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TDK Thesis

***In vitro* pharmacological characterization of antiviral
inhibitors of host matriptase/TMPRSS2**

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Table of Contents

1. List of Abbreviations	2
2. Introduction	4
3. Literature review.....	5
3.1. Role of TTSPs in influenza- and coronavirus infections	5
3.2. Matriptase/TMPRSS2 inhibitors.....	8
4. Aims.....	10
5. Materials and Methods	11
5.1. Inhibitors' effects on the activity of human microsomal CYP isoenzymes.....	11
5.2. Preparation of suspensions of primary human hepatocytes	12
5.3. Incubation of primary human hepatocytes with protease inhibitors .	12
5.4. Quantification of the unchanged protease inhibitors	14
5.5. Evaluation of the pharmacokinetic parameters of the protease inhibitors	15
5.6. Cells for virus propagation.....	15
5.7. Antiviral assay and analysis of viral multicycle replication	16
5.8. Cell viability assay	17
5.9. Statistical analysis.....	17
6. Results	18
6.1. Inhibitors' effects on human microsomal CYP isoenzyme activity ..	18
6.2. Cytotoxicity of MI-463 and MI-1900	19
6.3. Depletion rates of the protease inhibitors	20
6.4. Pharmacokinetic activity of the protease inhibitors.....	22
6.5. Inhibitory effect of protease inhibitors on matriptase-induced activation of H9N2 influenza virus.....	23
6.6. Effects of protease inhibitors on viral titers of H1N1 influenza virus	25
7. Discussion.....	26
8. Abstract.....	32
9. Hungarian Abstract.....	33
10. References	34
11. Acknowledgements.....	39

1. List of Abbreviations

Abcam	Monoclonal mouse anti-nucleoprotein antibody
ACE2	Angiotensin-converting enzyme 2
AECII	Type II alveolar epithelial cells
ANOVA	Analysis of variance
Calu-3	Human lung cancer cell line
Cl _H	Hepatic clearance
Cl _{int}	Intrinsic clearance
CYP	Cytochrome P450
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
E _H	Hepatic extraction ratio
F	Bioavailability
FCS	Fetal calf serum
H1N1pdm	H1N1 A/Hamburg/05/09
HA	Hemagglutinin
HAT	Human airway trypsin-like protease
HPAIV	Highly pathogenic avian influenza A viruses
HSA	Human serum albumin
IAV	Influenza A virus
INH	Inhibitor
KPL	TrueBlue Peroxidase substrate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MDCK-II	Madin-Darby canine kidney cells II
MERS-CoV	Middle East respiratory syndrome virus
MI	Matriptase/TMPRSS2 inhibitor
MOI	Multiplicity of infection
NA	Neuraminidase
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate-buffered saline
pfu	Plaque-forming units
Phe(3-Am)	3-amidinophenylalanine
PHH	Primary human hepatocyte

p.i.	Post-infection
PK	Pharmacokinetic
RFU	Relative fluorescence units
S	Spike protein
SARS-CoV	Severe acute respiratory syndrome coronavirus
SD	Standard deviation
SEM	Standard error of the mean
TMPRSS2	Transmembrane protease serine 2
TTSP	Type II transmembrane serine protease

2. Introduction

Influenza viruses possess notable morbidity and mortality rates and have caused numerous pandemics, resulting in a high number of severe infections and deaths. Notorious pandemics occurred in 1918-1919 (up to 50 million deaths), 1957-1958 (1-4 million deaths), 1968-1969 (1-4 million deaths), and one of the most recent, in 2009-2010 (100,000-400,000 deaths). Aside from these, human populations are subjected to seasonal influenza virus infections as well, causing severe, recurring disease each year due to constant evolution of the viral genome. These seasonal endemics result in an estimated annual one billion cases worldwide. Approximately three to five million of these are considered severe, and about 300,000-650,000 deaths occur due to complications of influenza virus infections yearly. The pandemic in 2009 caused by the H1N1 strain emphasized the fact that severe influenza pandemics can come about from avian- and swine-origin influenza viruses as zoonotic pathogens. It is clear that these viruses pose a great threat to public health systems and are a great economic burden, especially with increasing economic globalization and urbanization, which increases the potential for an influenza pandemic with more distant and faster spreading [1].

Many strains and subtypes of influenza viruses have been shown to be activated by type II transmembrane serine proteases (TTSPs) such as transmembrane protease serine 2 (TMPRSS2) and matriptase, indicating potential to prevent viral spread via inhibition of virus activation and cell entry [2-3]. However, TMPRSS2-mediated virus activation is not only limited to influenza viruses, but another family of viruses that induce respiratory infections, coronaviruses, namely the severe acute respiratory syndrome coronavirus (SARS-CoV), the Middle East respiratory syndrome virus (MERS-CoV), and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), also utilize host-cell proteases for cell entry [4-5]. Previous drugs for the treatment of influenza virus infections, such as the neuraminidase (NA) inhibitors, only have a few representatives that have been approved for treatment, and only two of them are available worldwide. Among these, oseltamivir phosphate is the only pharmaceutical in this drug

class that exhibits great efficacy and bioavailability and negligible toxicity. However, mutants of influenza viruses that are resistant to NA inhibitors have emerged in various influenza subtypes by NA substitution, which presents a great limitation in the clinical application of these drugs in the treatment of influenza virus infections [6]. Therefore, host-cell proteases such as TMPRSS2 and matriptase represent pharmacological targets that are of great interest for the prevention and treatment of respiratory virus-induced infections, especially those caused by influenza viruses.

In the present study, seven matriptase/TMPRSS2 inhibitors possessing a 3-amidinophenylalanine (Phe(3-Am))-based structure were evaluated for the determination of their pharmacokinetic (PK) properties. Inhibitory effects of the compounds on the activity of cytochrome P450 (CYP) enzymes were assessed using fluorometric assays. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), the depletion rates of the inhibitors were determined in primary human hepatocytes (PHH) over a designated timeframe. *In vivo* PK parameters were also predicted for further preliminary characterization of the compounds. Lastly, the antiviral efficacy of selected protease inhibitors against two influenza virus subtypes H1N1 and H9N2 were evaluated *in vitro* using an antiviral plaque assay. The *in vitro* PK characterization of these compounds provides useful preliminary results in the selection of novel drugs for further *in vivo* experimental phases during drug development.

3. Literature review

3.1. Role of TTSPs in influenza- and coronavirus infections

TMPRSS2 is classified as a TTSP, a family of host-cell proteases which also includes matriptase and human airway trypsin-like protease (HAT). Although the physiological function of TMPRSS2 is not fully understood, it is known to be expressed in numerous types of epithelial tissue, especially the prostate as well as tissue of the respiratory tract, such as the trachea, bronchi, and lungs [7]. Similarly, matriptase is also known to be a protease found strictly on epithelial tissue, and its physiological role is slightly better

known than that of TMPRSS2. Although matriptase is not omnipresent in epithelial tissue, it is still very widespread throughout the body, with great levels of expression in the respiratory, gastrointestinal, and urogenital tracts [8]. Despite the details of their physiological functions remaining somewhat unrevealed, multiple studies have already identified involvement of both TMPRSS2 and matriptase in various types of epithelial tumors, of which prostate tumors appear to greatly utilize TMPRSS2 for tumor growth and metastasis [8-9]. However, current interest has shifted towards these host-cell proteases' roles in the proteolytic cleavage of the surface proteins of influenza viruses, coronaviruses, and even human metapneumovirus, all of which can cause devastating respiratory disease [7,10-11].

Influenza viruses belong to the *Orthomyxoviridae* family and can be classified as type A, B, C, or D. They are negative-sense single-stranded RNA viruses that contain either eight (types A and B) or seven (types C and D) gene segments. Influenza A viruses (IAVs) are the main culprits behind influenza pandemics, especially those causing annual pandemics in humans. IAVs are enveloped and possess viral membrane proteins on their surface, namely hemagglutinin (HA) and NA. It is based on these surface proteins that we can differentiate subtypes of IAVs. Currently, there are 18 known HA (H1-H18) and 11 known NA (N1-N11) subtypes [12]. HA is of great relevance as it initiates viral envelope fusion to the cell's endosomal membrane after binding to receptors containing sialic acid. HA begins as a precursor (HA0) incapable of mediating fusion, and it must undergo cleavage by host-cell proteases into subunits HA1 and HA2 [2]. Host-cell proteases TMPRSS2 and matriptase, as well as other TTSPs like HAT, have been identified as proteolytic activators of influenza viruses by HA cleavage *in vitro* [3,11].

However, not all influenza viruses are dependent on these proteases for virus activation. The majority of IAVs, especially low pathogenic avian influenza A viruses, contain a monobasic cleavage site, for which TMPRSS2 has been identified as an integral virus-activating protease in human airway cells, but some IAVs, namely the highly pathogenic avian influenza A viruses (HPAIVs), possess multibasic cleavage sites which must

be cleaved by furin and other related host-cell proteases, and it is this difference that supports systemic infection in poultry by HPAIVs [13-14]. In mice, it has been shown that TMPRSS2 is the main proteolytic activator for the spread of human IAVs belonging to subtypes H1 and H2 as well as the zoonotic H7N9 virus and avian H10 subtypes. Yet for the human H3N2 virus and influenza B viruses, virus activation was supported by proteases other than TMPRSS2. Human virus H3N2 was also shown to be capable of multicyclic replication in primary type II alveolar epithelial cells (AECII) in both wild and TMPRSS2-deficient mice, whereas TMPRSS2 was essential for viral spread in primary human AECII, indicating that there are interspecies differences in viral entry mechanisms [2,15]. Based on the mechanism of cell entry of influenza viruses, it is evident that viral spread may be prevented through the application of host-cell protease inhibitors like those that inhibit matriptase and TMPRSS2, although specific knowledge of the mechanism of virus activation as well as the species affected must be known.

As mentioned previously, members of the *Coronaviridae* family also utilize TMPRSS2 for virus activation. Of chief importance are SARS-CoV, the culprit of the 2002/2003 outbreak, MERS-CoV, which caused an outbreak in 2012, and SARS-CoV-2, which prompted the COVID-19 pandemic. Due to the shared ability of some coronaviruses and influenza viruses to similarly potentiate infection in human airway cells, it is worth investigating the effects of host-cell protease inhibitors on their viral entry mechanism, since coinfection can possibly produce a pandemic characterized by airborne pathogens [4,16].

Coronaviruses utilize their structural spike (S) glycoprotein to initiate infection in cells possessing the angiotensin-converting enzyme 2 (ACE2) receptor, a characteristic which also contributes to the viruses' pathogenicity. The interaction between the S protein and the ACE2 receptor occurs by proteolytic cleavage in two steps. This begins with host-cell furin cleaving the S1/S2 site of the S protein and is followed by another cleavage at the S2' site inside the S2 subunit. The cleaving of the S2' site can be carried out either by TMPRSS2 on the surface of the cell or by cathepsin L

inside the endosomal compartment [5]. Each of the host enzymes catalyzing the reactions between the viruses and the host cell represent intriguing drug targets in the development of new treatments. Utilizing inhibitors of both furin and TMPRSS2 for treatment of SARS-CoV-2 infection is also of great interest, as both of these host-cell proteases each perform cleavage of the S protein at different sites, which would theoretically produce a synergistic effect in the blockage of virus cell entry [17-18].

In the present study, IAV strains H1N1 A/Hamburg/05/09 (H1N1pdm) and H9N2 A/quail/Shantou/782/00 were investigated in Calu-3 human airway epithelial cells, which are a human lung cancer cell line, and Madin-Darby canine kidney cells II (MDCK-II), respectively. TMPRSS2 has been proven to be expressed in Calu-3 cells and has also been identified as an essential host-cell protease for the activation of H1N1pdm [15,19]. Matriptase has been shown to be a key host-cell protease involved in the viral activation and spread of H9N2 viruses, including the strain A/quail/Shantou/782/00, in MDCK-II cells and other MDCK-related cell lines [3]. These findings validate the use of these cell lines in our investigation into the inhibition of replication of these virus strains.

3.2. Matriptase/TMPRSS2 inhibitors

Several matriptase/TMPRSS2 inhibitors based on a Phe(3-Am) structure and potentially possessing antiviral effects against the aforementioned pathogens have been evaluated in different cell models thus far [20]. There was no significant influence found from the inhibitors on redox homeostasis. Enzyme kinetic studies demonstrated that there was not only an affinity for matriptase/TMPRSS2 from the less selective inhibitors, but also for coagulation factors such as thrombin, which is indicative of a potential risk of bleeding. There was also no interaction found between human serum albumin (HSA) and the Phe(3-Am) derivatives in plasma protein binding assays, but complex formation was discovered between alpha-1 acidic glycoprotein and matriptase/TMPRSS2 inhibitors (MI) MI-472 and MI-477, of which the former is used in the present study [20]. Inhibitors MI-463 and MI-1900, which were subject to the present research, have also undergone spectroscopic studies to evaluate potential interactions

with HSA and CYP enzymes. The results showed that there were no significant interactions between the inhibitors and HSA, and among the investigated CYP enzymes, an inhibitory effect was only produced against CYP3A4 [21].

Investigation into interactions of novel drugs with CYP enzymes, especially those of the 1, 2, and 3 families, is of key importance in the drug discovery process as they are involved in the biotransformation of approximately 80% of clinical drugs [22]. Evaluation of these potential interactions provides researchers with important information on the mechanism of action, safety profile, and bioavailability of newly researched compounds [23-24]. In studies conducted on hepatocytes *in vitro*, CYP enzymes such as CYP3A4 were shown to be inhibited or downregulated by several inflammatory cytokines, including interleukin-1, interleukin-6, and tumor necrosis factor alpha, all of which elevate in concentration during infections, including those induced by SARS-CoV-2 and influenza viruses [22,25–27]. Various drugs used as antivirals in infections by SARS-CoV-2, namely remdesivir and Paxlovid (ritonavir-enhanced nirmatrelvir), possess inhibitory effects on the activity of CYP3A4, as well as CYP1A2, CYP2C9, CYP2C19, and CYP2D6 [22,28]. Therefore, virus-induced inflammation and the concomitant application of antiviral drugs for the treatment of viral infections yield potential for drug-induced hepatotoxicity during the course of SARS-CoV-2 infections [25]. Interestingly, in *in silico* studies done on several derivatives of oseltamivir, an inhibitor of the influenza virus NA surface protein which would lead to the prevention of viral shedding, have also been found to decrease the activity of various CYP enzymes [6]. Based on the findings, it is evident that the evaluation of drug interactions with CYP enzymes is imperative during the process of drug discovery. Primary hepatocytes, hepatic microsomes, and recombinant human CYP isoenzymes therefore exhibit extensive usage to unveil potential interactions between CYP enzymes and novel pharmaceuticals, which is the most prevalent type of drug-drug interaction [29].

4. Aims

Matriptase and TMPRSS2 are host-cell proteases utilized by various influenza and coronaviruses for host-cell entry, thus *in vitro* investigation into compounds inhibiting these proteases is of great interest in the initial phases of drug discovery. The goal of this study was to provide a PK characterization of selected Phe(3-Am) inhibitors (MI-21, MI-463, MI-472, MI-485, MI-1900, MI-1903, MI-1904) of matriptase/TMPRSS2 in order to isolate the compounds with ideal PK properties for further research. We conducted the following experiments:

- Firstly, modulation rates of CYP enzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 by the protease inhibitors were assessed in human hepatocyte models by determining % enzyme activity loss with fluorometric assays.
- Additionally, the depletion properties of the inhibitors in PHH were determined throughout a selected timeframe (0-120 min) using LC-MS/MS.
- Furthermore, *in vitro* intrinsic clearance (Cl_{int}) values were measured for each protease inhibitor, and *in vivo* PK parameters (predicted Cl [Cl_H], hepatic extraction ratio [E_H], and bioavailability [F]) were predicted as well.
- Lastly, the antiviral activity and cytotoxicity of selected inhibitors against IAV subtypes H1N1 and H9N2 was investigated with an *in vitro* post-infection (p.i.) antiviral plaque assay.

Several studies have already evaluated the PK properties and anti-influenza effects of various matriptase/TMPRSS2 inhibitors *in vitro*. Our study sought to characterize understudied Phe(3-Am)-derived protease inhibitors to further progress the research done on these intriguing compounds.

5. Materials and Methods

5.1. Inhibitors' effects on the function of CYP isoenzymes of human microsomes

The BioVision CYP450 fluorometric assay kits (BioVision, Inc., Kampenhout, Belgium) are based on the use of a fluorometer to detect highly fluorescent metabolites formed by CYP enzymes from non-fluorescent substrates selective for CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP3A4. Positive controls utilized the selective inhibitors of CYP1A2 (α -naphthoflavone, 6 μ M), CYP2C9 (tienilic acid, 60 μ M), CYP2C19 ((+)-N-3-benzylnirvanol, 30 μ M), CYP2D6 (quinidine, 3 μ M), and CYP3A4 (ketoconazole, 30 μ M).

For determination of the protein content of the human hepatic microsomes (Gibco, Biocenter, Szeged, Hungary), each assay used the bicinchoninic acid protein assay kit (Pierce BCA kit, Thermo Fisher Scientific, Waltham, MA, US) and a 100 μ g/well protein. A 2500 μ L human hepatic microsomal suspension containing human hepatic microsomes (30 μ L; 20 mg/mL) as well as 50 μ L of nicotinamide adenine dinucleotide phosphate (NADPH)-generating system in an assay buffer was used to produce aliquots of the suspension of microsomes (50 μ L), which then received either 20 μ L of the reference inhibitors of each CYP enzyme (α -naphthoflavone, 30 μ M; tienilic acid, 300 μ M; (+)- N-3-benzylnirvanol, 150 μ M; ketoconazole, 150 μ M; quinidine, 15 μ M) or 20 μ L of the protease inhibitors (MI-21, MI-463, MI-472, MI-485, MI-1903, and MI-1904), yielding a 250 μ M final concentration, except in the case of isoenzyme CYP3A4, which had 50, 125, and 250 μ M for its final concentrations. Control experiments consisted of 20 μ L of buffer not containing any inhibitors. Wells containing 70 μ L of the assay buffer alone were measured as background controls. Reactions were then initiated by mixing both the assay buffer and NADPH-generating system mixture with the microsomes and inhibitors. After a 15-minute incubation period at 37 °C, the final mixture containing 30 μ L of the appropriate CYP substrate/NADPH-generating system was pipetted into each well, resulting in a 100 μ L final reaction volume.

For the measurement of fluorescence intensities produced from the reactions, a fluorometer (Victor X2 2030, Perkin Elmer, Waltham, MA, US) set to wavelengths of $\lambda_{\text{ex/em}} = 406/468$ nm (CYP1A2, CYP2C19), $\lambda_{\text{ex/em}} = 415/502$ nm (CYP2C9), $\lambda_{\text{ex/em}} = 390/468$ nm (CYP2D6), and $\lambda_{\text{ex/em}} = 535/587$ nm (CYP3A4) was used.

5.2. Preparation of suspensions of primary human hepatocytes

Cryopreserved PHH were obtained from Primacyte (Primacyt, Schwerin, Germany). Cryopreserved hepatocyte-thawing medium (Primacyt, Schwerin, Germany) was used to thaw the hepatocytes, which were then centrifuged (10 min, $100\times$ g, 20 °C) to remove cell debris in the supernatants. Using the Trypan blue exclusion method, hepatocyte viability was assessed, and it confirmed that over 90% of cells were alive. Cells were then counted in a Bürker chamber until the appropriate concentration (original concentration of the viable hepatocytes was 10^7 cells/mL), and cell suspensions were subsequently diluted using serum-free maintenance medium (Primacyt, Schwerin, Germany).

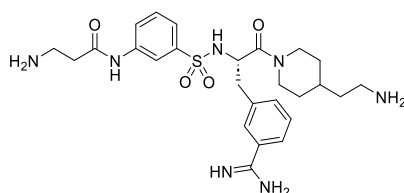
5.3. Incubation of primary human hepatocytes with protease inhibitors

Stock solutions of inhibitors MI-21, MI-463, MI-472, MI-485, MI-1900, MI-1903, and MI-1904 (10 mM) in dimethyl sulfoxide (DMSO) were kept at -20 °C. **Figure 1** demonstrates the structures of the protease inhibitors. Time courses of the unchanged amounts of the inhibitors (in %) were measured after incubation with human hepatocytes. Each compound was incubated in a cell suspension in 6-well plates at the concentration of 5×10^5 cells/mL. Incubation was maintained at constant and low-speed shaking at 37 °C in a humid atmosphere that contained 5% CO₂. Maintenance medium from the stock was used to freshly prepare the 10 μ M solutions of the inhibitors before each experiment. The concentration of DMSO was no more than 0.1% v/v in the final solutions. PHH viability was previously assessed after treatment with MI-21, MI-472, MI-485, MI-1903, and MI-1904 at 50 μ M for 24 hours [20]. In this study, the lack of cytotoxicity of

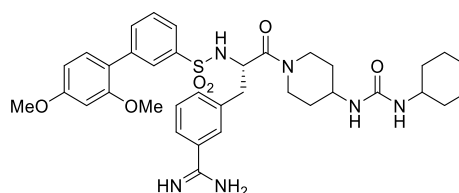
MI-463 and MI-1900 was confirmed in PHH at a concentration of 50 μ M with DMSO at 0.5% v/v as a solvent control for 24 hours.

Using serum-free maintenance medium either with or without each selected inhibitor, cells were incubated for 120 min. At multiple time points (0, 15, 30, 60, 120 min), each incubation mixture was sampled (aliquots: 0.15 mL) and then terminated using 0.1 mL of ice-cold acetonitrile. Cell debris was separated by centrifugation, and the supernatants were subsequently analyzed by LC-MS/MS to quantitate the remaining amount of the inhibitors.

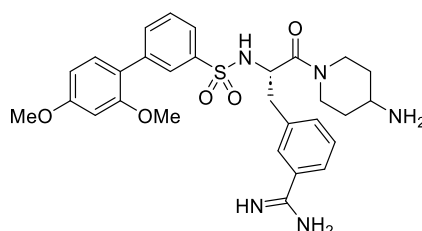
MI-21



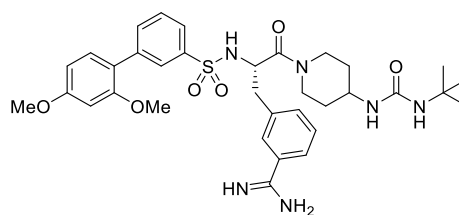
MI-463



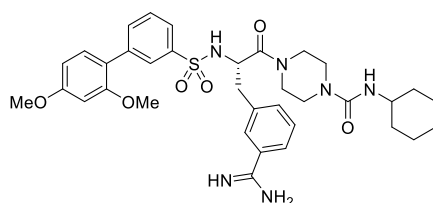
MI-472



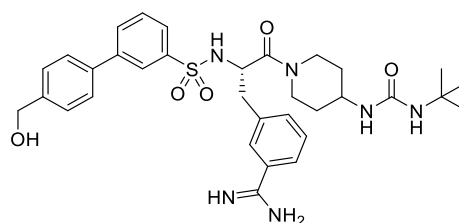
MI-485



MI-1900



MI-1903



MI-1904

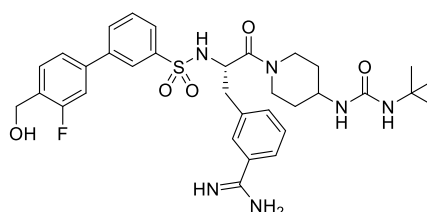


Figure 1. Chemical structure of the selected serine protease inhibitors.

5.4. Quantification of the unchanged protease inhibitors

150 μ L aliquots of the incubation mixture were utilized to evaluate parent compounds MI-463, MI-472, MI-485, MI-1900, MI-1903, and MI-1904 at various time points. Cold acetonitrile with triphenyl-phosphate (330 μ L; 12.000 ng/mL as internal standard for the quantitation) was then mixed with the aliquots. For inhibitor MI-21, an aliquot of 120 μ L was removed from the sample for evaluation after the necessary incubation period and cold acetonitrile (330 μ L) was added. Using a vortex, samples were mixed, and they were subsequently centrifuged (13.300 rpm) at 4 $^{\circ}$ C for 10 min. A polyvinylidene difluoride syringe filter with a pore diameter of 0.22 μ m was utilized to filter the supernatant into a chromatographic vial, and it was then analyzed with LC-MS/MS. A Shimadzu LCMS 8030+ system was used to analyze each sample, and it was run with an electrospray ion source set to positive multiple reaction monitoring mode. The following parameters were set in the MS interface: voltage 4.5 kV; interface temperature 350 $^{\circ}$ C; desolvation line 300 $^{\circ}$ C; heat block 400 $^{\circ}$ C (for MI-21 350 $^{\circ}$ C); detector 1.78 kV, nebulizing gas (N_2) 3 L/min, drying gas (N_2) 15 L/min (for MI-21 drying gas (N_2) was 10 L/min); collision gas (Ar) 230 kPa. For chromatographic separation, a Phenomenex Kinetex C18 EVO 100x4.6 mm column (Torrance, USA) with a particle size of 2.6 μ m was used. Alternatively, a Phenomenex Kinetex C18 EVO 50x4.6 mm column was used for MI-21. Eluents A: 50 mM ammonium acetate in water (pH 5 set by acetic acid) and B: 0.1 v/v% formic acid in acetonitrile were used for gradient elution. The flow rate and the injected volume were set to 0.4 mL/min and 10 μ L, respectively. The temperature of the column was set to 30 $^{\circ}$ C, and that of the autoinjector to 6 $^{\circ}$ C. Gradient elution was applied with eluents A: 0.1 v/v% formic acid in water and B: 0.1 v/v% formic acid in acetonitrile for compound MI-21. The flow rate and injected volume were set to 0.5 mL/min and 10 μ L, respectively. The temperature of the column was set to 40 $^{\circ}$ C, and that of the autoinjector to 6 $^{\circ}$ C.

5.5. Evaluation of the pharmacokinetic parameters of the protease inhibitors

The intrinsic clearance for hepatocytes (Cl_{int}) [$\text{ml min } 5 \times 10^5 \text{ cells}^{-1}$] was assessed based on the reduction in concentration of the protease inhibitors [30]. Predicted hepatic clearance Cl_H , hepatic extraction ratio E_H , and bioavailability F were calculated as *in vivo* PK parameters in accordance with what has been previously published [31]. For the prediction of Cl_{int} per whole liver (g)/bw (kg), the concentration of hepatocytes, the average liver weight, and average body weight in kg were used. The hepatic flow rate, plasma/blood ratio (**Table 1**), and $f_u=1$ were used for the calculation of Cl_H [32-33].

Table 1. Physiological parameters used to predict clearance values (human)

Parameter	Human
Number of hepatocytes in liver ($\times 10^6$ cells/g liver)	139
Liver weight (g)	1660
Body weight (kg)	70
Liver blood flow (mL/min/kg)	20.7
Plasma/blood ratio	0.57

5.6. Cells for virus propagation

The cell lines studied in the experiments were grown and incubated at 37 °C and 5% CO₂ in a humidified incubator. Dulbecco's modified Eagle medium (DMEM; Gibco), which contained the different supplements (10% fetal calf serum (FCS), 5% glutamine, and 5% penicillin/streptomycin) was used for the maintenance of MDCK-II cells. Calu-3 cells (ATCC number HTB55) were grown in DMEM/F-12 Ham (1:1) (Gibco), and also underwent supplement treatment (10% FCS, glutamine, and penicillin/streptomycin). Every 2 to 3 days, fresh culture medium was provided as replenishment. H9N2 IAV strain A/quail/Shantou/782/00 and H1N1 strain A/Hamburg/5/09 underwent cultivation in MDCK-II cells treated with infection medium (DMEM supplemented with 0.1% bovine serum albumin (Sigma-Aldrich), 5% glutamine, and 5% penicillin/

streptomycin), which contained 1 µg/mL tosyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma-Aldrich). Low-speed centrifugation was used to clear cell supernatants from cell debris, and the supernatants were stored at a temperature of -80 °C.

5.7. Antiviral assay and analysis of multicyclic replication of influenza viruses

MDCK-II cells were seeded in 24-well plates and cultivated until attainment of >95 % confluence. Cells were subsequently washed with phosphate-buffered saline (PBS), and they then underwent infection with H9N2 at a multiplicity of infection (MOI) of 0.01 for 1 hour at a temperature of 37 °C. Treating the cells with the protease inhibitors was done by removing the inoculum and washing the cells with PBS again. To cover the cells, fresh infection medium containing or not containing (control) protease inhibitors (20 and 50 µM as final concentrations) was used, and DMSO (≤5% v/v) was also used as a vehicle control. Cultures then underwent incubation at 37 °C and 5 % CO₂. 24 hours after infection, viral spread was analyzed by fixing cells with paraformaldehyde and then immunostaining them against the viral nucleoprotein through the use of a monoclonal mouse anti-nucleoprotein antibody (Abcam), peroxidase-conjugated secondary antibodies, and TrueBlue Peroxidase substrate (KPL), a method described in previous publications [11,34]. Immunostained cells were visualized using the ChemiDoc XRS+ system and the Image Lab software (Bio-Rad). To analyze the kinetics of virus replication, samples were taken from the supernatant and stored at -20 °C at 24 and 48 hours p.i., and subsequent virus titration was completed using a plaque assay on MDCK-II cells with Avicel overlay as described in previous publications [15,35]. To study the kinetics of H1N1pdm virus multicycle replication, Calu-3 cells were seeded in 24-well plates and grown until near-confluence was reached. H1N1 was inoculated into the cell culture at a MOI of 0.001 for 1 hour and then washed with PBS. The culture was then incubated in infection medium that either did or did not contain protease inhibitors at 20 and 50 µM or DMSO for 48 hours. Supernatants were collected at 24 and

48 hours p.i., and a plaque assay in MDCK-II cells was used to determine viral titers.

5.8. Cell viability assay

The CellTiterGlo® luminescent cell viability assay (Promega) measures cellular ATP content to assess cell viability. MDCK-II cells cultivated in 96-well plates were incubated in triplicates for 24 hours with 50 µM of the indicated inhibitor. Controls consisted of untreated and DMSO-treated cells. This was followed by incubation of the cells with the substrate in accordance with the manufacturer's protocol. Luminescence was measured using a 96-well plate (Nunc). The absorbance values of the inhibitor-treated cells were converted to percentages by comparing said values to those of the untreated control cells, whose cell viability was set at 100%.

5.9. Statistical analysis

R version 4.0.4 software package (Vienna, Austria, 2021) was used for confirmation of data of normal distribution and homogeneity of variances prior to the use of one-way analysis of variance (ANOVA) and Tukey's post hoc test. The results were presented as mean values $\pm$ standard error of the mean (SEM), and differences between results were considered significant if p was <0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6. Results

6.1. Inhibitors' effects on human microsomal CYP isoenzyme activity

Using liver microsomes, the protease inhibitors' effects on human CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) was examined. There was a significant ($***p < 0.001$) decrease in CYP enzyme activity following a 15-minute incubation period at 37 °C with each CYP enzyme's respective reference inhibitor (α -naphthoflavone at 30 μ M; tienilic acid at 300 μ M; (+)- N-3-benzylrivanol at 150 μ M; quinidine at 15 μ M; ketoconazole at 150 μ M). However, none of the tested protease inhibitors at 50 μ M ($p > 0.05$) demonstrated inhibition on the activities of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 enzymes (**Fig. 2**).

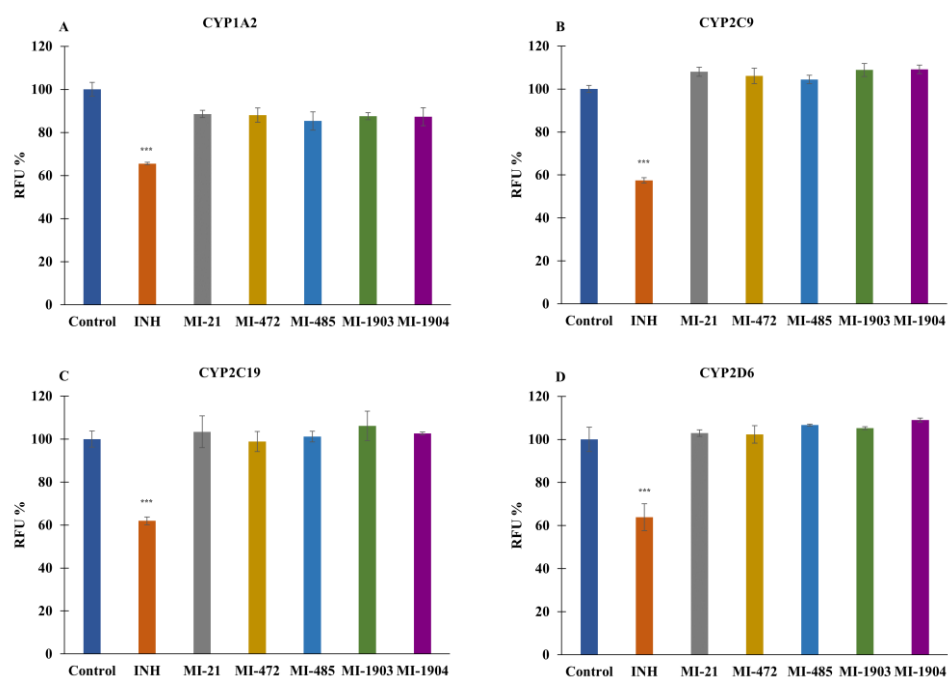


Figure 2. Inhibitory effects of protease inhibitors on CYP enzyme activities [CYP1A2 (A), CYP2C9 (B), CYP2C19 (C), and CYP2D6 (D)]. The reference inhibitors (INH) α -naphthoflavone (CYP1A2), tienilic acid (CYP2C9), (+)-N-3-benzylrivanol (CYP2C19), and quinidine (CYP2D6) (6 μ M, 60 μ M, 30 μ M, and 3 μ M, respectively) displayed significant inhibition on the activity of the CYP enzymes ($***p < 0.001$). Inhibition of each CYP isoenzyme exposed to the protease inhibitors was insignificant ($p > 0.05$). Relative fluorescence units (RFU) of the metabolites formed during the reactions (CYP1A2, CYP2C19: $\lambda_{ex/em}$ = 406/468 nm; CYP2C9: $\lambda_{ex/em}$ = 415/502 nm; CYP2D6: $\lambda_{ex/em}$ = 390/468 nm) are presented as means $\pm$ SEM. (n = 3).

On the contrary, the protease inhibitors induced significant inhibition on CYP3A4 function ($***p < 0.001$; 50 μ M MI-1904, $**p < 0.01$) at all three

concentrations (10, 25, and 50 μM), except for MI-21, which yielded no significant reduction in the activity of CYP3A4 at 10 μM , 25 μM and 50 μM concentrations ($p > 0.05$). Interestingly, MI-463 and MI-485 displayed potent inhibition of CYP3A4 even at the lowest concentration applied (**Fig. 3**).

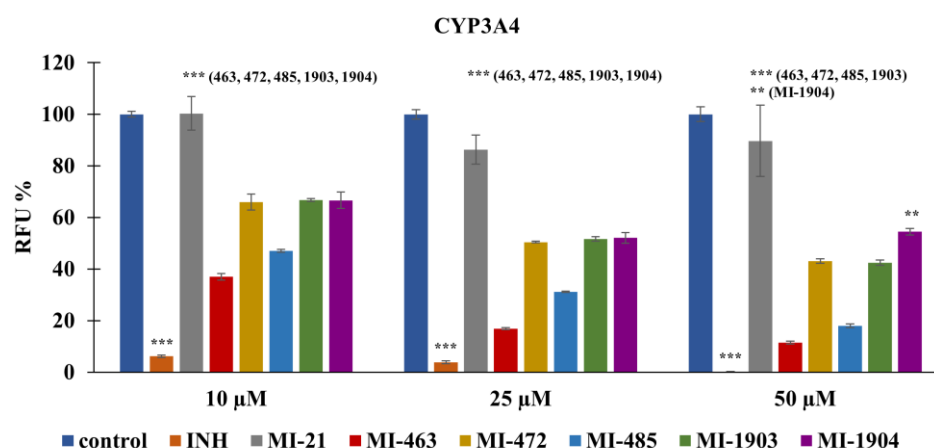


Figure 3. Effects of the protease inhibitors at concentrations of 10, 25, and 50 μM on the activity of isoenzyme CYP3A4. Reference inhibitor ketoconazole (30 μM) (INH) presented significant suppression on CYP3A4 function ($***p < 0.01$). Inhibitor MI-463 ($***p < 0.01$), MI-472 ($***p < 0.01$), MI-485 ($***p < 0.01$), MI-1903 ($***p < 0.01$), and MI-1904 (10 and 25 μM , $***p < 0.01$; 50 μM , $**p < 0.01$) each displayed significant inhibition on the activity of CYP3A4. Inhibitor MI-21 had no significant suppression on CYP3A4 activity at 10, 25 and 50 μM ($p > 0.05$). Relative fluorescence units (RFUs) of each compound ($\lambda_{\text{ex/em}} = 535/587 \text{ nm}$) are depicted as means $\pm$ SEM. ($n = 3$; MI-21, $n = 4$).

6.2. Cytotoxicity of MI-463 and MI-1900

The cell viability of PHH treated with 50 μM of protease inhibitors MI-463 and MI-1900 was assessed after a 24-hour incubation period. Compared to untreated and solvent (DMSO) controls, neither of the protease inhibitors induced any cytotoxic effects on the PHH (**Fig. 4**).

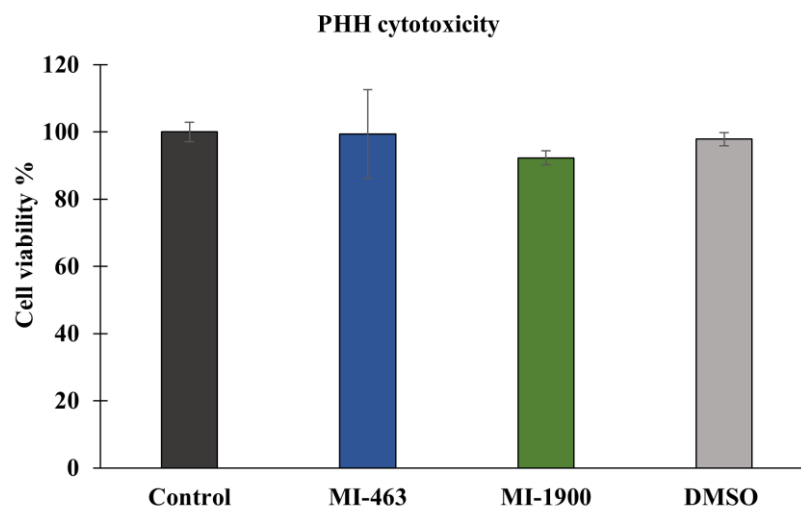


Figure 4. Cell viability of PHH after 24-hour treatment with MI-463 and MI-1900 at 50 μ M. Solvent control was DMSO at 0.5% for 24 hours. Data is represented with the mean absorbance values in comparison to an untreated control $\% \pm$ standard deviation (SD). The treated groups induced no significant differences in cell viabilities in comparison to the control groups ($p > 0.05$).

6.3. Depletion rates of the protease inhibitors

In PHH suspensions, the tested protease inhibitors displayed significant depletion rates already at the 30-minute sampling period ($p_{\text{MI-21, MI-463, MI-472, MI-485, MI-1900, MI-1903, 30 min}} < 0.001$ (**Fig. 5**). At this sampling interval, MI-21 exhibited significant biotransformation as there was a marked loss of its parent compounds in comparison to the other protease inhibitors, with the exception of MI-1903 ($p_{\text{MI-463, MI-472, MI-485, MI-1900, MI-1904, 30 min}} < 0.001$). The 60-minute and 120-minute sampling periods then showed that MI-21 was more significantly depleted than all other tested protease inhibitors ($p_{\text{MI-463, MI-472, MI-485, MI-1900, MI-1903, MI-1904, 60 and 120 min}} < 0.001$). Inhibitor MI-1903 was also more significantly depleted than the other protease inhibitors except MI-21 at the 30-minute ($p_{\text{MI-463, MI-472, MI-485, 30 min}} < 0.001$, $p_{\text{MI-1900, 30 min}} = 0.0050$, $p_{\text{MI-1904, 30 min}} < 0.001$), 60-minute ($p_{\text{MI-463, MI-472, MI-485, 60 min}} < 0.001$, $p_{\text{MI-1900, 60 min}} = 0.00251$, $p_{\text{MI-1904}} < 0.001$), and 120-minute ($p_{\text{MI-463, MI-472, MI-485, MI-1904, 120 min}} < 0.001$) sampling times. Compound MI-1900 was also extensively metabolized, being more significantly depleted than inhibitors MI-463 and MI-1904 at the 60-minute sampling interval ($p_{\text{MI-463, 60 min}} = 0.03246$, $p_{\text{MI-1904, 60 min}} = 0.00242$), and inhibitors MI-463, MI-472, MI-485,

and MI-1904 at the 120-minute sampling period ($p_{\text{MI-463, MI-472, MI-485, MI-1904, 120 min}} < 0.001$).

Inhibitor MI-21 possessed the highest rate of depletion in comparison to each of the protease inhibitors as a mere $16.6 \pm 1.6\%$ of the parent compounds were retained at the 120-minute sampling period ($p_{\text{MI-463, MI-472, MI-485, MI-1900, MI-1903, MI-1904, 120 min}} < 0.001$). Inhibitors MI-1900 and MI-1903 retained $48.6 \pm 1.5\%$ and $38.6 \pm 5.5\%$ of their parent compounds, respectively, which demonstrates that they were depleted at a significantly faster rate than compounds MI-463, MI-472, MI-485, and MI-1904 (MI-1900, MI-1903: $p_{\text{MI-463, MI-472, MI-485, MI-1904, 120 min}} < 0.001$). In comparison to the 0-min concentrations, inhibitors MI-463, MI-472, MI-485, and MI-1904 preserved the greatest amounts of their parent compounds ($66.7 \pm 5.5\%$, $65.1 \pm 5.5\%$, $64.9 \pm 5.3\%$ and $70.8 \pm 8.6\%$, respectively).

Although they share almost identical chemical structures, the simple presence of a fluorine atom in the N-terminal biphenyl side chain of MI-1904 appears to significantly affect its depletion rate in comparison to inhibitor MI-1903 ($p_{120 \text{ min}} < 0.001$). Inhibitor MI-1903 was rapidly depleted, whereas inhibitor MI-1904 was the compound possessing the lowest rate of depletion, having been significantly depleted only after 120 minutes ($p_{\text{MI-1904, 120 min}} = 0.00406$).

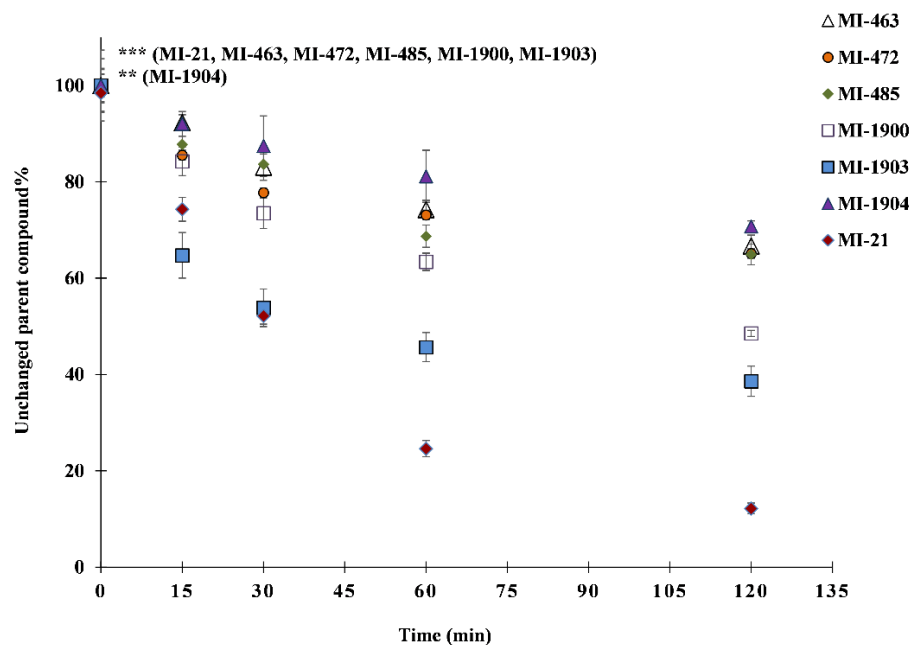


Figure 5. Depletion of the protease inhibitors in PHH until the 120-minute sampling interval. Relative percentages of the untransformed amount of the parent compounds were derived from division of the inhibitors' concentrations measured at each time point by the mean of the initial amounts of the inhibitors in the treatment solution (mean $\pm$ SEM.); $n = 3/\text{group}$. Asterisks are indicative of significance in the compounds' depletion rates at the 120-minute sampling time compared to their initial values (** $p < 0.01$, *** $p < 0.001$).

6.4. Pharmacokinetic activity of the protease inhibitors

All tested protease inhibitors possessed intermediate extraction properties based on predicted *in vivo* pharmacokinetic parameters (**Table 2**). There is, however, great variance among the *in vitro* pharmacokinetic parameters, namely half-life and intrinsic clearance. The compounds with the longest half-lives were MI-472 and MI-1904, whose values were 230.4 and 239.4 min, respectively. Compounds MI-21 (31.2 min) and MI-1903 (45.2 min) had half-lives which were notably shorter than those of the other protease inhibitors. In agreement with this, compounds MI-21 and MI-1903 displayed intrinsic clearance values of 146.56 ml/min/kg and 101.0261 ml/min/kg, respectively. These values were more than twice those of compound MI-1900, which was the inhibitor with the next highest intrinsic clearance value (42.34 ml/kg/min). Among the other parameters, there were only slight variations between the extraction ratios and bioavailability values, but compounds MI-21 and MI-1903 stood out as their extraction

ratios were the highest, and their bioavailability percentages were the lowest. Inhibitors MI-463, MI-472, and MI-1904 were the most stable compounds as they had extended half-lives, low intrinsic clearance values, and moderate extraction ratios and bioavailability percentages.

Table 2. *In vivo* PK classification of the protease inhibitors

Compound	Half-life $t_{1/2}$ (min)	CL_{int} (ml/min/kg)	Predicted CI CL_H	Extraction ratio E_H	Bioavailability F (%)	Classification (extraction type)
MI-21	31.2	146.56	10.92	0.53	47.25	Intermediate
MI-463	214.2	21.34	7.60	0.37	63.30	Intermediate
MI-472	230.4	19.83	7.40	0.36	64.26	Intermediate
MI-485	171.8	26.60	8.17	0.39	60.51	Intermediate
MI-1900	107.9	42.34	9.23	0.45	55.42	Intermediate
MI-1903	45.2	101.03	10.56	0.51	48.96	Intermediate
MI-1904	239.4	19.09	7.29	0.35	64.77	Intermediate

6.5. Inhibitory effect of protease inhibitors on matriptase-induced activation of H9N2 influenza virus

The A/quail/Shantou/782/00 strain of H9N2 influenza virus has been proven to be activated by host-cell protease matriptase in MDCK-II cells [3]. Therefore, the potential antiviral effects of protease inhibitors MI-463, MI-472, MI-1900, and MI-1903 were tested in MDCK-II cells infected by IAV H9N2. The MOI of the virus was set to 0.01, and cells were infected and then incubated with and without the inhibitors at three different concentrations (5, 20, and 50 μ M). After 24 hours, viral spread and proteolytic activation in the presence and absence of treatment with the protease inhibitors was determined by immunostaining the cells against the viral nucleoprotein (**Fig. 6A**).

Inhibitor MI-463 displayed the strongest antiviral effect among the inhibitors as it markedly reduced viral spread at 20 μ M and fully suppressed the formation of viral foci at 50 μ M. Inhibitor MI-1900 also strongly

prevented viral spread, whereas both MI-472 and MI-1903 only showed a modest reduction in the formation of viral foci.

The antiviral efficacy of inhibitor MI-463 was quantified as it presented the greatest inhibitory effect on H9N2 replication compared to the other four tested protease inhibitors. For this task, viral titers in the cell culture supernatants were determined using a plaque assay. After 24 hours and 48 hours viral titers were measured in the cell cultures treated with inhibitor MI-463 and were found to be decreased at both 20 μ M and 50 μ M significantly (**Fig. 6B**). Cell viability was unaffected by the protease inhibitors at 50 μ M (**Fig. 6C**).

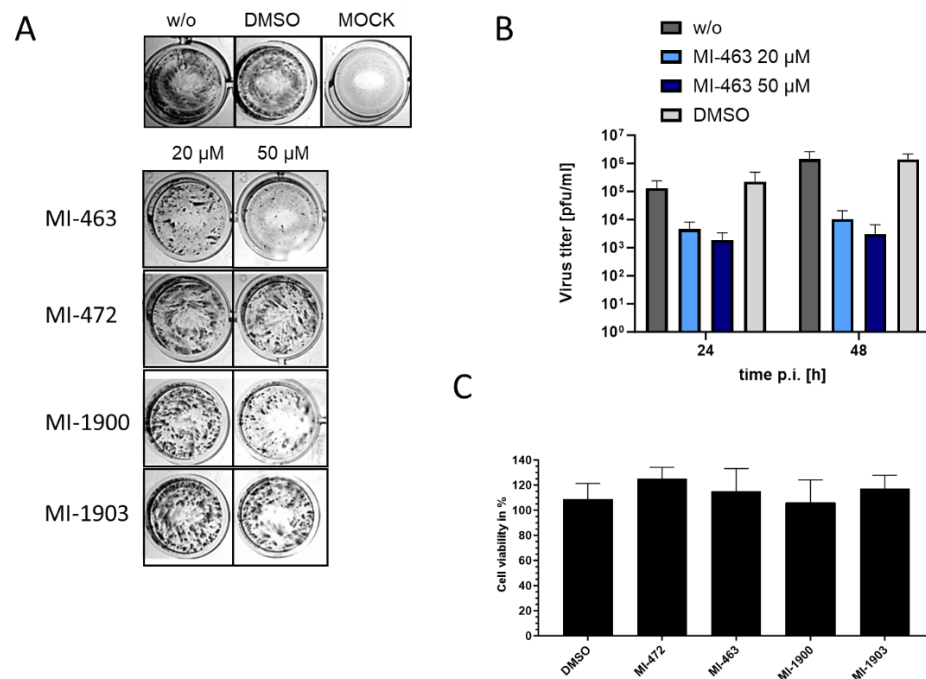


Figure 6. Inhibitory effects of the matriptase inhibitors on the multicyclic replication of H9N2 in MDCK-II cells. A) MDCK-II cells underwent infection by H9N2 IAV at an MOI of 0.01 and were incubated with the protease inhibitors for 24 hours. Controls were the cells without inhibitor treatment (w/o) and virus-free (MOCK) cells. Cells infected with the virus underwent immunostaining against the viral nucleoprotein after 24 hours. B) MDCK-II cells underwent infection with H9N2 IAV at an MOI of 0.01 and were incubated with inhibitor MI-463 for 48 hours. At 24 and 48 hours, plaque-forming units (pfu)/mL were used to determine virus titers in supernatants. Controls were cells without inhibitor treatment (w/o) and cells that received DMSO. Data are means + SD (n=4). C) Cell viability assessment after inhibitor treatment. MDCK-II cells received treatment with the indicated compound (50 μ M) for 24 hours. Controls were cells without inhibitor treatment (w/o) and cells that received DMSO. Viability of the cells without inhibitor treatment was set as 100%. Results are presented as means $\pm$ SD (n=3).

6.6. Effects of protease inhibitors on viral titers of H1N1 influenza virus

Host-cell protease TMPRSS2 has been recognized as an activator of IAV viral spread in human airway cells [2,15]. Both inhibitors MI-463 and MI-1900 were tested as they demonstrated inhibition of H9N2 viral replication in MDCK-II cells (**Fig. 6A**). The 2009 pandemic virus H1N1pdm was inoculated into human Calu-3 airway cells at a low MOI, and cells were subsequently incubated with or without inhibitors MI-463 or MI-1900 at 20 μ M and 50 μ M for 48 hours. At the 24-hour and 48-hour time points, plaque assays were carried out to determine viral titers from the collected cell supernatants. The antiviral efficacy of MI-463 against H1N1 was noteworthy as the viral titers were reduced at the different protease inhibitor concentrations at 24 and 48 hours p.i., although it had markedly lower efficacy in comparison to its effects against H9N2. Inhibitor MI-1900 demonstrated similar results to compound MI-463 while achieving a higher reduction of viral titers of H1N1 at 50 μ M after 48 hours (**Fig. 7**)

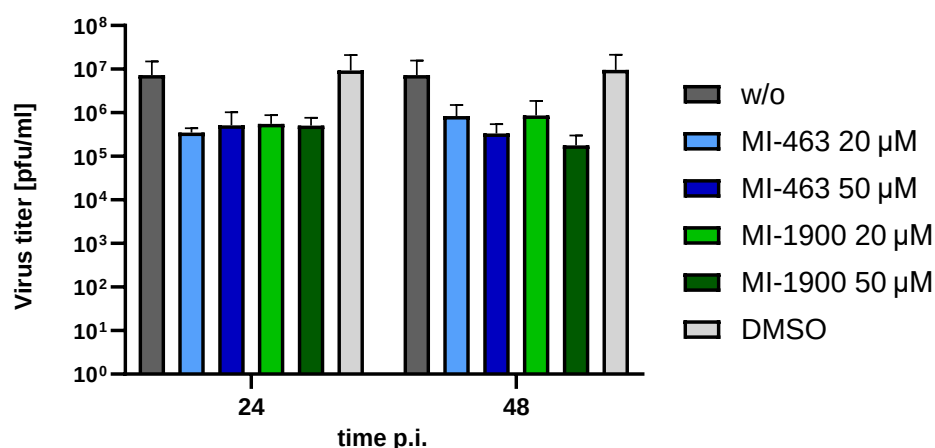


Figure 7. Inhibitory activity of protease inhibitors on H1N1pdm multicyclic replication in human airway cells. H1N1pdm virus was inoculated into Calu-3 human airway cells at an MOI of 0.001, and cells underwent incubation with the indicated compounds for 48 hours. Controls were cells without inhibitor treatment (w/o) and cells treated with DMSO. Plaque-forming units (pfu)/mL were used to determine virus titers in supernatants at 24 and 48 hours. Data are means $\pm$ SD (n=3).

7. Discussion

The potential inhibitory effects of the tested matriptase/TMPRSS2 inhibitors on activities of CYP enzymes were assessed in human liver microsomes for the evaluation of possible drug interactions. The present study is the first to investigate potential interactions between inhibitors MI-21, MI-472, MI-485, MI-1903, and MI-1904 and the drug-metabolizing CYP isoenzymes CYP1A2, CYP2C9, CYP2C19, and CYP2D6. None of the applied inhibitors demonstrated any significant inhibition on the activity of any of the four CYP isoenzymes, findings of which are similar to those observed with other Phe(3-Am) derivatives, such as MI-463 and MI-1900 [21]. However, another study produced compelling findings in which inhibitors MI-463 and MI-1900 significantly inhibited the activity of CYP1A2 derived from cynomolgus monkey and rat hepatic microsomes, yet in CYP1A2 of the beagle dog breed, no significant inhibition on enzyme activity was observed, indicating that the protease inhibitors possess species-dependent inhibition on the activity of CYP isoenzymes [36].

In the current study, inhibitors MI-463, MI-472, MI-485, MI-1903, and MI-1904 all significantly inhibited human microsomal CYP3A4 activity. On the contrary, inhibitor MI-21 was the only compound observed to lack substantial inhibitory effects on this isoenzyme. Inhibitor MI-1900 has previously demonstrated significant inhibitory effects only on CYP3A4 in studies conducted with fluorometric assays [21]. Moreover, inhibitors MI-463 and MI-485 displayed the most potent inhibition of CYP3A4 activity, which was additionally found to be concentration-dependent. These findings may be related to their chemical structures, as they both contain a dimethoxy-substituted biphenyl-3-sulfonyl group at the N-terminus, while their C-termini each contain a cyclohexyl-ureido moiety in MI-463 and a tert-butyl ureido moiety in MI-485. However, the presence of the N-terminus dimethoxy-substituted biphenyl-3-sulfonyl group did not appear to boost the inhibitory capacity of inhibitor MI-472 to the same levels of inhibitors MI-463 and MI-485, indicating that the inhibition rates of the individual compounds are likely linked to the presence of other structural moieties. In the case of MI-472, the absence of a ureido-derived entity at

the C-terminus appears to have diminished its inhibitory capacity in comparison to the MI-463 and MI-485 as this is its main structural difference. Moreover, the inhibition rate of inhibitor MI-1903 was similar to that of inhibitor MI-472, even though MI-1903 possesses the same C-terminus tert-butyl ureido moiety as inhibitor MI-485, but differs in that its N-terminus contains a hydroxy-methyl-biphenyl-3-sulfonyl group instead of a dimethoxy-substituted biphenyl-3-sulfonyl group, again indicating that different structural moieties in combination with one another yield different inhibitory capacities on CYP3A4. Our findings suggest that the concomitant possession of both an N-terminus dimethoxy-substituted biphenyl-3-sulfonyl group and a C-terminus ureido-derived structural group result in an increased capacity to inhibit the CYP3A4 isoenzyme.

The absence of either a dimethoxy-substituted- or hydroxy-methyl-biphenyl-3-sulfonyl group on the N-terminus and that of a ureido-based functional group on the C-terminus could explain the lack of inhibition on the activity of CYP3A4 by inhibitor MI-21, which contains two primary amines at both its N- and C-termini. The less efficient inhibition on CYP3A4 presented by inhibitor MI-1904 at 50 μ M may be attributed to the single fluorine atom which is the only structural characteristic that separates it from the more potent CYP3A4 inhibitor MI-1903, which inhibited CYP3A4 activities at a similar rate to inhibitor MI-472.

Prediction of *in vivo* PK parameters serves as a useful tool in the early, preclinical stages of drug discovery in order to characterize drugs for selection into later phases [37]. In our study, each of the evaluated inhibitors were characterized as intermediate extraction drugs. However, inhibitor MI-21 stood out with the shortest half-life (31.2 min) and the highest intrinsic clearance values (146.56 mL/min/kg). This feature may be explained by the structural differences that distinguish it from the other protease inhibitors applied in the study. Previously, inhibitor MI-21 stood out as a promising drug candidate due to negligible cell toxicity, lack of plasma-protein binding (e.g. HSA), absence of inhibitory effects on CYP3A4 activity, high affinity towards matriptase and TMPRSS2 (K_i = 5 nM and 10 nM, respectively), and even anti-H9N2 effects in Calu-3 cells

[3,20]. However, an Achilles' heel of this compound may be its potential lack of oral bioavailability due to its multibasic character, although synthesis of similarly structured analogues may yield compelling results in this inhibitor's journey through the process of drug discovery [38].

Following MI-21, inhibitor MI-1903 had the second shortest half-life (45.2 min) and the second longest intrinsic clearance values (101.03 ml/min/kg), although it possesses more structural similarities to the other protease inhibitors, especially MI-1904, rather than inhibitor MI-21. A quite compelling finding is that MI-1900 exhibited the third shortest half-life (107.9 min) at more than two times the value of MI-1903, and the third highest intrinsic clearance value (42.34 ml/min/kg) at less than half that of MI-1903, demonstrating a great gap in these values between both MI-21 and MI-1900 and the other inhibitors, despite the lack of structural similarities between the two compounds. The other PK parameters for all inhibitors expressed values that were in similar ranges without drastic differences (CL_H : 7.29-10.92; E_H : 0.35-0.53; F : 47.25-64.77%).

In this study using human hepatocytes, inhibitor MI-21 exhibited the most rapid depletion in comparison to the other protease inhibitors. This finding correlates well to what was observed in the predicted *in vivo* PK parameters. Inhibitors MI-1900 and MI-1903 also demonstrated a significantly faster depletion rate in comparison to the other tested compounds. Although this characteristic of the two inhibitors may potentially be linked to the ureido-derived group at their C-termini, this trait was not observed neither in inhibitor MI-485 nor in inhibitor MI-1904, both of which possess a C-terminus ureido group and exhibited a delayed rate of depletion. Based on these findings, one may deduce that other functional groups in each of the applied compounds play a pivotal role in each of their respective depletion rates.

Inhibitors MI-1903 and MI-1904 possess nearly identical structures, but a single fluorine substituent in the latter separates the two. Taking into consideration the lack of other structural differences between the two compounds, the findings of this depletion study suggest that the fluorine

atom of MI-1904 may provide benefits to the pharmacokinetics of inhibitor MI-1904. Increased efficacy, stability, and absorbability and a longer half-life are all characteristics known to be possessed by fluorinated compounds in comparison to their nonfluorinated counterparts. These features have made the incorporation fluorine atoms or fluorine-containing functional groups into organic compounds a commonly recurring procedure in the process of drug discovery [39–42].

The expression of CYP enzymes and the drug intake of hepatocytes in the hepatic metabolism of cynomolgus monkeys have a strong resemblance to those of humans, making cynomolgus monkeys great animal models for the prediction of *in vivo* hepatic clearance in humans [43–45]. In our depletion study in human hepatocytes, the biotransformation of the inhibitors was seemingly more substantial when compared with previously produced measurements of Phe(3-Am) derivatives in cynomolgus monkey hepatocytes. In hepatocytes derived from the cynomolgus monkey, inhibitors MI-463, MI-472, MI-485, and MI-1900 had all undergone significant biotransformation after the 60-120 min timepoints, whereas in human hepatocytes their depletion was significantly accelerated already within 30 min of treatment. Regardless of these variations, it remains clear that the depletion of these protease inhibitors in each of human and cynomolgus monkey hepatocytes are quite similar. Another compelling finding was that inhibitor MI-1900 exhibited a faster depletion rate than most of the other compounds in both human and cynomolgus monkey hepatocytes, whereas inhibitors MI-463, MI-472, and MI-485 were all depleted at a slower rate in cynomolgus monkey hepatocytes. On the contrary, less similarities related to the depletion rates of the compounds were discovered between human, beagle dog, and rat hepatocytes since the expression of hepatic CYP enzymes and the hepatic drug uptake in these species possess less similarities in comparison to those of human hepatocytes [17].

Previous studies have demonstrated the capacity of Phe(3-Am)-derived protease inhibitors to produce antiviral effects against IAVs and SARS-CoV-2. In Calu-3 cells, the proteolytic activation and replication of IAV

strains H1N1 and H3N2 were strongly inhibited by compound MI-432 [46]. Additionally, the multicyclic replication of SARS-CoV-2 underwent a dose-dependent inhibition by both compounds MI-432 and MI-1900 in Calu-3 cells. A significant reduction of viral titers was observed at a concentration of 50 μ M, as there was a 75-fold and a 35 to 280-fold decrease in the viral titers at 24 hours p.i. for each of inhibitors MI-432 and MI-1900, respectively, as compared to the control samples and without any significant effect on the viability of the Calu-3 cells [47]. A derivative of inhibitor MI-21, which was subject to the present study, has also demonstrated antiviral efficacy as it impeded the replication of H1N1 PR8 in Calu-3 cells [48]. Furthermore, an evaluation of the antiviral efficacy of a series of matriptase inhibitors against IAV strain H9N2 was conducted in MDCK-II cells. The series, which included inhibitors MI-485, MI-1900, and MI-1904, displayed a concentration-dependent effect on the replication of the virus, demonstrating robust inhibition at concentrations of 20 μ M and 50 μ M. Inhibitors MI-485 and MI-1904 presented the most pronounced antiviral effects and concurrently showed no cell cytotoxicity [49].

In this study, inhibitor MI-463 was proven to have antiviral activity against two IAV strains, H1N1pdm and H9N2 A/quail/Shantou/782/00, in Calu-3 and MDCK-II cells, respectively. However, MI-463 suppressed the replication of H1N1pdm less efficiently, than it did for H9N2. This may be due to the variations in expression of host-cell proteases matriptase and TMPRSS2 in the two different cell lines as well as different uptake of the inhibitors by the cells, despite the high affinity MI-463 possesses towards matriptase ($K_i = 0.004 \mu$ M) and TMPRSS2 ($K_i = 0.003 \mu$ M). Inhibitor MI-1900, which also has low K_i values for matriptase (0.038 μ M) and TMPRSS2 (0.079 μ M), also demonstrated significant antiviral activity against H1N1pdm [49].

The *in vitro* findings of these Phe(3-Am)-derived protease inhibitors shed light on their potential to reduce the replication and spread of SARS-CoV-2 and several IAVs in Calu-3 and MDCK-II cell lines. Despite the limitations of *in vitro* studies, they remain essential in the process of drug discovery as preliminary methods to assess the toxicity and antimicrobial and interactive

effects of prospective drugs. The full potential of these inhibitors is yet to be unveiled, as they not only can be utilized as antiviral drugs, but they may be repositioned for antibacterial effects as well [50]. Based on the current literature in the early stages of drug discovery for these compounds, it is evident that they provide yet another intriguing area to continue pharmaceutical research.

8. Abstract

It has been well established that various corona- and influenza viruses undergo activation by type II transmembrane serine proteases for entry into host cells, making these host-cell proteases attractive pharmacological targets for the treatment of respiratory infections caused by these viruses. Additionally, due to the shortage of other effective pharmaceutical compounds, virus mutations, and the looming threat of future airborne pandemics, novel drugs for the treatment of viral infections are imperative for public health. In this study, seven different 3-amidinophenylalanine-based inhibitors (MI-21, MI-463, MI-472, MI-485, MI-1903, and MI-1904) of host-cell proteases matriptase and TMPRSS2 were investigated. The cytotoxic effects and inhibitory capacity of the protease inhibitors on cytochrome P450 (CYP) enzymes were evaluated with fluorometric assays. Metabolic depletion rates of each inhibitor were determined over a 120-min period by liquid chromatography-tandem mass spectrometry in primary human hepatocytes, and various pharmacokinetic (PK) parameters, including half-life, intrinsic clearance, hepatic extraction ratio, and bioavailability, were calculated. Furthermore, the antiviral efficacy of selected protease inhibitors against influenza A virus subtypes H1N1 and H9N2 was evaluated *in vitro* as well. Except for MI-21, all other inhibitors significantly inhibited CYP3A4, and none of the compounds showed inhibitory activity on enzymes CYP1A2, CYP2C9, CYP2C19, and CYP2D6. Analysis of calculated PK parameters revealed that inhibitors MI-463, MI-472, MI-485, MI-1900, and MI-1904 boasted better stability than MI-21 and MI-1903, and these results were complimented by the similar findings of the depletion study, in which MI-21 and MI-1903 retained the lowest % of their parent compounds after 120 min. After a 24-hour incubation period, inhibitors MI-463 and MI-1900 displayed significant anti-H1N1 properties and MI-463 also showed robust antiviral effects against H9N2 at 20 and 50 μ M. Our findings suggest that MI-463 and MI-1900 can be used to prevent entry of these viruses into cells by suppression of host matriptase/TMPRSS2.

9. Hungarian Abstract

A korona- és influenzavírusok terjedésének egyik feltétele a kettes típusú transzmembrán szerin proteázok hatékony működése, így ezek az enzimek jelentős farmakológiai célpontok lehetnek a virális légúti fertőzések kezelésében. A vírusmutációk és az egyre gyorsabban kialakuló járványok miatt új antivirális gyógyszerek kifejlesztése kiemelkedően fontos közegészségügyi szempontból is.

Kutatásunkban hét különböző, 3- amidinofenilalanin típusú matriptáz /TMPRSS2 inhibitort (MI- 21, MI- 463, MI- 472, MI- 485, MI- 1903, és MI- 1904) vizsgáltunk. A vegyületek citotoxicitását lumineszcenciás és a citokróm P450 (CYP) izoenzimekre gyakorolt hatását fluorimetriás vizsgálatokkal mértük meg. Az egyes gátlók szerek metabolikus fogási arányát 120 perces időtartam alatt folyadékkromatográfiával- tandem tömegspektrometriával követtük nyomon primer humán hepatocitákban, és a mérési adatok alapján különböző farmakokinetikai (PK) paramétereket, mint a felezési időt, a máj extrakciós arányt és a biohasznosulást határoztuk meg. Ezenkívül *in vitro* jellemeztük a kiválasztott enzimgátlók antivirális hatását az influenza A vírus H1N1 és H9N2 altípusok ellen.

Eredményeink szerint az MI- 21 kivételével az összes többi inhibitor jelentősen gátolta a CYP3A4 izoenzimet, de egyik vizsgált vegyület sem mutatott gátló hatást a CYP1A2, CYP2C9, CYP2C19 és CYP2D6 aktivitásra. A farmakokinetikai paraméterek elemzése alapján az MI- 463, az MI- 472, az MI- 485, az MI- 1900 és az MI- 1904 vegyületek jobb stabilitással rendelkeztek, mint az MI- 21 és az MI- 1903 gátlók szerek, amelyek 120 percet követően jelentős mértékben bomlottak. A 24 órás inkubációs időszak után az MI- 463 és az MI- 1900 inhibitorok szignifikáns H1N1 ellenes hatást mutattak, valamint az MI- 463 H9N2 vírusfertőzés esetén is hatékonynak bizonyult 20 és 50 μ M koncentrációkban.

Összefoglalva, az MI-463 és az MI-1900 vegyületek hatékony vegyületek egyes influenza vírusok sejtekbe való belépésének megakadályozására a gazdasejt matriptáz/TMPRSS2-aktivitás számottevő gátlásának köszönhetően.

10. References

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