cell samples were mounted with Fluoromount and then analyzed using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). To quantify TOM20 positively stained area, 5-6 representative images from each group were taken by Zeiss LSM 880 Confocal Scannig Microscope (Carl Zeiss Technika Kft., Budaörs, Hungary). Image J software was used to convert images to gray scale (16 bit), threshold function was used to select the positively stained area. The fluorescence signal were calculated by the software (arbitary scale from 0-negative (white) to 255-maximal staining (black)) (Venglovecz *et al*,2018). Fluorescence intensity of the images were then normalized to the own total ductal or acinar area of the samples, which were measured in arbitary units. Fluorescence intensity was given in %, normalized to the total ductal or acinar total area.

AP was induced by caerulein (CER,10x50µg/kg); 4% sodium taurocholate (TAU, 2ml/kg,4%) (Niederau *et al*, 1985 ; Ding *et al*,2003 ; Perides *et al*,2010; Pallagi and Balla *et al*;2014 ;) or alcohol and fatty acid (intraperitonal injection of 1.75 g/kg ethanol and 750 mg/kg palmitic acid , EtOH+FA) as described earlier (Huang *et al*.,2014;Maleth *et al*, 2015). 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 AP, concentrations of NIM811 were 10 mg/kg or 5mg/kg. Dosage 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 (20ga x 38mm, Instech Laboratories, 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 hour 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 3rd injection of CER. The method for retrograde intraductal infusion of TAU has been described by Perides et al (Perides *et al*, 2010) . The surgery was performed on anesthetized mice (with ketamine-xylazine, dosage: 87.5 mg/kg ketamine-12.5 mg/kg xylazine). At the end of the procedure the mice were placed on a heating pad for 40 minutes and received buprenorphine i.p. injection (0.075 mg/kg) at once to reduce their occurrent pain. Following these mice were replaced into their cages for 24hours. They had free access to food and water. 24 hours after the TAU or EtOH +FA induced AP the mice were euthanized by 200 mg/kg pentobarbital i.p. (Bimeda MTC, Cambridge, Canada), . During the CER induced AP mice were euthanized with 200 mg/ kg pentobarbital i.p. (Bimeda MTC, Cambridge, and Canada) 2 hours after the last injections of CER. Mice were exsanguinated through cardiac puncture and the pancreas were removed. Blood from the cardiac puncture was placed on ice, then centrifuged with 2500 RCF for 15 mins 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 hematoxylin –eosin staining was performed. A colorimetric kit was used to measure serum amylase activity (Diagnosticum, Budapest, Hungary). Absorbance of the samples were detected at 405 nm with the use of 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 hematoxylin-eosin by using a standard laboratory method. To quantify oedema, necrosis and leukocyte infiltration grades a semiquantitative scoring system was used as Kui et al described previously (Kui and Balla *et al*, 2015) .

In vitro pancreatic ductal fluid secretion (luminal swelling) assays were developed by Fernández-Salazar et al, (Fernández-Salazar et al,2004) performed by videomicroscopy as described earlier (Balázs *et al*,2018). Briefly, stimulaton of pancreatic ductal fluid secretion was induced by 5 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX) , quantification were performed by Image J Software (Balázs *et al*,2018). In vivo fluid secretion measurements were performed on anesthetized (by i.p. 87.5 mg/kg ketamine-12.5 mg/kg xylazine) mice after CER or EtOH+FA induced AP prior to euthanasia. Animals were placed on warm pads (37◦ C) to maintain the body temperature. Briefly, the abdomen of the mice were opened and cannucaltion of the lumen of the common biliopancreatic duct was performed by a 30-gauge needle (Maléth *et al*, 2015). Then 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 PE-10 tube for 15 min. In vivo secretion was induced by i.p. administration of 0.75CU/kg secretin (Maléth *et al*, 2015).

Statistical Analysis

All data are expressed as means ± SEM. Data were compared by either one- or two-way analysis of variance (ANOVA) or Kruskal–Wallis tests followed by the Holm–Sidak Method as appropriate (Sigma Plot). The effects were considered significant when $p < 0.05$.

RESULTS

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

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 performed 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 CDC (BA) or co-administration of 100mM ethanol and 200μM 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 PDEC. Next, we investigated how the genetically preserved mitochondrial function affects the cellular function of PDEC (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 Cl/HCO_3^- exchangers (HCO_3^- efflux) and on Na/HCO_3^- co-transporters (HCO_3^- influx) are 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 PDEC

Both BA and EtOH+FA significantly decreased the ψ of PDEC (Fig. 2A). Importantly, 2μM 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 we could see during the genetic inhibition of mPTP (Fig. 2B). 2μM CYA decreased the extent of necrosis and apoptosis during the administration of BA or EtOH+FA in PDEC (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 PDEC

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 PDEC. Furthermore, it can strongly decrease BA- or EtOH-FA-evoked necrosis and apoptosis (Fig. 3C). NH_4Cl^- 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 PDEC (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 can not prevent BA or EtOH+FA induced fluid secretiory damage in isolated ducts (Fig.4 A-D, 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 we have seen in PDEC

(Fig. 4A). 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).

NIM811 has therapeutic benefits in caerulein, taurolithocholic acid sulfate and ethanol and fatty acid induced AP

Firstly, we confirmed that per os administration of either 5 or 10mg/kg NIM811 alone has no toxic effect on the pancreas (Fig 9.) . Secondly, we tested the compound in three different experimental AP models, the caerulein (CER) , alcohol and fatty acid (EtOH+FA) and the taurocholic (TAU)-induced ones (Niederau et al,1985; Huang et al, 2014; Perides et al,2010) . Importantly, both pretreatment 5 or 10mg/kg NIM811 significantly reduced the elevation of serum amlylase activity, as well as pancreatic oedema, necrosis and leukoctye infiltration in experimental AP models (Figs. 6–8). In our study we also confirmed, that post treatment of 5mg/kg or 10 mg/kg NIM811 has protective effects against pancreatic damage (Figs. 6-8.).

DISCUSSION

Acute pancreatitis is a multifactorial disease (Hegyi and Petersen ,2013; Sahin-Toth and Hegyi, 2017) involving several types of cell, including acinar and ductal cells. None of the therapeutic efforts targeting only one of them have been successful. Intravenous administration of secretin, which targeted ductal cells only, was found either to be slightly beneficial or natural in AP (Renner *et al*,1983; Lankisch *et al*, 1983; Keim *et al*, 1985). On the other hand, neither gabexate mesilate nor trasylol, which effectively inhibit trypsin activity, had beneficial effects in AP (Imrie *et al*,1978; Buchler *et al*, 1993) (Imrie, Benjamin et al. 1978, Buchler, Malfertheiner 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 (Maleth *et al*, 2013; Hegyi and Petersen, 2013 ; Maleth and Hegyi,2016) It decreases ATP production, causing 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; Maleth *et al*,

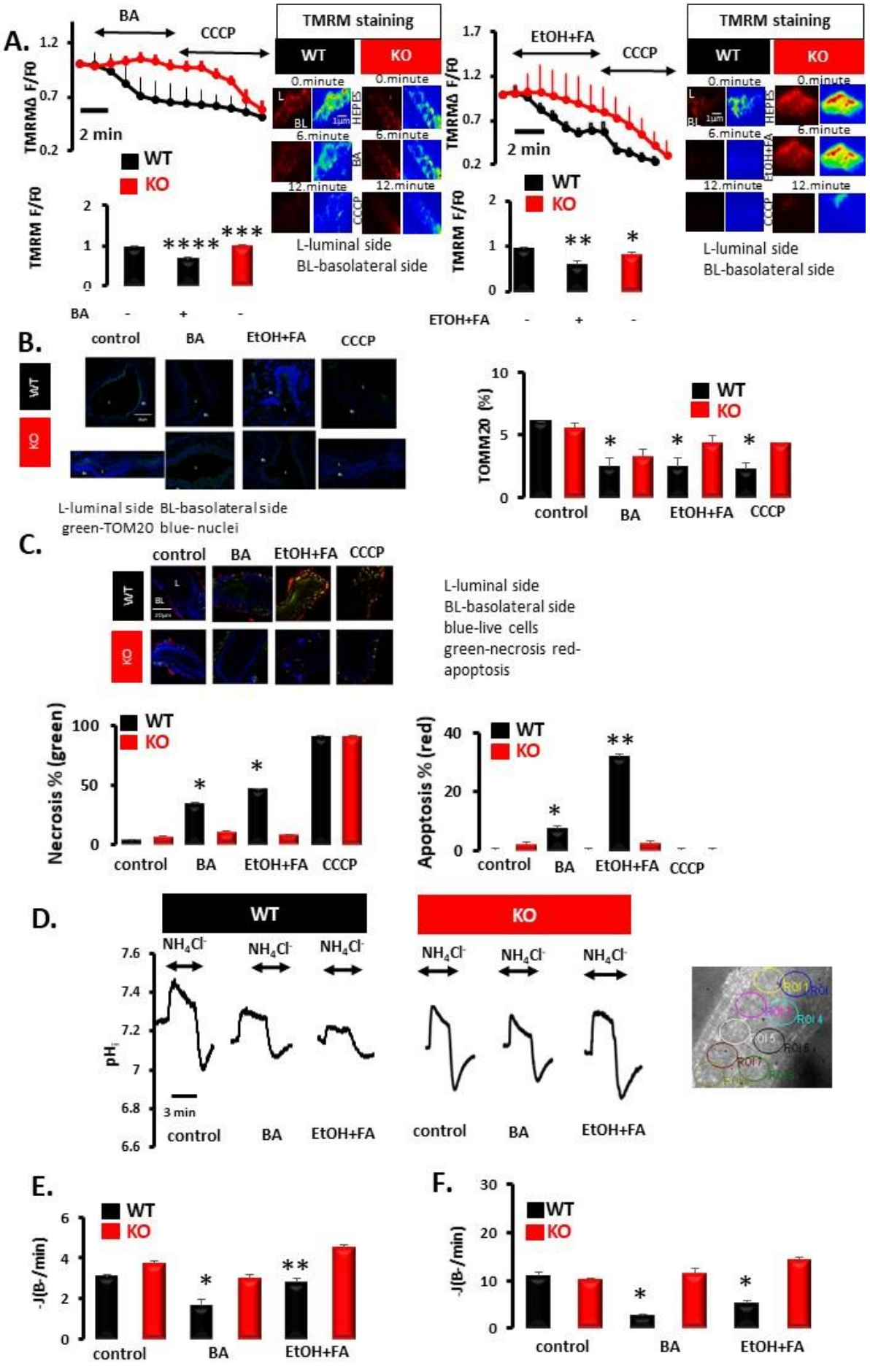
2013 ; Judak *et al*, 2014; Maleth *et al*,2015 ; Mukherjee *et al*,2016; Maleth and Hegyi,2016, Biczo and Vegh *et al*, 2018). In addition, mitochondrial damage is the main factor in determining cell death pathways necrosis and apoptosis. Release of mitochondrial cytochrome c into the cytosol causes 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 like cytochrome c to be released from the inner membrane to the cytosol (Tait *et al*, 2010; Maleth *et al*, 2016). On the other hand, the opening of the mPTP leads to loss of ψ , ATP depletion, increased inner membrane permeability, mitochondrial swelling and necrotic cell death (Golstein *et al*, 2007; Halestrap *et al*, 2009; Maleth *et al*, 2016). Very uniquely, inhibition of mPTP could prevent both cell death mechanisms in PDEC, which is different from that seen in acinar cells, where only necrosis could have been prevented. All in all, inhibition of mPTP seems to be highly beneficial in both cell types. In the last decade, it has been proved that genetic or pharmacological inhibition of mPTP reduces BA- or EtOH+FA-induced AC damage as well as augmenting the severity of AP (Sah et al,2011; Mukherjee et al, 2016 ; Gukovskaya et al, 2016; Biczo and Vegh 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 PDEC) (Maleth *et al*,. 2011, Maleth *et al*. 2015). 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 PDEC 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 have 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 the “proof of concept” phase 2 clinical trials in AP because of its inconsistent behavior in other trials due to the facts noted in the introduction. Recently, other new mPTP inhibitors have been introduced in experimental studies. Isoxazoles had inconsistent effects in myocardial infarction (Sileikyte *et al*, 2016). Benzamides resulted in impaired ATP generation (Sileikyte *et al*, 2016; Javed et al, 2018) . Cinnamic anilides were shown to be effective in myocardial infarction (Fancelli *et al*, 2010) ; however, lately it has turned out that it has an age-related toxicity (Fang *et al*, 2019). Besides unsuccessful attempts, NIM811 seemed 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 fluid secretion during BA or EtOH+FA treatment. These data suggest that rescuing intracellular ATP level 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 NIM811 treatment alone had no toxic effect, but significantly reduced the severity of AP. We found that NIM811 treatment was more beneficial in the TAU than the EtOH+FA induced AP model. One of the explanations could be that besides the direct toxic effect of EtOH and FA, the non-oxidative metabolites of FA namely FAEE has even higher toxicity on the mitochondria both in 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 the results to patient benefit is still missing (Maleth *et al*,2013 ;Mukherjee *et al*,2016 ;Maleth and Hegyi,2016 ; Gukovskaya *et al*, 2016; Biczo and Vegh *et al*,2018). In this study, we were the first to confirm that the mPTP inhibitor NIM811 is a highly suitable compound to be tested in clinical trials. As a next step, the companies should organize phase 2 clinical trials with the use of this novel and promising drug candidate.

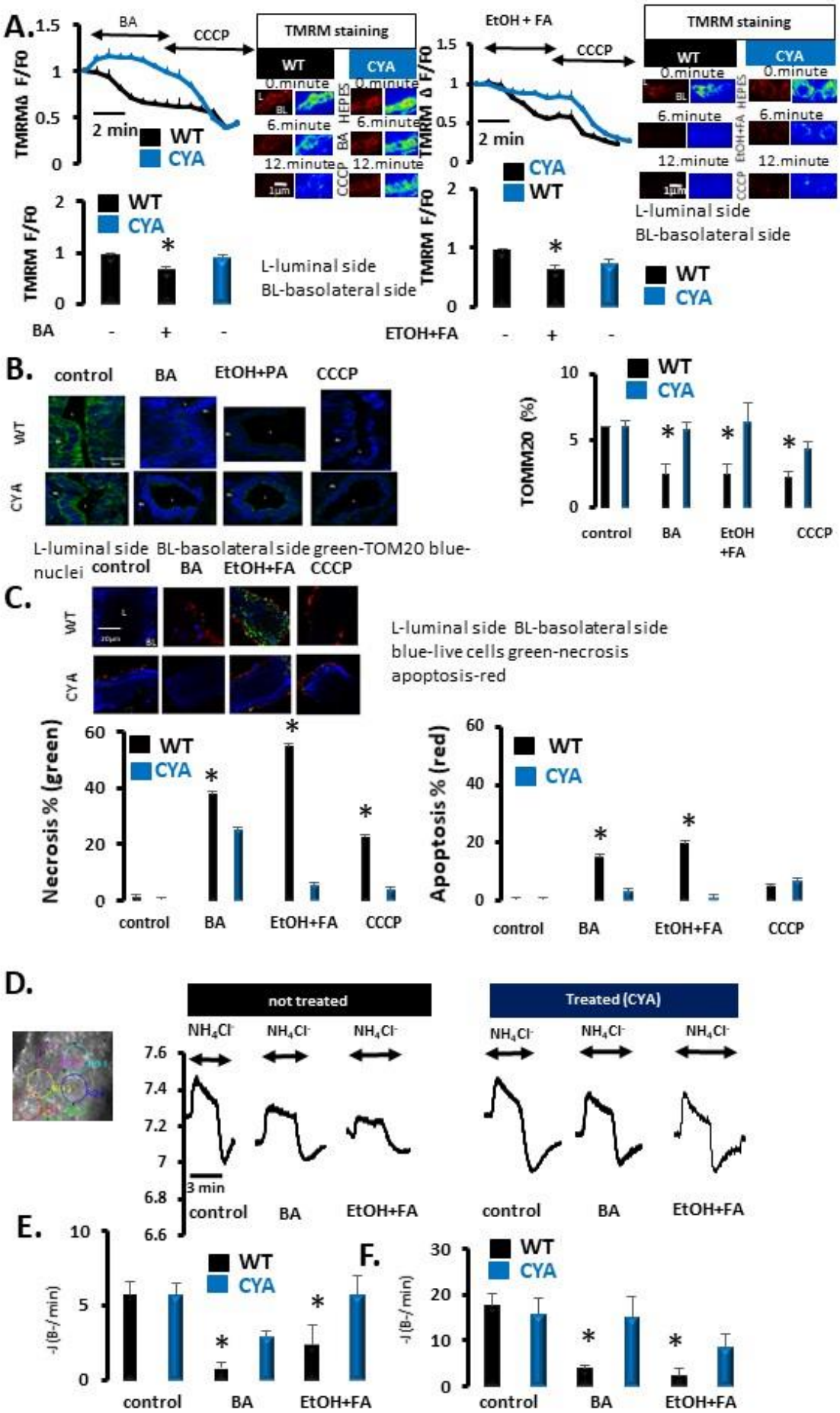
FIGURES AND FIGURE LEGENDS

Figure1. Genetic inhibition of Cyp D reduces the severity of bile acid or ethanol and fatty acid induced damage in PDEC


A.
BA
CCCP
TMRM staining
WT
KO
TMRMΔ F/F0
2 min
0.minute
6.minute
12.minute
HEPES
BA
CCCP
L-luminal side
BL-basolateral side
TMRM F/F0
EtOH+FA
ETOH+FA
B.
control
BA
EtOH+FA
CCCP
L-luminal side BL-basolateral side
green-TOM20 blue- nuclei
TOMM20 (%)
C.
L-luminal side
BL-basolateral side
blue-live cells
green-necrosis red-apoptosis
Necrosis % (green)
Apoptosis % (red)
D.
NH4Cl-
pHi
3 min
E.
-J(B-/min)
F.

Mitochondrial membrane potencial measurements revealed that genetic inhibition of mPTP significantly reduces the mitochondrial membrane potencial loss compared to WT controls during the administration of bile acid (500 µm CDC) or ethanol (100mM) and fatty acid (200µM FA) treatment (Fig1. A) (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=07.12,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/group, data means ±SEM. Results from the immunostainings revealed a significant decrease of the TOM20 stainings in BA; EtOH+PA or CCCP treated WT ducts , results were compared to Cyp D KO stainings . (Fig1.B) (*p<0.05). Genetic inhibition of mPTP also decreased the necrosis and apoptosis levels during bile acid; ethanol or fatty acid or CCCP treatment (Fig1.C). (*p<0.05)
Representative traces from the pancreatic ductal HCO_3- secretion measurements (Fig.1.D) Our data revealed that recovery from the alkalosis grades were significantly lower due to BA or ETOH+FA administration (*p<0.05) compared to the results from Cyp D KO ducts (Fig1.E). Recovery from the acidosis grades were significantly lower in the WT ducts due to the treatment with BA or EtOH and FA (*p<0.05), while in Cyp D KO ducts these grades were significantly higher (*p<0.05) . n=5-7 experiments/group, data means ±SEM.

Figure2. CYA reduces the severity of bile acid or ethanol and fatty acid induced pancreatic ductal damage

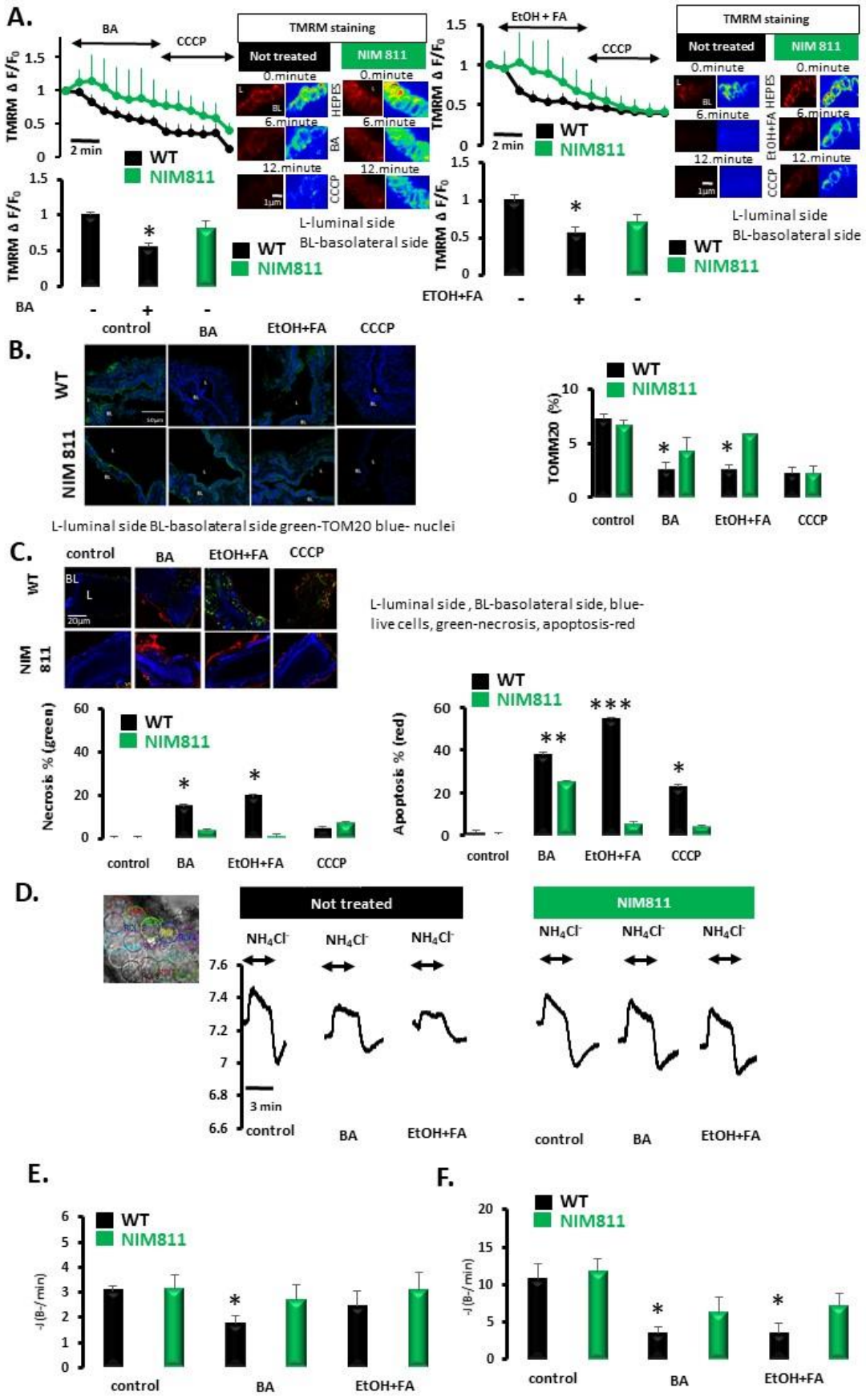

A.
BA
CCCP
TMRM staining
WT
CYA
0.minute
6.minute
12.minute
HEPES
BA
CCCP
TMRM Δ F/F0
2 min
WT
CYA
TMRM F/F0
L-luminal side
BL-basolateral side
BA
EtOH + FA
EtOH+FA
B.
control
BA
EtOH+PA
CCCP
WT
CYA
L-luminal side BL-basolateral side green-TOM20 blue-nuclei
TOMM20 (%)
EtOH +FA
C.
L-luminal side BL-basolateral side
blue-live cells green-necrosis
apoptosis-red
Necrosis % (green)
Apoptosis % (red)
D.
not treated
Treated (CYA)
NH4Cl-
3 min
E.
F.
-J (B-/min)

2 μM CYA treatment reduced the drop of mitochondrial membrane potencial loss which accured due to the BA or ETOH+FA treatment. (WT vs. CYA) (Fig.2.A). In WT ducts BA or ETOH+FA treatment resulted in significantly reduced mitochondrial membrane potencial (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 were no significant decrease. 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 were significantly higher (Fig2.B) *$p<0.05$. Between the control groups (WT control or only CYA treated samples) we found no significant alterations in the stainings. Necrosis levels were intensively elevated in BA or EtOH treated groups in WT ducts but not in CYA treated groups (Fig.2.C). Apoptosis levels were significantly higher as well in the not CYA treated groups compared to the CYA treated groups (Fig2. C).

Measurements of HCO_3^- secretion levels revealed a significant difference in WT and CYA treated ducts during the administration of BA ($p<0.05$ WT BA vs CYA BA) or EtOH+FA (*$p<0.05$). In WT ducts the levels of base flux (-J(B-/min) grades were significantly decreased (Fig2.E,F) due to BA (WT vs WT BA $p<0.05$) or ETOH+FA (WT vs WT EtOH+PA $p<0.05$) treatment (Fig2 E,F). Recovery from alkalosis (Figure 2. E) and recovery from acidosis values are presented in base flux ((-J(B-/min) grades respectively, with ±SEM. Comparison within CYA treated groups revealed no significant difference (CYA control vs CYA BA $p=0.644$).

473

474 **Figure3. NIM811 protects mitochondrial and cell function in PDEC**


A.
BA
CCCP
TMRM staining
Not treated
NIM 811
0.minute
6.minute
12.minute
HEPES
BA
CCCP
TMRM Δ F/F0
2 min
WT
NIM811
L-luminal side
BL-basolateral side
control
BA
EtOH+FA
CCCP
ETOH+FA
B.
WT
NIM 811
L-luminal side BL-basolateral side green-TOM20 blue- nuclei
TOMM20 (%)
C.
L-luminal side , BL-basolateral side, blue-live cells, green-necrosis, apoptosis-red
Necrosis % (green)
Apoptosis % (red)
D.
Not treated
NIM811
NH4Cl-
3 min
E.
-I(B-/min)
F.

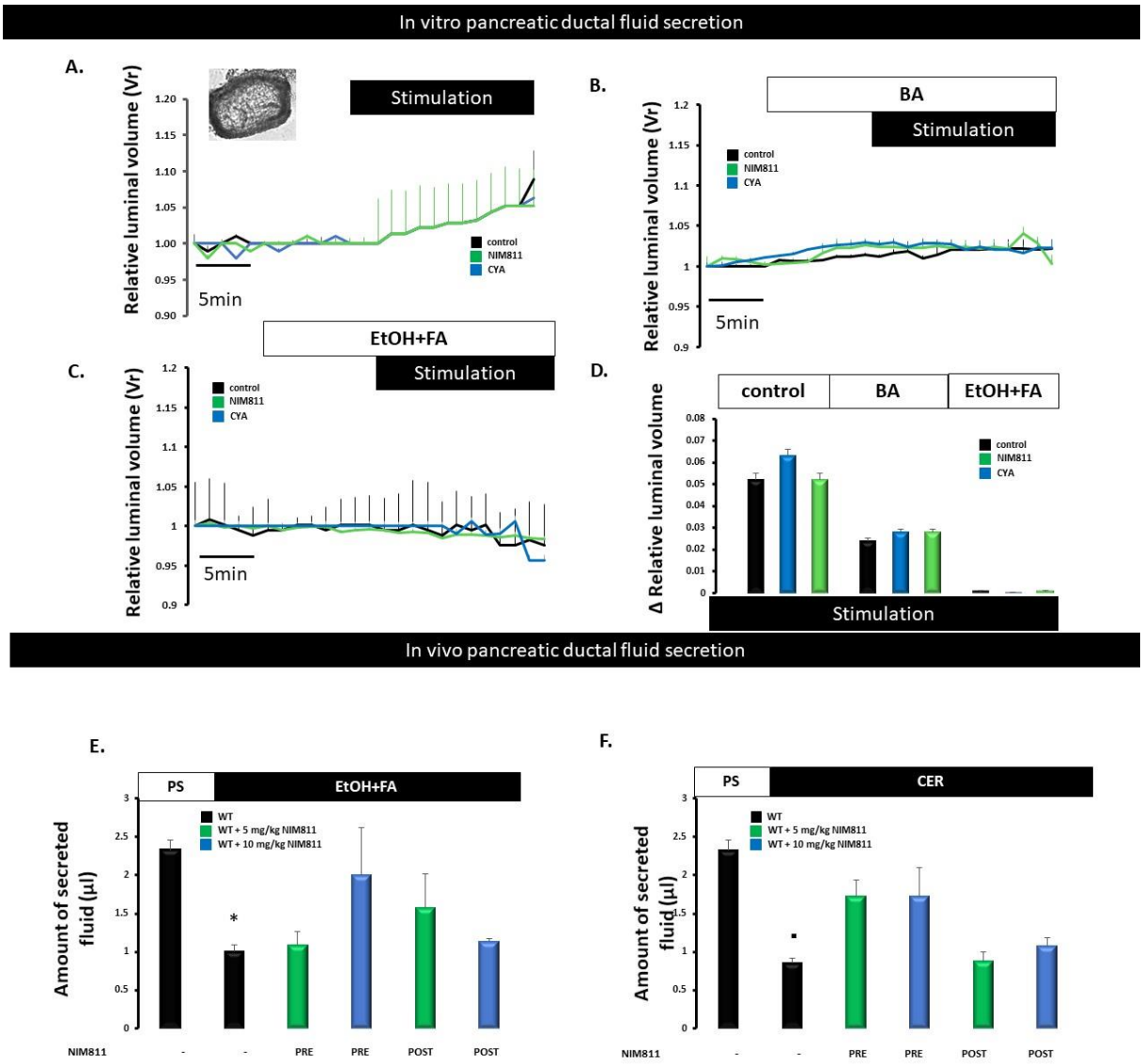
475

NIM811 treated ducts revealed a significantly consolidated loss of mitochondrial membrane potencial 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 (Fig.3A) . In NIM811 treated ducts the percentage of fluorescence intensity were significantly higher compared to not NIM811 treated ducts during BA or ETOH+FA administration. In CCCP treated ducts we found no significant difference in the amount of TOM20 stainings in the aspect of NIM811 treated or not treated groups. NIM811 itself did not alter the value of TOM20 stainings compared to the WT control samples (Fig.3B).

NIM811 decreased the numbers of apoptotic and necrotic cells during bile acid or ethanol and fatty acid treatment (Fig.3C) (WT BA vs NIM811 BA *p<0.05, WT EtOH+FA vs NIM811 *p<0.05). While during the administration of CCCP the apoptosis and necrosis grades were not significantly different in the comparative groups (Fig.3.C).

NIM811 treatment did not decreased the HCO_3^- secretion grades (control, Fig.3 D,E,F) , while during the adminsitration of BA or ETOH+FA treatment it had a protective effect against the reduction of HCO_3^- secretory levels (Fig.3E/F) (WT BA vs NIM811 BA *p<0.05, WT EtOH+FA vs NIM811 EtOH+FA *p<0.05). In the aspect of recovery levels from alkali load during EtOH and FA treatment , the difference were not sigfnificant in WT EtOH+FA compared to the NIM811 and EtOH+FA treated groups (Fig.3E).

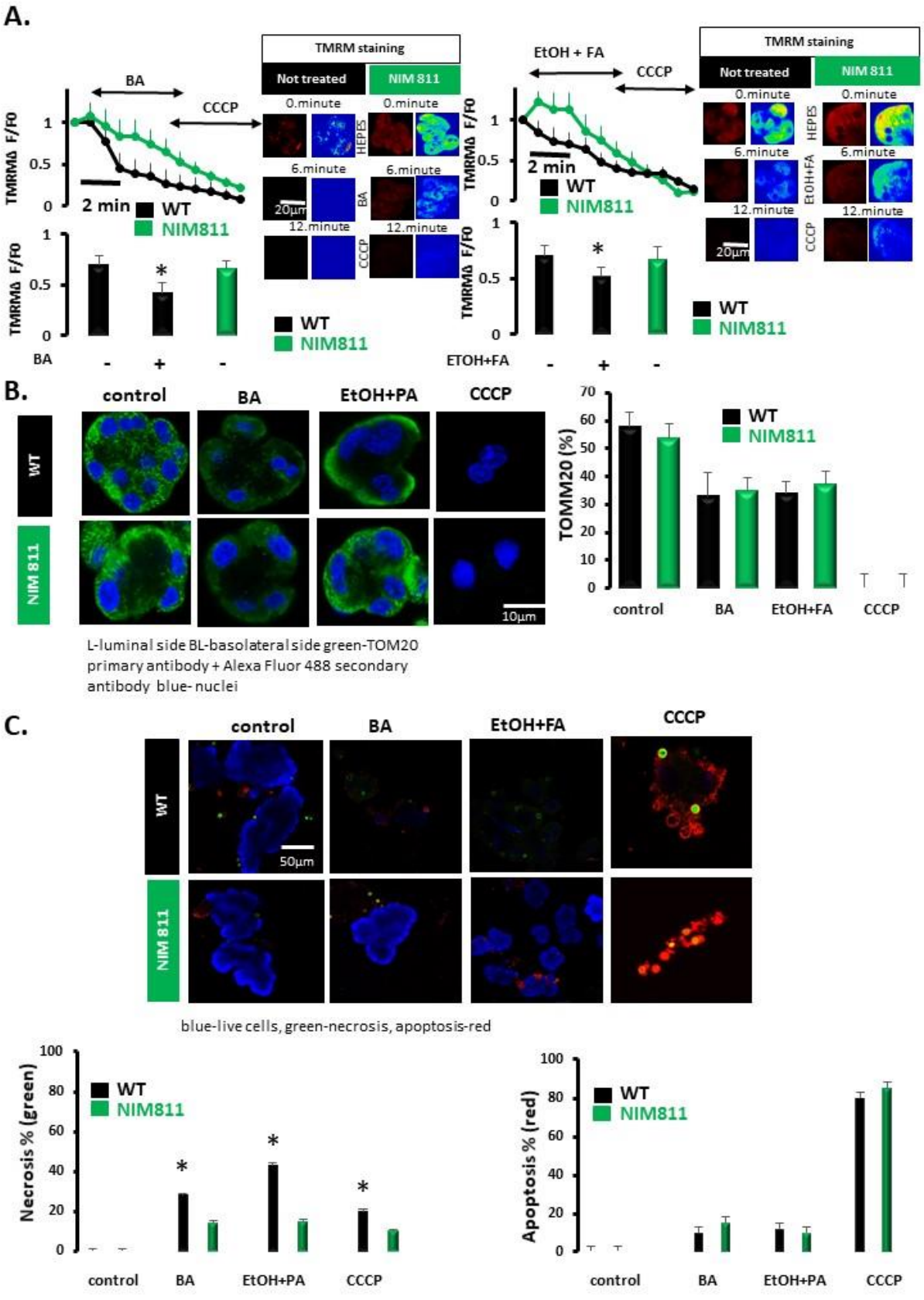
Figure 4. Pancreatic ductal fluid secretion is not altered by NIM811 or CYA treatment



In vitro fluid secretion was stimulated by 5µM forskolin and 100µM IBMX (stimulation). BA or EtOH+PA treatment inhibited the luminal swelling (Fig.4.B-C). Figure 4D represents the relative luminal volume changes during forskolin and IBMX stimulation (Figure4.D). Means ±SEM. n= 5-10 ducts/group. In vivo fluid secretion measurements were performed after the induction of CER or EtOH+FA induced AP (Fig.4.E-F.). These experiments confirmed that

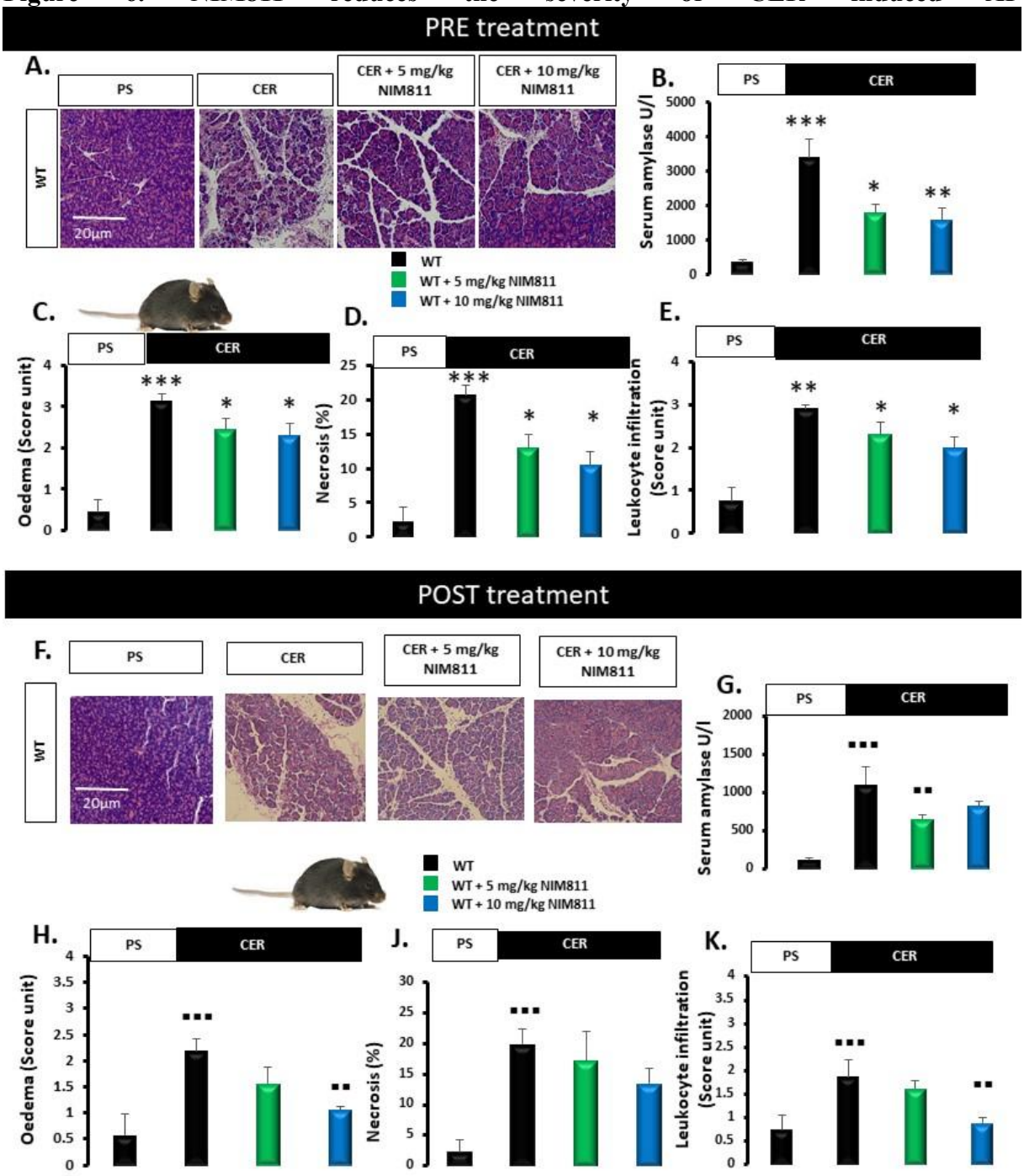
502 pancreatic ductal fluid secretion is not affected by NIM811 or CyA. (Fig.4.E-F). *p<0.05 WT
503 PS vs. WT EtOH+FA, ▪p<0.05 WT PS vs. WT CER n=4-7 animal/group
504

Figure5. NIM811 treatment protects mitochondrial function in pancreatic acinar cells



Mitochondrial membrane potencial measurements revealed a significant difference between WT not NIM811 treated and the NIM811 treated acinar cell response due to bile acid or ethanol and fatty acid treatment (Fig.5A) (WT BA vs NIM811 BA *$p<0.05$; WT EtOH+FA vs NIm811 ETOH+FA * $p<0.05$). Significant difference was detected between the NIM811 treated acinar cells and the groups which were not treated with NIM811 (Fig.5A) during BA or ETOH+FA treatment. Mitochondrial protein TOM20 levels did not show difference in the NIM811 treated or not treated groups after BA, ETOH+FA or CCCP treatment (Fig.5B) ($p>0.05$). In necrosis levels we found significant difference between NIM811 treated and not treated groups in BA or ETOH+FA (Fig.5C) (*$p<0.05$). However, in CCCP treated groups we found no difference (Fig.5C). Apoptosis levels were not altered significantly by NIM811 during BA or ETOH+FA treatment.

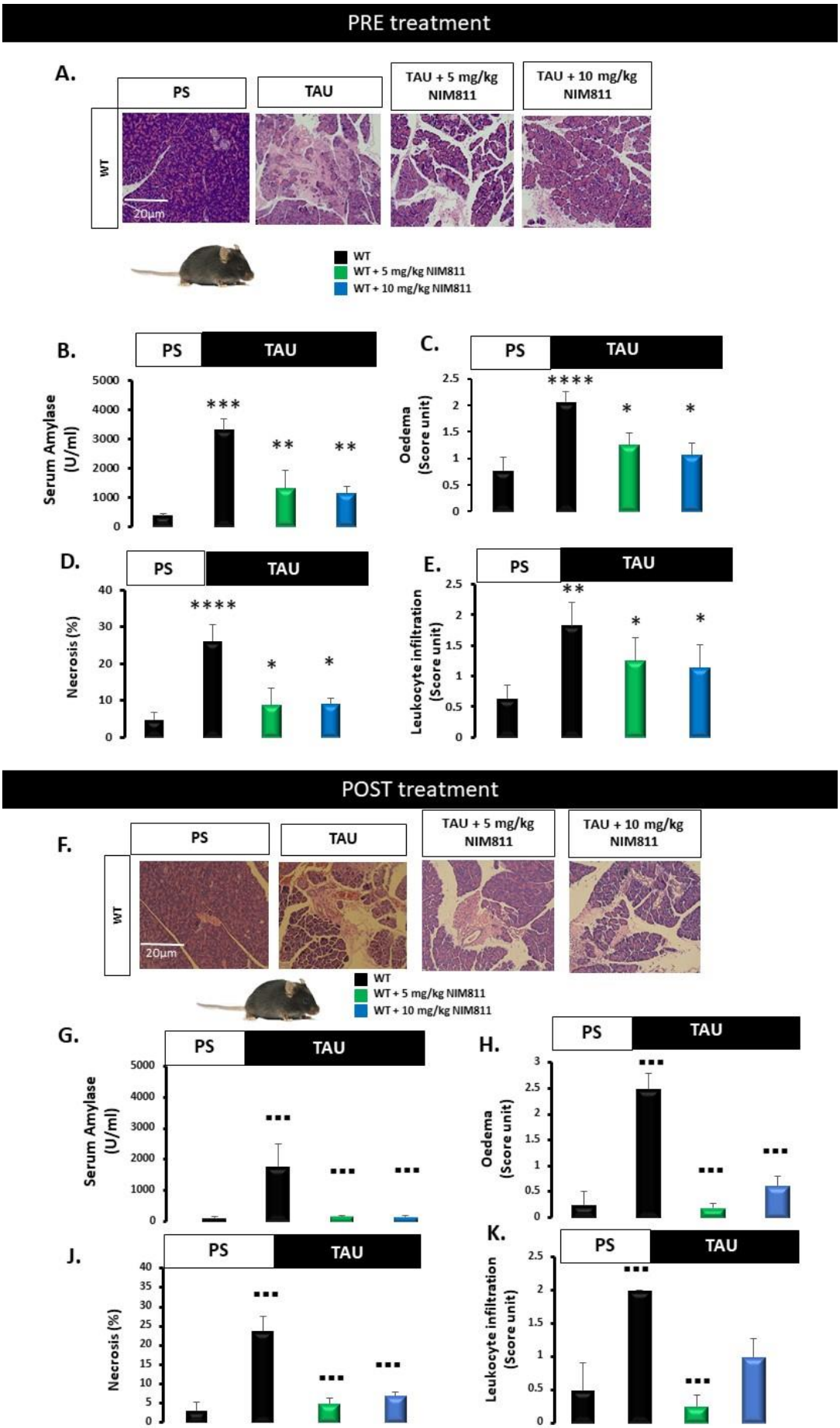
Figure 6. NIM811 reduces the severity of CER induced AP



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 WT CER group (Fig. 6B ***p<0.01 WT PS vs WT CER, **p<0.02 WT CER vs pre10mg/kg NIM811 CER, *p<0.05 WT CER vs pre 5mg/kg NIM811 CER, p=0.717 CER+ pre 5mg/kg NIM811 vs CER + pre

526 10mg/kg NIM811). In the aspect of CER induced pancreatitis both 5 mg/bwkg NIM811 (Fig.6
527 A-F, $p<0.05$ WT CER vs. pre 5mg/bwkg NIM811 CER) and pre 10 mg/bwkg NIM811 (Fig.6
528 A-F, $p<0.05$ WT CER vs. Pre 10mg/bwkg NIM811 CER)) treatment reduced the CER-induced
529 damage. Post 5mg/kg NIM811 treatment significantly reduced serum amylase levels compared
530 to WT CER ▪$p<0.05$, ▪▪▪$p<0.001$ WT PS vs WT CER (Fig.6G-E). Post insult administration
531 of 10mg/kg NIM811 significantly reduced oedema and leukocyte infiltration levels compared
532 to WT CER treated groups ▪▪$p<0.05$ (Fig.6H), n=8-10 animals per group, data means ±SEM).
533

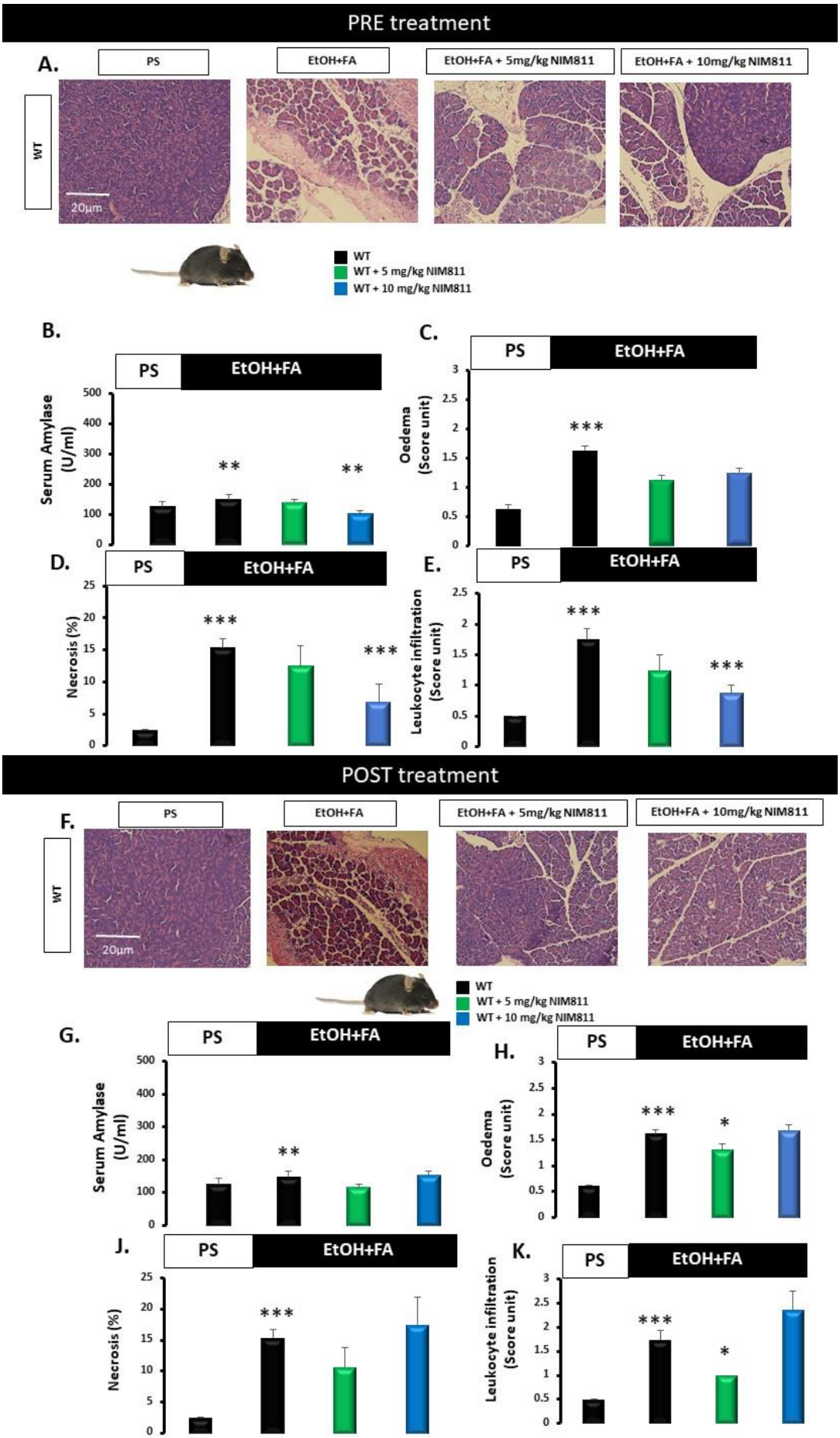
Figure7. NIM811 reduces the severity of TAU induced AP in mice


PRE treatment
A.
PS
TAU
TAU + 5 mg/kg NIM811
TAU + 10 mg/kg NIM811
WT
20µm
WT
WT + 5 mg/kg NIM811
WT + 10 mg/kg NIM811
B.
PS
TAU
Serum Amylase (U/ml)
5000
4000
3000
2000
1000
0
***
**
**
C.
PS
TAU
Oedema (Score unit)
2.5
2
1.5
1
0.5
0
****
*
*
D.
PS
TAU
Necrosis (%)
40
30
20
10
0
****
*
*
E.
PS
TAU
Leukocyte infiltration (Score unit)
2.5
2
1.5
1
0.5
0
**
*
*
POST treatment
F.
PS
TAU
TAU + 5 mg/kg NIM811
TAU + 10 mg/kg NIM811
WT
20µm
WT
WT + 5 mg/kg NIM811
WT + 10 mg/kg NIM811
G.
PS
TAU
Serum Amylase (U/ml)
5000
4000
3000
2000
1000
0
H.
PS
TAU
Oedema (Score unit)
3
2.5
2
1.5
1
0.5
0
J.
PS
TAU
Necrosis (%)
40
35
30
25
20
15
10
5
0
K.
PS
TAU
Leukocyte infiltration (Score unit)
2.5
2
1.5
1
0.5
0

535

We performed TAU induced pancreatitis(Fig.7A-K), serum amylase measurements revealed that due to retrograde infusion of TAU elevated serum amylase levels occured (***p<0.01 WT PS vs WT TAU Fig.6.B, ▪▪▪p<0.001 WT PS vs WT TAU Fig.7G) however 5 mg/bwkg or 10 mg/bwkg NIM811 treatment significantly reduced the enzyme levels both in the pre and post treatment (Fig. 7B **p<0.02 WT TAU vs pre 5mg/kg NIM811+TAU , ** p<0.02 WT TAU vs pre 10mg/kg NIM811+TAU, , ▪▪▪p<0.001 WT TAU vs. post 5mg/kg NIM811 TAU, ▪▪▪p<0.001 WT TAU vs post 10mg/kg NIM811 +TAU) the serum amylase levels were reduced compared to WT TAU treated groups (Fig.7B. and 7G *p<0.01 WT TAU vs. WT 5mg/bwkg NIM811 TAU and *p<0.01 WT TAU vs WT 10 mg/bwkg NIM811 TAU) . During pre NIM811 treatment oedema, necrosis and leukocyte infiltration scores were significantly decreased compared to the only TAU treated groups (Fig.7A,C,D,E p<0.05 WT TAU vs pre 5mg/bwkg NIM811 TAU/10mg/bwkg NIM811 TAU). Post insult administration of NIM811 decreased oedema, leukocyte infiltration and necrosis levels in the TAU group (▪▪▪p<0.001 Fig.7G-K) n=4-6 animals per group, data means ±SEM).

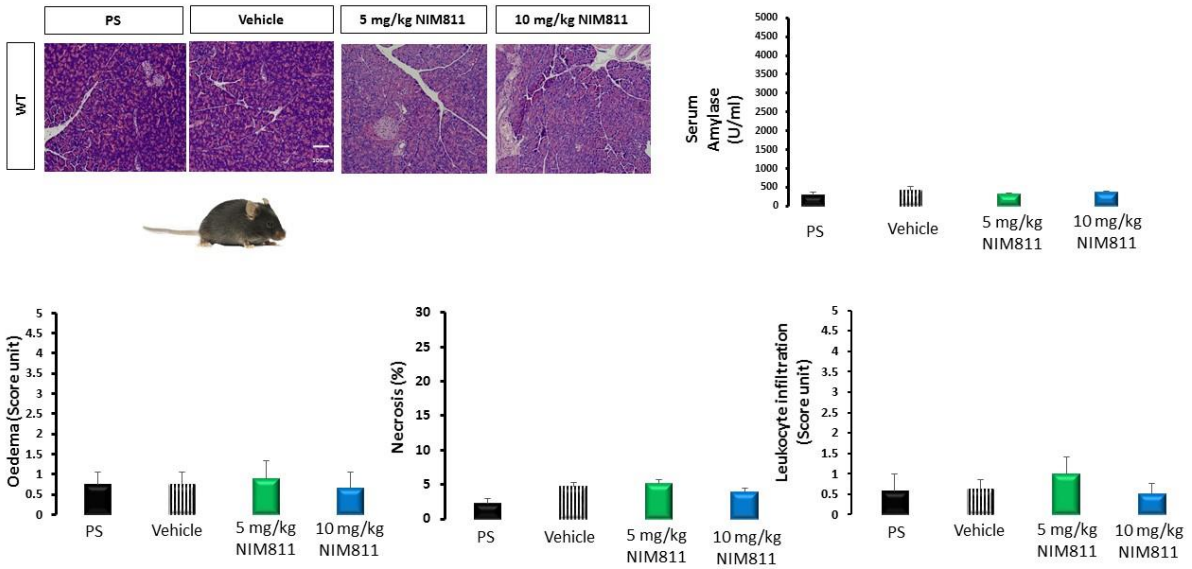
552 **Figure8. NIM811 has protective effect against EtOH+FA induced pancreatic damage**


PRE treatment
A.
PS
EtOH+FA
EtOH+FA + 5mg/kg NIM811
EtOH+FA + 10mg/kg NIM811
WT
20µm
WT
WT + 5 mg/kg NIM811
WT + 10 mg/kg NIM811
B.
PS
EtOH+FA
Serum Amylase (U/ml)
C.
Oedema (Score unit)
D.
Necrosis (%)
E.
Leukocyte infiltration (Score unit)
POST treatment
F.
G.
H.
J.
K.

553

We performed EtOH+FA induced pancreatitis (Fig.8A-K). Serum amylase measurements revealed that in pre treatment of 10 mg/kg NIM811 significantly reduced serum amylase levels **p<0.002 WT EtOH+FA vs pre 10mg/kg NIM811 +EtOH+FA (Fig.8B), **p<0.002 WT PS vs Wt EtOH+FA (Fig.8B and G), in post NIM811 treatment serum amylase levels did not differ significantly compared to its ETOH+FA control (Fig.8G). In pre 10mg/kg NIM811 treatment leukocyte infiltration (***p<0.001 WT EtOH+FA vs 10mg/kg NIM811) and necrosis levels (***p<0.001 Wt EtOH+FA vs 10 mg/kg NIM811) were significantly reduced compared to EtOH+FA AP group (Fig.8D-E). ***p<0.001 WT PS vs Wt EtOH+FA in Fig.8C-E. Oedema and leukocyte infiltration levels were significantly reduced in post 5mg/kg NIM811 treated groups compared to WT EtOH+FA groups (*p<0.05 WT EtOH+FA vs post 5 mg/kg NIM811) (Fig.8H and K) n=4-7 animals per group, data means ±SEM).

Figure9. 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 animal/group

571 **Table1. Solutions used in our study**

	HEPES (Standard) mM	**HCO_3-** (Standard) mM	**NH_4Cl-HCO_3- mM**	**1xTBS 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	**-**	**-**	**-**	**-**	**-**
$NaHCO_3$-	**-**	**25**	**25**	**-**	**4.2**
NH_4Cl-	**-**	**-**	**20**	**-**	**-**
Trisma Base	**-**	**-**	**-**	**50**	**-**
Na_2HPO_4	**-**	**-**	**-**	**-**	**0.25**
KH_2PO_4	**-**	**-**	**-**	**-**	**0.44**
$MgSO_4$	**-**	**-**	**-**	**-**	**1.03**

572

573

574 **Table 2. Oligonucleotide primers used in genotyping**

Primers	
F-null2	TTCTCACCAGTGCATAGGGCTCTG
LoxP1f	AAACTTCTCAGTCAGCTGTTGCCTCTG
CyPuP2	GCTTTGTTATCCCAGCTGGCG

575

576

577

ADDITIONAL INFORMATION SECTION

COMPETING INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTION

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 to us and were involved in the 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 the authors approved the final manuscript.

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AUTHORS' 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 the disease initiation is the mitochondrial dysfunction and ATP depletion. It has been shown that the pancreatitis-inducing factors namely ethanol, fatty acids and bile acids open the membrane transition pore (mPTP) channel, keeping the channel continuously opened resulting in mitochondrial depolarization, lower ATP synthesis and cell necrosis both in pancreatic acinar and ductal cells. In this study,

609 we provided strong evidence that one of the mPTP inhibitors, namely NIM811 is highly
610 effective in different experimental pancreatitis models. Since NIM811 had no side-effects and
611 passed the important phase 1 stage in the clinical trial process, companies should organize phase
612 2 clinical trials with the use of this novel and promising drug candidate.

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