GENETIC BASIS OF CHRONIC PANCREATITIS: COHORT ANALYSIS AND PRECLINICAL DRUG TESTING

Ph.D. Thesis

Zsófia Gabriella Pesei M.D.

Doctoral School of Theoretical Medicine, Faculty of Medicine, University of Szeged, Szeged

Department of Medical Genetics, Albert Szent-Györgyi Health Centre, Szeged

Supervisors:

Eszter Hegyi M.D.,Ph.D.

Doctoral School of Theoretical Medicine, Faculty of Medicine, University of Szeged, Szeged

Institute for Translational Medicine, Medical School, University of Pécs, Pécs

Prof. Miklós Sahin-Tóth M.D., Ph.D.

Doctoral School of Theoretical Medicine, Faculty of Medicine, University of Szeged, Szeged

Division of General Surgery, Department of Surgery, David Geffen School of Medicine at University of California Los Angeles; Los Angeles

Doctoral School of Theoretical Medicine, Faculty of Medicine, University of Szeged, Szeged

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List of publications related to the subject of the thesis

I. **Zsófia Gabriella Pesei**, Zsanett Jancsó, Alexandra Demcsák, Balázs Csaba Németh, Sandor Vajda, Miklós Sahin-Tóth: Preclinical testing of dabigatran in trypsin-dependent pancreatitis. *JCI Insight.* 2022; 7(21), e161145. doi:10.1172/jci.insight.161145. **IF$_{2021}$: 9.484, D1**

II. **Zsófia Gabriella Pesei***, Balázs Csaba Németh*, Eszter Hegyi, Ákos Szücs, Andrea Szentesi, Péter Hegyi, Mark E. Lowe, Miklós Sahin-Tóth: The common truncation variant in pancreatic lipase related protein 2 (*PNLIPRP2*) is expressed poorly and does not alter risk for chronic pancreatitis. *PloS One.* 2018;13(11),e0206869. doi:10.1371/journal.pone.0206869. **IF$_{2018}$: 2.776, Q1**

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List of publications not related to the subject of the thesis

I. András Salamon, Rita Török, Evelin Sümegi, Fanni Boros, **Zsófia Gabriella Pesei**, Máté Fort Molnár, Gábor Veres, Dénes Zádori, László Vécsei, Péter Klivényi: The effect of physical stimuli on the expression level of key elements in mitochondrial biogenesis. *Neurosci Lett.* 2019; 698:13-18. doi: 10.1016/j.neulet.2019.01.003. **IF$_{2019}$: 2.274, Q3**

II. Fanni Annamária Boros, Rita Török, Evelin Vágvölgyi-Sümegi, **Zsófia Gabriella Pesei**, Péter Klivényi, László Vécsei. Assessment of risk factor variants of LRRK2, MAPT, SNCA and TCEANC2 genes in Hungarian sporadic Parkinson's disease patients. *Neurosci Lett.* 2019; 706:140-145. doi: 10.1016/j.neulet.2019.05.014. **IF$_{2019}$: 2.274, Q3**

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INTRODUCTION

The inflammatory diseases of the pancreas include acute pancreatitis (AP), recurrent acute pancreatitis (RAP), and chronic pancreatitis (CP). These clinical syndromes form a disease continuum, and CP often presents as a progressive, relapsing-recurring disorder, starting with an episode of AP, followed by RAP, and eventually progressing to end-stage CP. The AP-RAP-CP progression is driven by environmental or genetic risk factors. Modifiable environmental risk factors include chronic alcohol consumption and smoking, while genetic alterations mostly affect genes encoding pancreatic digestive enzymes. Genetic variants that modify risk for CP can be classified into mechanistic pathways that explain their pathogenic effect. There are at least 3 major categories recognized to date, the trypsin-dependent, the misfolding-dependent and the ductal pathways. The present thesis focuses on the misfolding and trypsin-dependent pathways.

The misfolding-dependent pathway of genetic risk includes genes and variants that induce misfolding of digestive enzymes of the pancreas, and result in harmful endoplasmic reticulum (ER) stress in the pancreatic acinar cells, which indicates maladaptive activation of the unfolded protein response pathways. A number of genetic variants that induce enzyme misfolding affect lipase genes, such as the *CEL* (carboxyl ester lipase) gene and *PNLIP* (pancreatic lipase) gene. Similarly to *PNLIP*, variants in the pancreatic lipase related protein 2 (*PNLIPRP2*) gene might increase the risk for CP. The relatively common nonsense *PNLIPRP2* variant p.W358X results in a truncated protein. When expressed in transfected HEK 293T cells, the mutant PNLIPRP2 protein was secreted poorly, and it formed detergent-insoluble aggregates inside the cells and activated the unfolded protein response. These findings suggested that the p.W358X *PNLIPRP2* variant might be a risk factor for CP, however, genetic evidence has been lacking.

The trypsin-dependent pathway of genetic risk comprises gene variants of digestive enzymes and their inhibitor that regulate intrapancreatic trypsin activity. These contribute to pancreatitis onset and progression through increasing the risk of ectopic, intrapancreatic activation of the digestive protease precursor trypsinogen to its active form trypsin. Gain-of–function mutations in the serine protease 1 (*PRSS1)* gene encoding human cationic trypsinogen block or diminish protective trypsinogen degradation or accelerate trypsinogen autoactivation. Loss-of-function mutations in *SPINK1* and *CTRC* compromise the antitrypsin defenses, i.e. trypsinogen degradation by CTRC and trypsin inhibition by SPINK1. The pathogenic role of trypsinogen in CP has been confirmed by multiple mouse models from the Sahin-Toth laboratory. These mice carry mutations in mouse cationic trypsinogen (isoform T7). The *T7D23A* and *T7K24R*

strains harbor mutations p.D23A and p.K24R in the trypsinogen activation peptide that directly accelerate autoactivation by 50- and 5-fold, respectively. Heterozygous *T7D23A* mice develop spontaneous, early-onset AP with rapid progression to CP. Homozygous *T7K24R* mice do not have spontaneous AP or CP, but exhibit more severe disease than C57BL/6N control mice when AP is induced experimentally by repeated cerulein injections, and continue to progress to CP while control C57BL/6N mice recover rapidly. In addition to the knock-in models described above, the laboratory of Dr. Baoan Ji developed several transgenic lines with human *PRSS1* and *PRSS2* genes. Together, the availability of these models sets the stage for preclinical drug testing for the treatment of CP.

The genetic, biochemical, and animal modeling evidence identify trypsin as a clear therapeutic target in CP. Although early preclinical testing of trypsin inhibitors yielded promising results in rodents, human clinical trials did not provide convincing evidence for their efficacy. In 2019, the Baoan Ji laboratory reported that the anticoagulant dabigatran etexilate cured/reversed cerulein-induced pancreatitis in transgenic mice carrying human *PRSS1* with the p.R122H mutation, which is the most commonly found *PRSS1* variant in hereditary and familial forms of CP. Dabigatran etexilate (brand name PRADAXA) is used worldwide as an orally active, reversible thrombin inhibitor for long-term anticoagulation of patients with atrial fibrillation. After absorption from the gastrointestinal tract, the prodrug is converted by nonspecific esterases in the blood to its active form, dabigatran. Dr. Ji and coworkers speculated that the anti-coagulation activity of dabigatran may explain its beneficial effect in pancreatitis. However, dabigatran is a benzamidine derivative and it has been reported to inhibit bovine trypsin with an inhibitory constant (K_i) of 50.3 nM. Therefore, we hypothesized that the observed therapeutic effect of dabigatran in transgenic mice with the p.R122H mutation was due to trypsin inhibition in the pancreas rather than inhibition of thrombin in the circulation.

AIMS

Aim 1. *Genetic analysis of the c.1074G>A (p.W358X) variant in CP*. Our goal was to test the hypothesis that the common *PNLIPRP2* truncation variant p.W358X increases risk for CP.

Aim 2. *Preclinical testing of dabigatran in trypsin-dependent pancreatitis*. Our goal was to test the anticoagulant dabigatran as a trypsin inhibitor against human and mouse trypsin isoforms, and to evaluate the therapeutic efficacy of the prodrug dabigatran etexilate in the *T7K24R* and *T7D23A* mouse models of trypsin-dependent pancreatitis.

MATERIALS AND METHODS

Genetic analysis of the c.1074G>A (p.W358X) PNLIPRP2 variant in CP

Nomenclature

Nucleotide numbering follows coding DNA numbering with the first nucleotide of the ATG translation initiation codon designated as +1. Amino acids are numbered starting with the initiator methionine of the primary translation product of *PNLIPRP2*. The NCBI genomic reference sequence for *PNLIPRP2* (NC_000010.11, Homo sapiens chromosome 10, GRCh38.p12 primary assembly) and the NCBI coding DNA reference sequence (NM_005396.4) correspond to the minor truncation allele. We used the major full-length *PNLIPRP2* allele as reference for the designation of all *PNLIPRP2* variants. In this manner, the nonsense p.W358X variant becomes the "effect" allele, which is the biologically meaningful representation.

Study subjects

For this study, we used de-identified genomic DNA samples from the registry of the Hungarian Pancreatic Study Group (ethical approval number TUKEB 22254-1/2012/EKU; biobanking approval number IF702-19/2012). A total of 256 unrelated patients with CP, including 152 with alcoholic CP and 104 with non-alcoholic CP and 200 control subjects with no pancreatic disease were analyzed. De-identified pancreatic cDNA and matching genomic DNA samples (n = 9) from cadaveric donors were obtained from the University of Szeged, Hungary.

DNA sequencing

The region of interest was PCR amplified and Sanger sequencing was performed using the forward and/or reverse PCR primers as sequencing primer.

Preclinical testing of dabigatran in trypsin-dependent pancreatitis

Modeling dabigatran binding to trypsin

To model dabigatran binding to trypsin, dabigatran chemical structure was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), the human mesotrypsin structure 1H4W was downloaded from the Protein Data Bank (PDB). The docking was carried out by the docking server ClusPro LigTBM (https://ligtbm.cluspro.org/).

Enzyme kinetic measurements

Recombinant human and mouse trypsinogens were expressed *in Escherichia coli* BL21(DE3), refolded in vitro, and purified by ecotin affinity chromatography, according to published

protocols. The concentration of trypsin solutions was determined by titration with the trypsin inhibitor ecotin. Michaelis-Menten kinetic parameters of trypsin isoforms were determined with the chromogenic substrate GPR-pNA. The K_m, k_{cat} values were calculated from hyperbolic fits to plots of reaction velocity versus substrate concentration.

To measure competitive inhibition of different trypsin isoforms with benzamidine or dabigatran trypsin isoforms were preincubated with increasing concentrations of these inhibitors.

Cerulein-induced pancreatitis in *T7K24R* mice

Animal experiments were performed at the University of California Los Angeles (UCLA) with the approval and oversight of the Animal Research Committee, including protocol review and post-approval monitoring.

Pancreatitis was induced in homozygous *T7K24R* mice and C57BL/6N mice with 8 hourly intraperitoneal injections of cerulein used in a dose of 50 μg/kg. Mice were sacrificed 96 hours from the first cerulein injection and the pancreas and blood were harvested. Trypsin and chymotrypsin activities were measured from freshly prepared pancreas extracts of C57BL/6N and *T7K24R* mice.

Dabigatran etexilate treatment of *T7D23A* and *T7K24R* mice

Heterozygous *T7D23A* and homozygous *T7K24R* mice were administered dabigatran etexilate orally either by intragastric gavage to a final dose of 100 or 200 mg/kg or by feeding with solid chow containing the prodrug in 10 mg/g concentration. Control mice were given gavage of the vehicle solution or regular chow. Dabigatran in the blood plasma was quantified using the HEMOCLOT Thrombin Inhibitors kit.

Statistics

Differences between means were analyzed by unpaired 2-tailed t test for 2 groups and by 1-way ANOVA for multiple groups, with Tukey-Kramer post hoc analysis for pairwise comparison using Prism 8 (GraphPad). Statistical significance was defined as $P < 0.05$.

RESULTS

Genetic analysis of the c.1074G>A (p.W358X) PNLIPRP2 variant in CP

We genotyped 104 subjects with non-alcoholic CP, 152 subjects with alcoholic CP, and 200 control subjects, recruited from the registry of the Hungarian Pancreatic Study Group. Within the amplified 793 nt sequence including exon 11 and flanking intronic regions of *PNLIPRP2*, we found 6 nucleotide variants, which included three intronic variants (c.1070-379delG,

c.1070-321T>C, and c.1181+55A>C), one synonymous variant (c.1161G>A, p.S387=), one missense variant (c.1084G>A, p.V362I), and the truncation variant c.1074G>A (p.W358X). The commonly occurring variants c.1070-321T>C, p.W358X, p.V362I, p.S387=, and c.1181+55A>C were found in linkage disequilibrium as a conserved haplotype (CAAAC). Another common haplotype (CGGAA) was formed by variants c.1070-321T>C and p.S387=.
When allele frequency was considered, distribution of the variants between patients and controls showed no significant difference. Subgroup analysis for non-alcoholic and alcoholic CP patients versus controls revealed no association either. We also analyzed genotypes using dominant and recessive models but found no significant differences in genotype frequencies between all CP patients or the non-alcoholic and alcoholic cohorts versus controls.
Comparison of the three haplotypes between patients and controls yielded no significant differences with the exception of the CGGAA haplotype, which was overrepresented in the non-alcoholic CP cohort relative to controls (OR 1.6, P 0.04). We consider this a spurious association due to the limited sample size.

To estimate the relative mRNA expression of the full-length and truncation alleles of *PNLIPRP2*, we used direct sequencing of 9 pancreatic cDNA samples with matching genomic DNA from cadaveric donors. Sequencing of the genomic DNA identified five heterozygous samples and one sample homozygous for the truncation allele. The electropherograms of the heterozygous genomic sequences showed two signals at the position of variants p. W358X and p.V362I, with comparable peak heights. When heterozygous cDNA samples were sequenced, only one peak was visible at these positions, which corresponded to the major full-length allele, whereas no signal was apparent for the minor truncation allele. PCR amplification of the pancreatic cDNA sample with the homozygous truncation allele confirmed the absence of detectable mRNA expression. We also consulted the Genotype-Tissue Expression (GTEx) Portal and found that all five common variants within the truncation haplotype were associated with diminished *PNLIPRP2* mRNA expression.

Preclinical testing of dabigatran in trypsin-dependent pancreatitis

We hypothesized that the reported efficacy of dabigatran etexilate in pancreatitis is related to the trypsin-inhibitory activity of dabigatran. As a benzamidine derivative, dabigatran is expected to inhibit trypsin-like enzymes competitively; however, this inhibitory activity against human and mouse trypsin isoforms has not been demonstrated before to our knowledge. To verify the trypsin inhibitory effect of dabigatran, first, we used homology-based docking to demonstrate that dabigatran can bind to the specificity pocket of trypsin. In our model, showing

dabigatran docked to human mesotrypsin (PDB structure 1H4W), the amidine moiety of dabigatran interacts with the side chain of Asp194 at the bottom of the specificity pocket, and the N-methyl-benzimidazole scaffold that bridges the benzamidine and the distal pyridine ring and propanoic acid end is positioned above the catalytic triad. The benzamidine moiety of dabigatran overlaps with the bound benzamidine of the 1H4W mesotrypsin structure. Next, we performed enzymatic measurements to compare the effect of benzamidine and dabigatran against human trypsin isoforms PRSS1, PRSS2, and PRSS3, and mouse trypsin isoforms T7 (cationic trypsin) and T8, T9, and T20 (anionic trypsins). Benzamidine inhibited trypsin with micromolar K_i values (range 3.3–20.6 μM and 4.2–22.6 μM by individual and global fit analysis, respectively), while dabigatran was an about 200- to 400-fold stronger inhibitor, exhibiting nanomolar K_i values (range 10–65 nM and 10.3–78.9 nM by individual and global fit analysis, respectively). Anionic trypsin isoforms were inhibited slightly stronger by benzamidine than cationic trypsins; however, this trend was less conspicuous with dabigatran. The K_i values reported earlier for dabigatran against bovine trypsin and measured in our experiments were essentially identical. Dabigatran inhibited human trypsins as well as or slightly better than mouse trypsins, suggesting that results from preclinical mouse experiments should be relevant to human clinical trials. The experiments demonstrate that derivatives of benzamidine, such as dabigatran, can have highly improved inhibitory activity against trypsin and are universally effective against various trypsin paralogs.

We measured plasma concentrations of dabigatran in C57BL/6N mice after oral administration of the prodrug dabigatran etexilate. First, we performed intragastric gavage of a single dose (100 mg/kg) and followed plasma levels up to 8 hours. Dabigatran levels sharply rose to micromolar values within 30 minutes of oral gavage and peaked around 1 hour, after which time levels steadily decreased, with very little dabigatran measurable at the 4- and 8-hour time points. Importantly, peak concentrations of dabigatran were more than 2 orders of magnitude above the K_i values measured for trypsin inhibition. Second, we fed mice with solid chow containing dabigatran etexilate (10 mg/g) for 1 week and measured their plasma dabigatran concentration. Chronic feeding resulted in lower but steadier plasma concentrations with most values falling in the 600–800 nM range. This drug level is still more than 10-fold higher than K_i values of dabigatran against mouse trypsins. Third, we measured the dabigatran plasma concentration in 3 week-old *T7D23A* and C57BL/6N mice after consuming dabigatran-etexilate laced chow (10 mg/g) for one week. We detected no significant difference between the plasma dabigatran levels from C57BL/6N and *T7D23A* mice, indicating that the two strains consumed

the prodrug-containing chow similarly, and the spontaneous CP of *T7D23A* mice had no impact on the bioavailability of dabigatran.

The *T7K24R* mouse strain carries the p.K24R mutation in mouse cationic trypsinogen (isoform T7), which is analogous to the p.K23R pancreatitis-associated human *PRSS1* mutation. We recently demonstrated that cerulein-induced pancreatitis in *T7K24R* mice is progressive; and after the acute episode, marked acinar atrophy develops with fibrosis and macrophage infiltration. Before testing the effect of dabigatran etexilate, we characterized intrapancreatic trypsin and chymotrypsin activity in *T7K24R* mice after 8 hourly injections of saline or cerulein. Protease activities were measured at 1 hour, 1 day, 2 days, and 3 days after the cerulein injections. Relative to cerulein-treated C57BL/6N mice, pancreatic trypsin activity in *T7K24R* mice was at least 10-fold elevated, and this high value persisted on days 1 and 2, finally diminishing on day 3, as acinar atrophy develops. Pancreatic chymotrypsin activity was also significantly higher in *T7K24R* mice, with peak activity (20-fold higher than in C57BL/6N mice) seen on day 1, which sharply declined by days 2 and 3. As expected, no intrapancreatic protease activation was observed in saline-treated control mice. The high trypsin activity in the pancreas of cerulein-treated *T7K24R* mice suggests that trypsin-inhibitory therapy should be efficacious against pancreatitis in this model. Therefore, in our experiments, we induced pancreatitis in *T7K24R* mice by 8 hourly injections of cerulein and euthanized the mice 96 hours later. To test the effect of dabigatran etexilate, a single dose of the prodrug was administered 30 minutes after the last injection. Negative control mice without pancreatitis and vehicle-treated positive control mice with pancreatitis served for comparison. When the body weight of mice at the beginning and at the end of the experiment was compared, vehicle-treated mice with pancreatitis showed a slight decrease. This phenomenon is due to a transient digestive dysfunction associated with the rapid development of acinar atrophy. In contrast, dabigatran etexilate–treated *T7K24R* mice with pancreatitis showed no change in body weight by the end of the experiment, suggesting a protective effect of the drug. The pancreas weight of vehicle-treated *T7K24R* mice with pancreatitis was significantly reduced, to almost half the normal pancreas size. The atrophic weight loss of the pancreas remained prominent even after the pancreas weight was normalized to body weight. Remarkably, however, the pancreas weight of the dabigatran etexilate–treated mice with pancreatitis was significantly higher, in some cases approaching the values of control mice with no pancreatitis, suggesting that the drug prevented and/or reversed acinar atrophy to a large extent. *T7K24R* mice exhibited low plasma amylase activity 4 days after the induction of cerulein-induced pancreatitis, close to the levels seen in

control mice without pancreatitis. Interestingly, in a subset of dabigatran etexilate–treated mice with pancreatitis (4 of 15), we observed more than 3-fold higher plasma amylase activity values, suggesting ongoing acinar cell injury. Histological analysis of pancreata from 10 vehicle-treated and 15 dabigatran etexilate–treated mice with hematoxylin-eosin staining demonstrated widespread loss of intact acini in vehicle-treated mice. A dramatic, complete protective effect of dabigatran etexilate was observed in almost 50% of the drug-treated mice. A significant yet incomplete (30%–50% normal histology) effect was seen in about 20% of the mice, whereas in the remaining 30% of mice dabigatran showed limited efficacy, with less than 25% of normal acini preserved, including 2 cases with no detectable effect. The 4 drug-treated mice with the elevated plasma amylase activity all had partial histological responses, with 13%, 15%, 35%, and 45% intact acini visible on pancreas sections. Overall, the proportion of intact acini in the dabigatran etexilate–treated group was significantly higher than in the vehicle-treated group, indicating that dabigatran is effective in this model of trypsin-dependent pancreatitis.

Next, we tested the effect of dabigatran etexilate in a more aggressive, spontaneous pancreatitis model. The *T7D23A* mouse strain carries the p.D23A mutation in mouse cationic trypsinogen (isoform T7), which is analogous to the p.D22G pancreatitis-associated human *PRSS1* mutation. The mutation increases autoactivation of trypsinogen about 50-fold and elicits spontaneous, early-onset (3–5 weeks of age), and progressive pancreatitis. In the first experiment, we treated 3-week-old *T7D23A* mice with various doses of dabigatran etexilate (once daily 100 mg/kg, twice daily 100 mg/ kg, and once daily 200 mg/kg) via intragastric gavage for 2 weeks. As controls, untreated *T7D23A* and C57BL/6N mice were used. Mice were euthanized at 5 weeks of age. Conversely, by 5 weeks of age, all *T7D23A* mice were expected to have developed early CP. During this period, mice of both strains gained weight, and this was unaffected by gavage treatment. The weight gain of *T7D23A* mice was slightly lower relative to the C57BL/6N parent strain. Compared with untreated C57BL/6N mice, the pancreas weight of untreated *T7D23A* mice was markedly lower, due to the massive pancreas atrophy associated with their early CP, even after normalization of the pancreas weight to the body weight of the mice. In stark contrast to the effect seen with *T7K24R* mice, dabigatran etexilate treatment did not improve the pancreas weight of *T7D23A* mice. Curiously, a clear trend of worsening atrophy emerged with increasing dabigatran dosages, even though the differences did not reach statistical significance. As expected, plasma amylase activity was reduced in untreated *T7D23A* mice relative to C57BL/6N mice, though the difference did not reach statistical significance. In agreement with their smaller pancreas weights, drug-treated *T7D23A*

mice had significantly lower amylase levels relative to the untreated *T7D23A* controls. Histological analysis of pancreata revealed comparable CP-like disease in all groups of *T7D23A* mice whereas C57BL/6N controls showed normal pancreas morphology. The results from this experiment indicated that dabigatran etexilate introduced by intragastric gavage did not ameliorate the spontaneous pancreatitis of *T7D23A* mice. Based on these results we speculated that the *T7D23A* mouse model may require sustained drug levels in the blood to achieve full inhibition of pancreatic trypsins and prevention/reversal of disease. Therefore, we tested whether feeding the mice with solid chow containing dabigatran etexilate would be efficacious. We fed 3 week-old-mice for 1 week and euthanized the mice at the age of 4 weeks. There were 4 experimental groups, treated and untreated C57BL/6N controls, and treated and untreated *T7D23A* mice. Each group gained weight similarly during the 1-week treatment, indicating that mice readily consumed the dabigatran etexilate–containing chow. We observed a small increase regarding pancreas weight in the drug-treated groups of both strains indicating that this change is likely unrelated to a drug effect on pancreatitis. Long-term feeding of mice with trypsin inhibitors causes the pancreas weight to increase, due to luminal trypsin inhibition and a feedback mechanism that increases plasma cholecystokinin levels. Plasma amylase activities were comparable in all 4 groups of mice. Histological analysis showed normal pancreata in C57BL/6N mice and early CP in *T7D23A* mice without any effect of dabigatran etexilate treatment on pancreatitis severity. Taken together, the results indicate that feeding *T7D23A* mice with solid chow containing dabigatran etexilate did not prevent or improve their spontaneous pancreatitis.

DISCUSSION

In our work, we investigated a potential new genetic risk factor for CP, and performed preclinical testing of a drug candidate in mouse models of hereditary pancreatitis.

Genetic analysis of the c.1074G>A (p.W358X) PNLIPRP2 variant in CP

We investigated whether a common genetic variant in *PNLIPRP2* increased risk for CP. The p.W358X variant introduces a premature stop codon that causes early translation termination, resulting in a truncated PNLIPRP2 protein. We found no association of variant p.W358X with CP when all cases were considered as a group or in subgroup analyses when alcoholic and non-alcoholic CP were examined separately. These observations demonstrate that the *PNLIPRP2* variant p.W358X is not a genetic risk factor for CP. Published data indicated that variant p.W358X might cause acinar cell damage and increase risk to CP by inducing ER stress and

activating cell-death pathways. However, this scenario would occur only if expression levels of the truncated PNLIPRP2 protein were high enough to cause ER stress. To characterize expression of the p.W358X allele relative to the full-length, wild-type *PNLIPRP2* allele, we PCR amplified *PNLIPRP2* from pancreatic cDNA of heterozygous and homozygous p.W358X carriers, and estimated mRNA expression. Our results indicated that mRNA levels encoding the p.W358X *PNLIPRP2* variant are diminished relative to those of full-length *PNLIPRP2*. In all likelihood, the mRNA encoding the p.W358X variant suffers nonsense-mediated decay, an mRNA degradation mechanism. Our new data convincingly demonstrates that mRNA expression of *PNLIPRP2* variant p.W358X is diminished. Thus, given the low levels of mRNA expression, it is unlikely that the p.W358X *PNLIPRP2* variant causes CP or increases disease risk through a gain of function, as suggested by prior studies in transfected tissue culture cells.

The very high prevalence of the *PNLIPRP2* p.W358X allele (allele frequency 48%) in the general population should suggest that this variant is unlikely to be relevant for CP or any other human disease. Even if CP risk is unaffected by *PNLIPRP2* p.W358X variant, it remains possible that loss of lipase function might have an impact on other human health conditions. Alternatively, the p.W358X *PNLIPRP2* variant in humans may represent a completely benign loss-of-function lipase variant compensated by other lipases, a protective allele, or an allele that modifies adaptations to diet.

Preclinical testing of dabigatran in trypsin-dependent pancreatitis

The aim of this study was to demonstrate that pancreatic trypsin inhibition is a viable therapeutic approach to treat, cure or prevent pancreatitis. Specifically, we tested the hypothesis that the recently reported therapeutic effect of dabigatran etexilate in experimental pancreatitis of transgenic PRSS1^{R122H} mice was due to the trypsin inhibitory activity of dabigatran. First, we modeled the binding of the dabigatran molecule to trypsin using homology-based docking, and demonstrated that dabigatran was readily docked into the substrate binding pocket of trypsin, and it inhibited all trypsin isoforms potently and with similar efficacy. Dabigatran exhibited several hundred-fold higher comptetitive inhibitory activity compared to its parent compound benzamidine. The experiments demonstrate that derivatives of benzamidine, such as dabigatran, can have highly improved inhibitory activity against trypsin and are universally effective against various trypsin paralogs. In mouse experiments, we confirmed that oral administration of dabigatran etexilate produced high enough blood concentrations of dabigatran that can achieve full trypsin inhibition. A single gavage of dabigatran etexilate (100 mg/kg dose) yielded dabigatran concentrations (peak ~2.5 μg/mL) that were more than 100-fold higher

than the K_i values against the various trypsin isoforms. However, under these conditions, plasma dabigatran levels decreased rapidly, and the drug was almost completely eliminated within a few hours. When dabigatran etexilate was administered to mice by feeding of solid chow containing the prodrug (10 mg/g), more steady plasma concentrations were observed (~300-400 ng/mL), which were about 10-fold higher than the K_i values against trypsins. In clinical practice, the typical dosing of Pradaxa is two 150 mg capsules daily, which is unlikely to produce high enough dabigatran plasma concentrations that exert significant trypsin inhibitory activity in the pancreas.

To characterize the effect of dabigatran-etexilate on trypsin-dependent pancreatitis, we used two mouse models, the homozygous *T7K24R* (cerulein induced-pancreatitis) and heterozygous *T7D23A* (spontaneous pancreatitis), that were developed recently in the Sahin-Tóth laboratory. Our results with *T7K24R* mice confirmed the published efficacy of dabigatran etexilate against cerulein-induced progressive CP. We found that a single gavage of dabigatran etexilate given shortly after the last cerulein injection was sufficient for therapeutic effect. Interestingly, we observed some variability with respect to the therapeutic activity of dabigatran etexilate. We speculate that efficient inhibition of intrapancreatic trypsin requires high enough plasma levels of dabigatran, which, in turn, may be determined by the success of the gavage procedure, and the intestinal absorption of the prodrug. The plasma dabigatran concentration after a single gavage of dabigatran etexilate showed significant variability at the 1 h peak, reinforcing our view that bioavailability and peak drug concentrations may be the critical factors in therapeutic efficacy.

In the next set of experiments, we used heterozygous *T7D23A* mice that exhibit rapid, spontaneous CP development. We tested the effect of dabigatran etexilate at an early age with incipient CP, utilizing 1- and 2-week drug dosing schemes, using either daily gavages of dabigatran etexilate or feeding with solid chow containing the prodrug. Surprisingly, neither method of drug treatment showed any therapeutic effect. It is likely that dabigatran concentrations achieved in the pancreas of *T7D23A* mice were insufficient to exert a significant trypsin inhibitory effect. With the gavage treatment, only the short-lived peak concentrations might have been adequately high, whereas the use of drug-laced chow yielded much lower blood concentrations of dabigatran. We also note, that relative to *T7K24R* mice, *T7D23A* mice carry a trypsinogen mutation with a stronger biochemical effect and exhibit more aggressive pancreatitis phenotype. Therefore, *T7D23A* mice may require higher blood concentrations of dabigatran than *T7K24R* mice for successful inhibition of intrapancreatic trypsin activity and

treatment of pancreatitis. Also suggesting a dose dependence, the largest effect was seen with twice-daily doses.

The aim of our studies was to show that dabigatran inhibits trypsin, and thereby confers therapeutic benefit in pancreatitis. Although our results are fully consistent with this notion, we cannot rule out that dabigatran also acts via other pathways that may reduce pancreatic inflammation and/or ameliorate pancreas regeneration. In their original paper, the Ji laboratory proposed that the anticoagulant activity of dabigatran may be partly or largely responsible for its therapeutic efficacy in pancreatitis. Their intriguing data showed that the factor Xa inhibitor apixaban (100 mg/kg), which is devoid of trypsin inhibitory activity, had no therapeutic activity. However, when apixaban and camostat (200 mg/kg) were administered together, the combination was highly effective, even though camostat showed only a partial effect when given alone. A possible interpretation of these published results is that anticoagulation may improve tissue penetration of the trypsin inhibitor camostat, and thereby renders it more effective. Other prior rodent and clinical cohort studies demonstrated that use of anticoagulants are beneficial in pancreatitis therapy, possibly by eliminating fibrin deposits and improving circulation and oxygenation.

The goal of our study was to offer proof of concept that inhibition of trypsin activity in the pancreas can have a therapeutic effect in pancreatitis. Our experiments demonstrated that benzamidine derivatives such as dabigatran are effective trypsin inhibitors with therapeutic efficacy in pancreatitis. Translation of the preclinical studies to human clinical trials in the future should focus on the utility of trypsin inhibitors against trypsin-mediated forms of CP, such as hereditary pancreatitis associated with trypsinogen mutations.

CONCLUSIONS AND NEW FINDINGS

Genetic analysis of the c.1074G>A (p.W358X) PNLIPRP2 variant in CP

1. Our genetic case-control study found no association between the p.W358X *PNLIPRP2* variant and CP.

2. We demonstrated that the mRNA expression of the p.W358X *PNLIPRP2* variant allele was diminished in the human pancreas, relative to the full-length, wild-type *PNLIPRP2* allele. We propose that the variant allele mRNA undergoes nonsense-mediated decay.

Preclinical testing of dabigatran in trypsin-dependent pancreatitis

1. We demonstrated that the anticoagulant dabigatran inhibited the enzyme activity of all human and mouse trypsin isoforms.

2. We showed that oral administration of dabigatran etexilate to mice either by gavage or by solid chow resulted in potentially therapeutic dabigatran concentrations in the blood.

3. We demonstrated that in the *T7K24R* mouse model of trypsin-dependent pancreatitis, a single oral dose of dabigatran etexilate prevented progression of cerulein-induced acute pancreatitis, and resulted in histologically verified healing of the pancreas.

4. We found that the development of spontaneous, trypsin-dependent CP in *T7D23A* mice was unaffected by chronic oral administration of dabigatran etexilate. The lack of efficacy in this model was likely due to the more aggressive CP phenotype, and the insufficient dabigatran levels attained in the pancreas.

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