Theses of doctoral (PhD) dissertation

In vitro study of matriptase modulators

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Introduction

Serin proteases are one of the oldest and largest families of proteases. Its members are involved in several physiological and pathological processes, such as digestion, blood clotting, wound healing, blood pressure regulation or virus-cell interaction. Type II serin proteases (TTSPs) play important roles in a wide variety of physiological and pathological pathways. They are located on the cell surface due to their N-terminal plasma membrane-bound domain and many of their functions include mediating signaling between the cell and the extracellular environment, regulating various cellular responses, tissue morphogenesis, barrier function, water and ion transport. Since many members of the TTSP family have been extensively studied in recent years, we have selected such part of their diverse and multifaceted mechanisms that remains unknown. This thesis is focusing on an activator and certain inhibitors which can modulate the effect that matriptase-1 and matriptase-2 possess on different kind of cell cultures. The present study may contribute to the future use of the investigated modulators as drug targets.

Matriptase-1 is required for postnatal survival by modulating cell junctions, although its increased activity can promote the metastasis formation of certain tumors of epithelial origin. It is involved in the lysis of the articular cartilage in osteoarthritis, has a role in the development of pulmonary fibrosis and helps the cellular entry of influenza viruses. Matriptase-2 plays a central role in iron metabolism, and, in connection with this, it is involved in the pathogenesis of obesity, insulin resistance and type 2 diabetes. Decreasing levels are associated with poor prognosis of several types of tumors. Therefore, a precise understanding of their function may help to elucidate a wide range of pathomechanisms, and their regulation may also be a target for drug discovery.

Our goal was to extend the information available on matriptase enzymes in several directions. In our experiments, a known matriptase-1 activator, the sphingosine-1-phosphate (S1P) and an inhibitor family, the 3-amidinophenylalanine (3-APhA) based inhibitors were tested *in vitro*. The 3-APhA based compounds described to be effective in inhibiting enzymes in a number of *in vitro* and *in vivo* studies, but little data is available on their further cellular effects. Since most research to date has been carried out mainly on mammalian cells or individuals, little is known about vertebrate species belonging to other taxonomic categories. The very wide range of effects and the modulator molecules that already exist or will be developed in the future, also call for a well-organised *in vitro* test system where the various activating and inhibitory effects in different species and organs can be compared in the future. Therefore, it was aimed to develop a species- and organ-specific *in vitro* assay system to test the effects and applicability of these molecules.

Aims of the study

In the present research, it was aimed to extend our knowledge about the activators and inhibitors of matriptase-1 and matriptase-2 in several aspects, so the further goals were set for the experiments:

1. In the first study, effects of S1P, previously described as a matriptase-1 activator, have been investigated. Our aim was to find out whether S1P could affect hepcidin production of hepatocytes and thus induce a decrease in hepcidin concentration, presumably by stimulating the enzyme matriptase-2. An important part of the experiment was to see whether the concentrations of S1P applied would affect cell viability and induce oxidative stress response. For this experiments, primary rat hepatocyte mono-cultures were used to mimic the physiological *in vivo* conditions of the mammalian liver.

2. During the second study, two inhibitors that have been analyzed in several *in vitro* experiments for inhibition of both matriptase-1 and matriptase-2 were used. Since MI432 and MI460 inhibitors have been already tested in mammalian cell lines, chicken primary hepatic cell cultures were applied in the experiment. As, based on the literature, these compounds may emerge as drug candidates in the future, their effects on the inflammatory and oxidative stress response were investigated in the liver models. The two agents were tested on hepatocyte mono-cultures, modeling healthy liver, as well as in hepatocyte – non-parenchymal (NP) cell co-cultures representing mild inflammation. Different markers of inflammation and oxidative stress were monitored along with specific elements of the cell's oxidative defense system.

3. In the third study, matriptase-1 inhibitors, previously not used in cell cultures, were tested in a well-established model system, the porcine small intestine-derived IPEC-J2 cell line. Our objectives were to investigate how the applied inhibitors affect cell viability, whether they reduce monolayer integrity through inhibition of matriptase-1, and whether they induce an oxidative stress response in intestinal epithelial cells.

4. In addition, to investigate the effects of specific inhibitory or activating molecules, it was also aimed to develop *in vitro,* species- and organ-specific model systems that will be suitable for comparing the effects of different activating and inhibitory agents in the future.

Materials and methods

Investigation of a matriptase activator on rat primary hepatocyte mono-culture (Study 1)

In this study, S1P, a natural matriptase-1 activator, was examined whether it could also activate matriptase-2 enzyme. The effects of S1P were monitored by investigating cell viability (MTS assay), extracellular hydrogen peroxide production (Amplex Red Hydrogen Peroxidase Assay Kit) and hepcidin levels (sandwich ELISA) on rat primary hepatocyte mono-culture. To monitor cell viability, cells were seeded on a 96-well culture plate, and for collecting culture media samples, cells were seeded on 6-well inserts. S1P was added to the apical compartment of the mono-culture at a concentration of 50, 200, 1000 ng/ml for 72 hours, and the apical supernatant was sampled after 24, 48, and 72 hours of incubation. Viability testing was performed at 24 and 72 hours.

Effects of matriptase inhibitors on chicken primary hepatic mono- and co-cultures (Study 2)

In Study 2, it was aimed to assess the effects of the 3-AphA-type matriptase inhibitors MI432 and MI460 on the inflammatory and oxidative state of chicken primary hepatocyte mono-cultures and hepatocyte – NP cell co-cultures, the latter providing a proper model of hepatic inflammation in birds as previously described by our research group. Three-week-old Ross-308 broiler chickens were used to establish the primary cell cultures. Briefly, a multi-step perfusion of the liver was followed by digestion with collagenase enzyme, after which hepatocyte and NP cell enriched fractions were gained by differential centrifugation. The NP cell fraction contains mainly macrophages, predominantly Kupffer cells, and the currently used co-culture with a 6:1 cell ratio (hepatocyte to NP cells) can mimic mild hepatitis with moderate macrophage infiltration. Following cell isolation, hepatocyte mono-culture and hepatocyte - NP cell co-cultures were established.

The inhibitors MI432 and MI460 were applied at the concentrations of 10, 25 and 50 µmol/l for 4 or 24 h incubation time. The metabolic activity of cells was assessed by the Cell Counting Kit-8 (CCK-8) assay, IL-6 and IL-8 concentrations of the supernatant were quantified by ELISA, while malondialdehyde and H_2O_2 were measured by specific colorimetric assays (Lipid Peroxidation MDA Assay Kit and Amplex Red Hydrogen Peroxidase Assay Kit, respectively). After 24 h sampling, cells were lysed using a special buffer (M-PER). To standardize the results, the total protein concentration of cell lysates was determined using Pierce Bicinchoninic Acid (BCA) Protein Assay. Finally, the activity

of glutathione peroxidase (Glutathione Peroxidase Cellular Activity Assay Kit) was measured from the cell lysate. CCK-8 assay was performed on 96-well cell culture plates, while all other measurements were carried out from cultures seeded on 6-well cell culture dishes.

Matriptase inhibitory assay on porcine IPEC-J2 intestinal epithelial cell line (Study 3)

In this study, inhibitors MI439 and MI476 were tested on the IPEC-J2 non-tumorigenic neonatal porcine jejunal epithelial cell line. Based on previous experiments, it was investigated how the inhibitors affect cell viability (MTS), intestinal barrier integrity (TER) and extracellular redox status *in vitro* (Amplex Red Hydrogen Peroxidase Assay Kit). Assays were performed after 24 h incubation at concentrations of 10, 25, 50 µmol/l. Cells were plated on 96-well culture dishes for viability assays, while 6-well membrane inoculants were used for all other experiments. Both apical and basolateral compartments were used for media sampling.

Statistics

Data was analyzed with one-way ANOVA, Tukey's post hoc test and Spearman's test of correlation. The results are expressed as mean ± standard error of mean (SEM). Statistical significance was set at $p < 0.05$.

Results and discussion

Investigation of a matriptase activator on rat primary hepatocyte mono-cultures (Study 1)

Matriptase-2 has the ability to inhibit the transcription of hepcidin by splitting the membrane-bound hemojuvelin. If the TMPRSS6 gene encoding matriptase-2 is deficient or matriptase-2 is proteolytically inactive, iron-deficient anaemia develops due to extremely high concentration of hepcidin in the blood and the consequent decreased expression of ferroportin in the intestinal epithelial cells. Based on the above-mentioned facts, activation of matriptase-2 may represent a novel therapeutic approach for iron-deficient non-regenerative anaemia of chronic inflammation.

S1P has previously been shown to activate matriptase-1 in nanomolar amounts, acting as both an extracellular regulator and an intracellular second messenger. Since matriptase-1 and matriptase-2 share numerous properties regarding their structures, and S1P can be found throughout the organism possessing a variety of regulatory functions, the question arises whether it is able to activate matriptase-2 in addition to matriptase-1.

In the present study, the effect of S1P on matriptase-2 has been investigated indirectly by monitoring the quantitative changes in hepcidin concentration. Since matriptase-2 is produced predominantly by the liver, our research was carried out on hepatocyte mono-cultures. Our results indicate that as a response to S1P exposure, hepcidin concentration is reduced after 24, 48 and 72 h, respectively. Thus, S1P treatment resulted in a decrease in hepcidin concentration of the cell supernatant, presumably mediated by the activation of matriptase-2. These data suggest that S1P exerts its regulatory function as an activator of matriptase-2 in the liver, hence it may have an impact on iron metabolism by altering hepcidin levels.

In our experiments, the selected 50, 200 and 1000 ng/ml concentrations of S1P could be safely applied on rat primary hepatocyte mono-cultures, using 72 h incubation time, since it has not affected either the metabolic activity and viability of the cells, or their extracellular redox state.

Effects of matriptase inhibitors on chicken primary hepatic mono- and co-cultures (Study 2)

In Study 2, the effects of matriptase inhibition were investigated in healthy and inflammatory liver cell models of chicken origin, with a focus on the potential regulatory role of matriptase in cellular metabolic, inflammatory and redox processes.

Limited information is available on matriptase-1 and matriptase-2 enzymes in different animal species. The orthologue of matriptase-1 has been detected in the genomes of several animal species, including chicken, but no information is available on the exact protein structure of matriptase in various animal species. The differences between the data obtained from mammalian cell studies and the results of the present experiments on chicken liver cell cultures highlight the importance of species-dependent differences in matriptase activity.

The results of this study showed that certain concentrations of both MI432 and MI460 inhibitors moderately reduced the aerobic catabolic activity of both types of cell cultures after 4 h incubation time. However, the extent of reduction indicated that the inhibitors were not cytotoxic. After a longer incubation period of 24 h, there was no significant difference between control and inhibitor-treated cells, suggesting that liver cells adapt metabolically rapidly to the inhibitors used.

The present study revealed that matriptase inhibition by MI432 triggered intense and substantive proinflammatory IL-6 and IL-8 production in chicken-derived hepatic cell culture models, which was not accompanied with enhanced oxidative stress and lipid peroxidation as indicated by cellular ROS production and MDA levels. In contrast to the MI432 inhibitor, MI460 was not shown to be pro-inflammatory based on IL-6 and IL-8 concentrations. Despite the fact that MI432 and MI460 are both 3-APhA-based inhibitors with similar structures, our results confirm that they may have widely different effects on cell function, probably due to their different Ki values. These results may suggest that physiological matriptase activity can play a key role in maintaining hepatic metabolic and inflammatory homeostasis in chicken liver without being a major regulator of the hepatocellular redox state.

No significant difference was found between hepatocyte mono-culture and hepatocyte – NP cell co-culture in terms of the changes associated to the matriptase inhibitors we used. This finding may suggest that the presence of hepatic NP cells is not a critical factor in determining the effects of matriptase inhibition. 3-AphA-based matriptase inhibitors may act similarly under physiological and mildly inflamed conditions on chicken-derived liver cells. Our results suggest that these inhibitors do not greatly affect the oxidative state of chicken liver cells and can be safely used without causing oxidative stress and lipid peroxidation.

Matriptase inhibitory assay on porcine IPEC-J2 intestinal epithelial cell line (Study 3)

The inhibition of matriptase-1 and matriptase-2 is being intensively researched with a focus on potential therapeutic application. However, there are currently only rather limited data available on 3-APhA-based inhibitors with respect to their effects on cellular redox status, oxidative stress response and cell layer integrity. In Study 3, we used inhibitors that have not been previously tested in cellular media. In the present experiment, the non-tumorigenic porcine jejunal epithelial cell line IPEC-J2 was used as it is more similar to physiological epithelium than any cell line of tumor origin.

Based on our results, MI439 and MI476 had no effect on cell viability during the 24 h incubation period tested, confirming that these inhibitors can be safely used at a concentration of 50 µmol/l for 24 h on IPEC-J2 intestinal epithelial cells. This is consistent with what has been observed with other inhibitors tested previously.

In Study 3, it was found that both MI439 and MI476 significantly reduced TER values at 25 and 50 µmol/l applying 24 h incubation time. Consistent with this, in other studies on IPEC-J2 cells, MI432 at concentrations of 10, 25 and 50 µmol/l reduced TER values at 48 h of application, suggesting that matriptase inhibitors may affect intestinal integrity in pigs.

In the present study on IPEC-J2 cells, both MI439 and MI476 were able to significantly reduce the amount of extracellular H_2O_2. These data may point to a novel beneficial effect of matriptase inhibition by improving the redox state of the intestinal epithelium. The results of this study suggest that certain matriptase inhibitors may also have antioxidative potential, thus reducing the release of extracellular ROS from the intestinal epithelial cells, contributing to intestinal health. Since oxidative stress plays a key role in the pathogenesis of many intestinal diseases through a multifaceted mode of action, the putative potential of the 3-APhA inhibitors used (MI439 and MI476) in attenuating intestinal ROS formation may be of great relevance for new therapies targeting gastrointestinal disorders.

In conclusion, the 3-APhA inhibitor MI439 and MI476 are promising candidates for establishing and maintaining redox homeostasis and gut health. The present results, in conjunction with previous studies, highlight the key function of the enzyme matriptase-1 in regulating intestinal barrier function and also suggest that proper matriptase inhibition may be a useful tool to improve intestinal health by maintaining physiological oxidative balance.

Conclusion

Summarizing the results of all studies, the cell cultures used in our research can provide appropriate species- and organ-specific models to investigate the effects of

matriptase modulators. The description of the cellular effects of the tested activator and inhibitory compounds in liver and intestinal cell cultures of different animal species may contribute to the possible future pharmacological development of the therapeutic agents, taking into account the therapeutic purposes as well as the safety of use.

New scientific results

Ad 1,

Sphingosine-1-phosphate, as an activator of matriptase-2, has shown to be able to decrease the extracellular concentration of hepcidin on rat primary hepatocyte mono-cultures, presumably having an impact on the regulation of iron metabolism.

Ad 2,

3-AphA type matriptase inhibitors (MI432, MI460) were examined on chicken primary hepatocyte mono- and co-cultures for the first time, thereby investigating the role of matriptase in metabolic, inflammatory and redox processes. Albeit MI432 has exerted a significant production of pro-inflammatory cytokines, this effect has been connected neither to oxidative stress nor lipid peroxidation, furthermore, none of the inhibitors has been shown to reduce the metabolic activity of the cells. Our results suggest that the physiological activity of matriptase may have an important role in maintaining the metabolic and inflammatory homeostasis of the liver in birds.

Ad 3,

Novel effects of matriptase inhibitors (MI439, MI476) have been described on porcine IPEC-J2 intestinal epithelial cell line. Inhibitors MI439 and MI476 have not reduced cell viability, however at higher concentrations TER reduction has been observed and extracellular H_2O_2 concentration has been diminished. These results suggest that in addition to having impact on the intestinal barrier, some matriptase inhibitors may also have antioxidant potential, thus reducing the release of H_2O_2 from intestinal epithelial cells, thereby contributing to intestinal health.

Own scientific publications

Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Barna, R.F., Mackei, M., Pászti-Gere, E., Neogrády, Zs., Jerzsele, Á., Mátis, G.: **The effects of matriptase inhibition on the inflammatory and redox homeostasis of chicken hepatic cell culture models.** *Biomedicines*, *9*(5), 450, 2021. **Impact factor: 5,612**

Barna, R.F., Pomothy, J.M., Pásztiné Gere, E., Mátis, G., Jerzsele, Á.: **A matriptáz enzimek élettani és patológiás szerepe.** *Magyar Állatorvosok Lapja, 142*(11), 673-680, 2020. **Impact factor: 0,12**

Barna, R.F**.,** Pomothy, J.M., Paréj, Zs., Pásztiné Gere, E.: **Investigation of sphingosin-1-phosphate-triggered matriptase activation using a rat primary hepatocyte model.** *Acta Veterinaria Hungarica 67*(4), 578-587, 2019. **Impact factor: 0,955**

Pásztiné Gere, E., Barna, R.F., Szombath, G., Rokonál, P., Gálfi, P.: **A vasanyagcsere-zavarok kezelésének lehetőségei, új perspektívák.** *Magyar Állatorvosok Lapja 138*(10), 559-564, 2016. **Impact factor: 0,12**

Oral and poster presentations of Hungarian national conferences

Pomothy Judit Mercédesz, Barna Réka Fanni, Pásztiné Gere Erzsébet:
Matriptáz enzim aktivátor és inhibitor tesztelése humán és patkány primer májsejt modelleken,
Akadémiai Beszámolók, Budapest, 2020.

Barna Réka Fanni, Pomothy Judit Mercédesz, Pásztiné Gere Erzsébet:
Matriptáz modulátorok hatásának vizsgálata az extracelluláris hidrogén-peroxid-szintre primer májsejtmodellen,
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Matriptáz inhibitor tesztelése primer májsejteken és bélhámsejtvonalon
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Bélhámsejteken tesztelt új matriptáz inhibitorok jellemzése,
Akadémiai beszámolók, Budapest, 2019.

Pomothy Judit Mercédesz, Barna Réka Fanni, Pásztiné Gere Erzsébet:
Matriptáz enzim aktivátor és inhibitor tesztelése patkány és humán primer májsejt modelleken

49. Membrán-transzport Konferencia, Sümeg, 2019.

Barna Réka Fanni, Pomothy Judit Mercédesz, Pásztiné Gere Erzsébet:
Szelektív matriptáz inhibitorok biztonságos alkalmazásának vizsgálata IPEC-J2 bélhámsejteken
TOX'2018 Tudományos Konferencia Program, Lillafüred, 2018.

Barna Réka Fanni, Pomothy Judit Mercédesz, Rokonál Patrik, Szombath Gergely, Pásztiné Gere Erzsébet:**Matriptáz modulátorok vizsgálata in vitro primer májsejteken**, MTA Akadémiai beszámolók: Élettan és Biokémia Patológia Gyógyszertan és Toxikológia Morfológia, Budapest, 2018.

Publications not related to the topic of the present dissertation

Pomothy J.M., Barna R.F., Pászti E.A., Babiczky Á., Szóládi Á., Jerzsele Á., Pásztiné Gere E.: **Beneficial Effects of Rosmarinic Acid on IPEC-J2 Cells Exposed to the Combination of Deoxynivalenol and T-2 Toxin**. *Mediators of Inflammation*, 2020. **Impact factor: 4,711**

Pomothy J.M., Barna R.F., Czimmermann Á.E., Szóládi Á., Pásztiné Gere E.: **A deoxinivalenol mikotoxin toxikus hatásai a gazdasági haszonállatokra**, *Magyar Állatorvosok Lapja*, 142(2), 117-127, 2020. **Impact factor: 0,12**

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Pomothy J.M., Barna R.F., Szóládi Á., Pásztiné, Gere E.: **The benefical effects of rosmarinic acid on a non-tumorgenic epithelial cell line**, *GRADUS,* 7(1), 79-83, 2020.

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