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TDK thesis

**Repositioning of matriptase inhibitors against *Streptococcus suis*
infection**

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1. List of abbreviations

ACN: Acetonitrile

AMEG: Antimicrobial advice ad hoc expert group

ANOVA: Analysis of variance

B. cepacia: *Burkholderia cepacia*

Caco-2: Human colorectal adenocarcinoma cells

CAMHB: Cation-adjusted Mueller–Hinton broth

CFU: Colony-forming unit

CPS: Capsular polysaccharides

CYP: Cytochrome P450 isoenzyme

DCF: Dichloro-fluorescein

DCFH-DA: 2'-7'-dichlorodihydrofluorescein diacetate

DME: Drug metabolising enzymes

DMEM: Dulbecco's modified Eagle's medium

DMEM/F12: Dulbecco's modified Eagle's medium and Ham's F-12
Nutrient

DMSO: Dimethyl sulfoxide

EC: Extracellular

ELISA: Enzyme-linked immunosorbent assay

EGF: Epidermal growth factor

E. coli: *Escherichia coli*

E. faecalis: *Enterococcus faecalis*

HA: Influenza hemagglutinin

HepG2: Hepatoblastoma cell line

HT-29: Human colon cancer cell line

H₂O₂: Hydrogen peroxide

IC: Intracellular

IL: Interleukin

IPEC-J2: Intestinal porcine jejunal enterocytes

INH: Inhibitor

MERS-CoV: Middle East respiratory syndrome coronavirus

MI: Matriptase inhibitor

MIC: Minimum inhibitory concentration

NAD⁺: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NR: Neutral red
Phe(3-Am): 3-amidinophenylalanine
P. aeruginosa: *Pseudomonas aeruginosa*
P. multocida: *Pasteurella multocida*
ROS: Reactive oxygen species
RFU: Mean relative fluorescence intensities
S: Spike protein
SARS-CoV: Severe acute respiratory syndrome coronavirus
SD: Standard deviation
SEM: Standard errors of mean
S. aureus: *Staphylococcus aureus*
S. enterica: *Salmonella enterica*
S. suis: *Streptococcus suis*
TGN: Trans-Golgi network
TMPRSS2: Transmembrane serine protease 2
TNF- α : Tumour necrosis factor alpha
TSB: Tryptic soy broth
T84: Human epithelial cell line

2. Introduction

In industrial pig farming, piglets are most susceptible to environmental pathogens during the post weaning period due to their weakened immune systems [1]. Among these environmental pathogens, *Streptococcus suis* (*S. suis*) is one of the most detrimental bacterial species harmful to swine health, resulting in extensive economic loss globally [2-3]. Moreover, *S. suis* is a major public health concern in many countries due to its zoonotic potential. This encapsulated bacterium possesses a high degree of invasiveness. *S. suis* can enter the bloodstream through the hosts mucosal barriers, infiltrating multiple organs including the lungs, heart, spleen, and liver. In healthy pigs, *S. suis* resides in the nasopharynx and tonsils of the upper respiratory tract [4].

Based on capsular polysaccharide (CPS) antigens, 35 serotypes of *S. suis* have been identified [2,5]. Among the identified serotypes, type 2 is most commonly isolated from both diseased humans and pigs [6]. Both serotypes 2 and 9 harbour different CPS with regards to the primary structure, which contributes to the adhesion of bacterial cells to intestinal epithelial cells-jejunum 2 (IPEC-J2) and human colorectal adenocarcinoma cells (Caco-2). Moreover, serotype 9 showed higher affinity to porcine IPEC-J2 cells, whereas serotype 2 demonstrated higher adhesion to human Caco-2 cells [7]. *S. suis* infections are often treated with antimicrobial agents, however resistant strains can emerge due to improper use of these agents. Broad spectrum beta-lactams are often used to eliminate *S. suis* infections. Nevertheless, in some studies, varying degrees of resistance have been found against these antimicrobials, up to 0-25%. Furthermore, in many countries, *S. suis* has shown relatively high resistance to tetracyclines, macrolides, sulphonamides, and macrolides [8]. *S. suis* has developed several mechanisms of resistance to modulate antibiotic activity, including preventing antibiotics to bind to its target through target mutation, enzyme target alteration, antibiotic modification, and removal of antibiotics out of the cell by intensified efflux pump production [9]. Therefore, there is growing demand to replace the use of antimicrobial treatment with alternative methods.

3-amidinophenylalanine (Phe(3-Am))-derived inhibitors potentially produce antiviral effects through the inhibition of host-cell proteases such as matriptase and transmembrane serine protease 2 (TMPRSS2), which are crucial in the activation and initiation of host-cell entry of certain influenza and coronaviruses. These host proteases are located in the trans-Golgi network (TGN) and/or on the surfaces of host cells and proteolytically cleave haemagglutinin precursor (HA0) of influenza viruses H1N1 and H7N9 as well as the spike (S) protein of Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 [10-16]. Anti-SARS-CoV-2 effects of the Phe(3-Am)-derived matriptase/TMPRSS2 inhibitors MI-432 and MI-1900 have already been demonstrated in human airway cells in synergism with host cell furin inhibitor MI-1851 [14]. In the present study, the minimum inhibitory concentration (MIC) of ten Phe(3-Am)-derived inhibitors were evaluated against eight bacterial strains of *S. suis*. For the assessment of potential interactions of the inhibitors with liver microsomal cytochrome P450 (CYP) enzymes, alterations in the activities of isoenzymes CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 in the presence of the protease inhibitors were measured with fluorometric assays. Cytotoxicity of *S. suis* in the presence and absence of the proteases inhibitors with antibacterial efficacy were evaluated with cell viability measurements of IPEC-J2 cells and primary porcine hepatocytes. Furthermore, extracellular (EC) hydrogen peroxide (H₂O₂) was measured following 24 h exposure of IPEC-J2 cells or swine hepatocytes to *S. suis* alone or in the presence of inhibitors. For the detection of *S. suis*-induced inflammation, levels of the proinflammatory cytokine interleukin (IL)-6 were measured in the presence of the protease inhibitors.

3. Literature overview

3.1 Evaluation of IPEC-J2 cells and pig hepatocytes

The IPEC-J2 cell line is a non-tumorigenic and non-transforming, derived from intestinal enterocytes isolated from the jejunum of neonatal piglets before the intake of colostrum milk [17]. These cells can produce mucin, release cytokines and exhibit Toll-like receptors similar to the original tissue. It maintains its epithelial characteristics and can be utilised as an effective model to replicate innate immune function of the intestinal epithelium. [18]. IPEC-J2 cells have been proven as a valuable model in offering a comprehensive understanding of the host-pathogen interaction by characterising cell interactions between epithelial cells and enteric bacteria [19]. In addition, it can simulate the human gut physiology due to the close resemblance between the pig and human intestines [20].

The liver is an important organ to investigate for drug distribution, especially with regards to CYP activity. CYP450 may be the most critical enzyme in hepatocytes, and it is observed that minipig hepatocytes closely resemble human hepatocytes [21-22]. Additionally, minor differences were found in the activity of enzymes CYP1A2, CYP2A6, CYP2C9, CYP2E1 and CYP3A4 compared to humans whereas medium variations were seen with enzymes CYP2C19 and CYP2D19 [23].

3.2 Examination of extracellular oxidative stress

Oxidative stress is a result of an imbalance between superoxides and antioxidants which leads to the production of reactive oxygen species (ROS) [24]. ROS molecules are produced by the epithelial cells, macrophages and granulocytes which are cells of the innate immune system responsible for protecting the immune system against pathogens [25]. The most stable ROS molecule is H_2O_2 and plays a crucial role in regulation of protein function [26]. The production and elimination of H_2O_2 is balanced under healthy conditions, however in response to oxidative stress, H_2O_2 is excessively

produced [27]. As a result, the superoxide ion ($O_2^{\cdot-}$) is also over secreted. Superoxide is highly reactive and therefore severely toxic [28]. Ultimately, disruption in ROS regulation can trigger apoptosis in hepatocytes [29].

3.3 Production of pro-inflammatory cytokine IL-6 expression

Interleukin-6 (IL-6) is a cytokine which regulates the development and function of the immune system through responding to inflammation and infections. Cytokines of the IL-6 family have proven to exhibit multiple functions such as activation of hepatic acute phase proteins and B cell stimulation. Moreover, IL-6 plays an important role in liver regeneration. IL-6 not only activates the immune system, but also has anti-inflammatory responses as well as regulating metabolic processes [30-31]. Previous studies have demonstrated that *S. suis* induces IL-6 in whole blood systems, indicating infections caused by *S. suis* in the blood leads to increased IL-6 production [32].

3.4 Characterization of CYP isoenzymes

CYP superfamilies of human and porcine origin are categorised in 18 families based on similarities in their amino acid sequence, where CYP families 1, 2 and 3 are related to xenobiotic metabolism [33]. The most important drug metabolising enzymes (DME) in the human liver are CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A7 [34]. In humans, CYP450 enzymes are often found in the mitochondria or the membranes of the endoplasmic reticulum and are responsible for the detoxification process typically carried out in two phases, where phase 1 metabolism is regulated by CYP450 [35]. In mammals, CYP450 enzymes are present in various tissues including the liver, kidney, brain, intestine, and gonads. The highest expression for CYP450 enzymes is mostly in the liver [36]. Up to 50% of prescribed medications are metabolised by the CYP 2 family [37].

4. Aim of the study

S. suis negatively affects the physiological function of the intestines and the liver of pigs and has become resistant to certain antimicrobial medications. Therefore, investigating the damaging effects of this bacteria in porcine IPEC-J2 cells and pig hepatocytes is of fundamental importance to develop alternative solutions in the treatment of enterohepatic infections. Our main goal was to investigate the antibacterial efficacy of protease inhibitors on *S. suis* in porcine IPEC-J2 cells and hepatocytes. In our study we aimed to conduct the following experiments:

- The drug interaction potential of the protease inhibitors and changes in activities of liver microsomal cytochrome P 450 isoenzymes such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were measured.
- Markers of oxidative stress such as EC H₂O₂ production and IC ROS status were measured after 24 h exposure of IPEC-J2 cells and hepatocytes to *S. suis* alone or in combination with the protease inhibitors.
- Additionally, changes in pro-inflammatory cytokine production such as IL-6 were measured after 24 h exposure of IPEC-J2 cells and hepatocytes to *S. suis* alone or in combination with the protease inhibitors.

There have been some studies on the efficiency of protease inhibitors on bacterial infections, however little is known about the effects of protease inhibitors on improving *S. suis* infections. This *in vitro* study was created to predict the attributes of protease inhibitors to alleviate intestinal and hepatic damage produced by *S. suis* infections.

5. Materials and methods

5.1 Chemicals

All chemicals, including dimethyl sulfoxide (DMSO), IL-6 porcine enzyme-linked immunosorbent assay (ELISA), Neutral Red (NR) dye and an Amplex red hydrogen peroxide assay kit were purchased from Merck (Darmstadt, Germany). Mueller-Hinton liquid broth was acquired from Biolab Ltd (Budapest, Hungary). The Phe(3-Am) derivatives were prepared at the Faculty of Pharmacy, Institute of Pharmaceutical Chemistry, Philipps, University Marburg, Germany.

5.2 Preparation of inhibitor solutions for IPEC-J2, microsomal and hepatocyte assays

The chemical structures of the protease inhibitors MI-432, MI-471 and MI-476 are shown in **Figure 2**. A stock solution of 10 mM in DMSO was prepared and stored at -20 °C. Prior to each study, freshly made working solutions were prepared for the inhibitors. After incubation of the hepatocytes, microsomes and IPEC-J2 cells with inhibitors at 37 °C in a humidified atmosphere of 5% CO₂, the solutions underwent additional procedures including fluorometric (CYP assays, Amplex red method) and spectrophotometric (NR, IL-6 ELISA) assays.

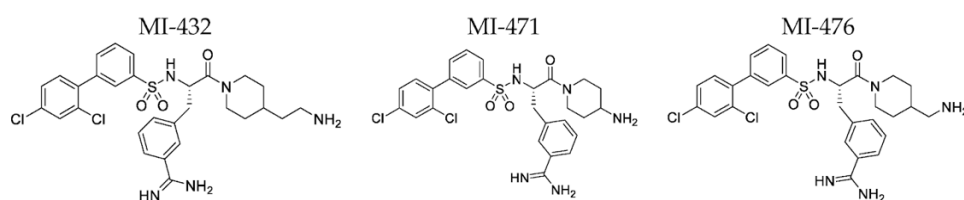


Figure 2. Chemical structures of inhibitors MI-432, MI-471 and MI-476

5.3 MIC measurements

The bacterial strains examined in this study included: *S. suis* serotype 2 (ATCC 958242) *Staphylococcus (S.) aureus* (ATCC 10832), *Pseudomonas (P.) aeruginosa* (ATCC 27853), *Pasteurella (P.) multocida* (clinical isolate), *Escherichia (E.) coli* (ATCC 11775), *Burkholderia (B.) cepacia* (clinical isolate), *Salmonella (S.) enterica* (clinical isolate), and *Enterococcus (E.) faecalis* (clinical isolate). Of the strains used in this study, *P. multocida* is derived from porcine origin, *B. cepacia* from human origin, and *S. enterica* and *E. faecalis* are from poultry origin. The bacterial strains were preserved at a temperature of -80°C in the Microbiology Laboratory of the University of Veterinary Medicine, Budapest. One day prior to the tests, bacterial strains were administered in 5 mL of cation-adjusted Mueller–Hinton broth (CAMHB) and incubated at 37°C for 18-24 h. For the MIC assay, the starting inoculum concentration was set at $5 \times 10^5/\text{mL}$. All wells other than the first column of the working plates were loaded with 90 μL CAMHB. After which a stock solution of inhibitors at 800 μM was prepared, and 180 μL of this solution was transferred into the first column of the plates to create a series of two-fold dilutions across the 96-well plates. The tips together with 90 μL of the surplus solution were disposed of to ensure that all the columns contained 90 μL of the solution. A single row was used on the working plates to test each bacterial strain. A 25x diluted bacterial suspension was prepared for bacterial inoculation on an auxiliary plate by adding 240 μL of tryptic soy broth (TSB) per column of plates and adding 10–10 μL of bacterial strain suspension per well. Subsequently, the isolated bacterial strains were added onto the plates. Starting from the 11th column (positive control) of the plates containing dilution row 2, 10 μL of bacterial suspension was added to each well, working backward. The 11th column contained only bacterial suspension and broth whereas column 12 contained only the broth serving as the negative control. The microplates were then incubated for 18-24 h at 37°C and the MIC values were evaluated relative to the positive controls.

5.4 IPEC-J2 cell culture and cytotoxicity assay

IPEC-J2 cells obtained from the intestinal enterocytes isolated from the jejunum of unsuckled pigs were used for this experiment. The IPEC-J2 cells were kindly offered by Dr. Jody Gookin (Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA). The cells were cultured in a medium containing Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 Nutrient (1:1, DMEM/F12), supplemented with 1% insulin/selenium/transferrin, 5 ng/mL epidermal growth factor (EGF), 5% foetal bovine serum, and 1% penicillin–streptomycin. The cells were passaged around 50 times. IPEC-J2 cells were incorporated on 96-well plates using the 2 h lasting NR method in order to measure the potential cytotoxic effects of *S. suis* (10^4 CFU/mL), inhibitors (at 50 μ M), and their combinations. DMEM/F12 medium was used as control in this experiment. After 24 h treatments, the cell viability ratio was measured by absorbance at 540 nm wavelength with a SpectraMax iD3 microplate reader.

5.5 Cytotoxicity assays in porcine hepatocytes

Porcine cryopreserved primary hepatocytes were supplied by Primacyt Cell Culture Company (Schwerin, Germany). The plating, thawing and maintenance media were supplied by Lonza Group Ltd. (Biocenter Ltd., Szeged, Hungary). The NR method was used to determine the cytotoxic effects of inhibitors MI-432, MI-471, and MI-476 which were tested on the viability of primary hepatocytes. The hepatocytes were propagated for 24 h on a 96-well plate followed by incubation with the inhibitors at 0, 50, and 100 μ M for an additional 24 h. For the incubation of the control cells, only maintenance medium was used. Together with NR dye the plate underwent incubation for 2 h. Hepatocyte viability was measured at 540 nm with a SpectraMax iD3 microplate reader.

5.6 CYP enzyme fluorometric activity measurements

The Biovision CYP assays (BioVision, Inc., Kampenhout, Belgium) employ non-fluorescent CYP1A2, 2C9, 2C19, 2D6, or 3A4 substrates able to undergo transformation into highly fluorescent detectable metabolites. In these experiments, positive controls such as quinidine (CYP2D6, 3 μ M), tienilic acid (CYP2C9, 60 μ M), (+)-N-3-benzylnirvanol (CYP2C19, 30 μ M), α -naphthoflavone (CYP1A2, 6 μ M), and ketoconazole (CYP3A4, 5 μ M) were used. Human hepatic microsomal supernatants were produced (Gibco, Biocentre, Szeged, Hungary protein concentration: 20 mg/mL) separately by combining 30 μ L with 2425 μ L assay buffer and with 50 μ L nicotinamide adenine dinucleotide phosphate (NADPH) generating system (100 $\times$). 20 μ L of protease inhibitor solutions (250 μ M for all CYP enzymes except CYP3A4, where 50, 125, and 250 μ M have been used) were introduced to 50 μ L aliquots of microsomal suspensions. Control measurements were conducted without the addition of inhibitors. In negative controls, 20 μ L assay buffer and in positive controls, the reference inhibitors (each 20 μ L; α -naphthoflavone at 30 μ M, tienilic acid at 300 μ M; (+)- N-3-benzylnirvanol at 150 μ M; ketoconazole at 150 μ M, quinidine at 15 μ M) were supplemented to the 50 μ L buffer containing microsomes. 70 μ L of assay buffer without microsomes, combined with test compounds were used in background controls. Following incubation at 37 $^{\circ}$ C for 15 min, 30 μ L of appropriate CYP substrate/NADP $^{+}$ mixture was added into each well, resulting in a final volume of 100 μ L/well. A bicinchoninic acid protein assay kit (Pierce BCA kit, Thermo Fisher Scientific, Waltham, MA, USA) was used to measure microsomal protein content. The microsomal protein concentration was calibrated to 100 μ g/well in each assay. The fluorescence intensities were measured with a fluorometer (Victor X2 2030, Perkin Elmer, Waltham, MA, USA) using $\lambda_{\text{ex/em}}$ = 406/468 nm for CYP1A2 and for CYP2C19, $\lambda_{\text{ex/em}}$ = 415/502 nm for CYP2C9, $\lambda_{\text{ex/em}}$ = 390/468 nm for CYP2D6, and $\lambda_{\text{ex/em}}$ = 535/587 nm for CYP3A4 assays. The solvents applied ($\leq$ 0.5%DMSO and $\leq$ 1% acetonitrile (ACN)) had no significant effect on the inhibition of the function of the tested CYP enzymes.

5.7 Examination of oxidation status in IPEC-J2 cells and pig hepatocytes

Effects of inhibitors MI-432, MI-471 and MI-476 (50 μ M) alone or in combination with *S. suis* were tested on oxidative status of IPEC-J2 cells and primary porcine hepatocytes for 24 h on 24-well plates. An Amplex red hydrogen peroxide assay kit was used to measure EC H₂O₂ production in IPEC-J2 cells and in hepatocytes. Following the manufacturer's instructions, Amplex red working solution was mixed with cell-free supernatants (in a 1:1 ratio) which resulted in a red fluorescent product in the presence of horseradish peroxidase, namely resorufin. A Victor X2 2030 fluorometer was used to detect fluorescence intensity at wavelengths 530 nm (excitation) and wavelengths 590 nm (emission). The control served as cells treated only with plain medium.

For intracellular (IC) oxidative status measurements, 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) dye (Merck, Budapest, Hungary) was used. DCFH-DA can be oxidized by IC ROS into the fluorescent compound dichloro-fluorescein (DCF). Consequently, elevated fluorescence levels indicate an increase in IC ROS levels. The dye was mixed with the cells for 60 min, proceeded by washing the medium and scraping the centrifuged cells (3000 x g) for 10 min. Fluorescence of the samples was measured with a Victor X2 2030 fluorometer at wavelengths 480 nm (excitation) and 530 nm (emission).

5.8 Evaluation of proinflammatory cytokine IL-6 expression

Supernatants free of cells exposed to MI-432, MI-471, MI-476 were sampled from IPEC-J2 cells and porcine hepatocytes after a 24 h incubation time. Porcine IL-6 ELISA kits were used to evaluate the alterations in cytokine levels. Following the manufacturer's instructions, supernatants were treated and measured by a SpectraMax iD3 microplate reader at 450 nm.

5.9 Statistical analysis

The statistical evaluation of the data was executed using version 2023 R Core Team. One-way ANOVA was used to determine differences between groups. Post hoc Tukey was applied for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate statistically significant differences.

6. Results

6.1 Determination of MIC values of inhibitors

The test substances were unable to inhibit bacterial growth against *E. coli*, *P. aeruginosa*, *S. enterica* and *E. faecalis* (MIC > 800 µM). *B. cepacia* isolates were moderately inhibited by test substances 471 and 476 (MIC at 400 µM). Test substance 432 significantly inhibited the *S. aureus* clinical isolate at MIC values of 50 µM, whereas test substances 471, 476, and 485 only moderately inhibited bacterial growth (MIC 100–200 µM). While no significant effect was observed on bacterial growth by the other test substances. *P. multocoda* clinical isolates were significantly inhibited by inhibitors 432, 471 and 476 (MIC at 50 µM) whereas test substances 472 and 477 only moderately inhibited bacterial growth at MIC values of 400 µM, and no significant effect was observed on bacterial growth by the other test substances. The highest efficacy of the screened inhibitors was observed against *S. suis*, where test substances 432, 471, and 476 inhibited bacterial growth at 1.5 µM, 3 µM, and 6 µM, respectively. The results are summarised in **Table 1**.

Table 1. Impact of the tested substances against various bacterial isolates. Sensitive isolates are indicated by the green colour. The orange colour shows moderately sensitive isolates, and the grey colour applies to isolates resistant to the administered inhibitors.

Bacterial Strains	Sources	MIC (μ M) of Phe(3-Am) - derived protease inhibitors									
		432	463	471	472	476	477	485	490	1903	1904
<i>P. aeruginosa</i>	ATCC	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800
<i>E. coli</i>	ATCC	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800
<i>S. enterica</i>	204/22	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800
<i>P. multocida</i>	380/22	50	>800	50	400	50	400	>800	800	800	800
<i>S. aureus</i>	ATCC	50	>800	100	800	100	800	200	800	800	800
<i>S. suis</i>	672/22	<1,5625	>800	3,125	50	6,25	50	50	400	200	200
<i>B. cepacia</i>	20-10299	800	>800	400	800	400	800	800	800	800	400
<i>E. fecalis</i>	442	>800	>800	>800	>800	>800	>800	>800	>800	>800	>800

6.2 Effect on human CYP3A4, 1A2, 2D6, 2C9, and 2C19 activities

Only the three inhibitors MI-432, MI-471, and MI-476, which demonstrated the highest antibacterial activity against *S. suis*, were used in the subsequent experiments. Inhibitor concentrations of 10 μ M, 25 μ M, and 50 μ M were added for 15 min to human microsomal preparations, using ketoconazole as a CYP3A4 reference inhibitor. It was shown that the addition of all three inhibitors reduced CYP3A4 isoenzyme activities respectively at 10 μ M ($p < 0.0001$), 25 μ M ($p < 0.0001$), and 50 μ M ($p < 0.0001$), as shown in **Figure 2**.

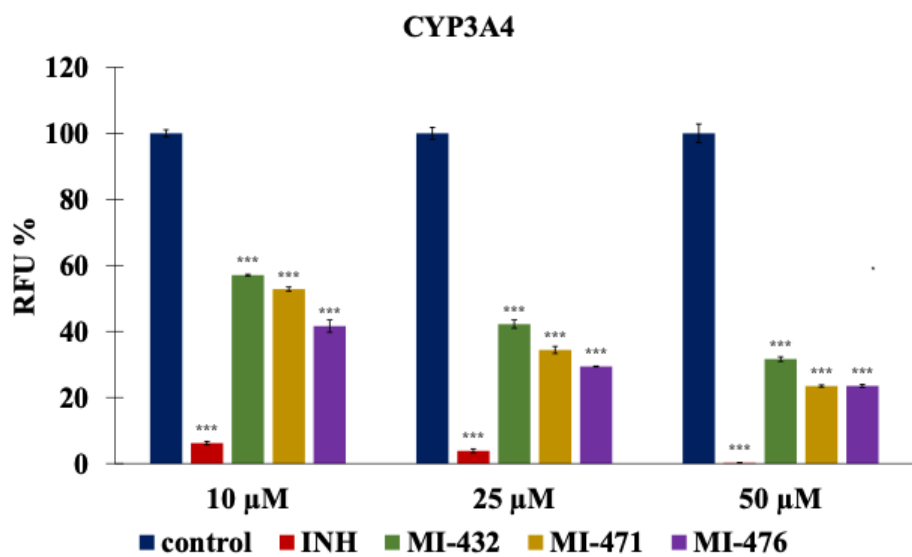
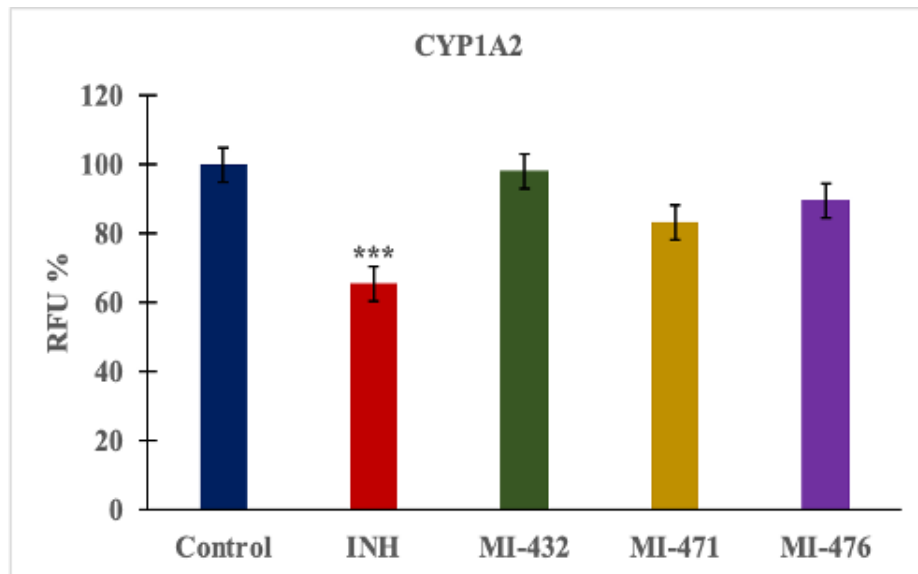


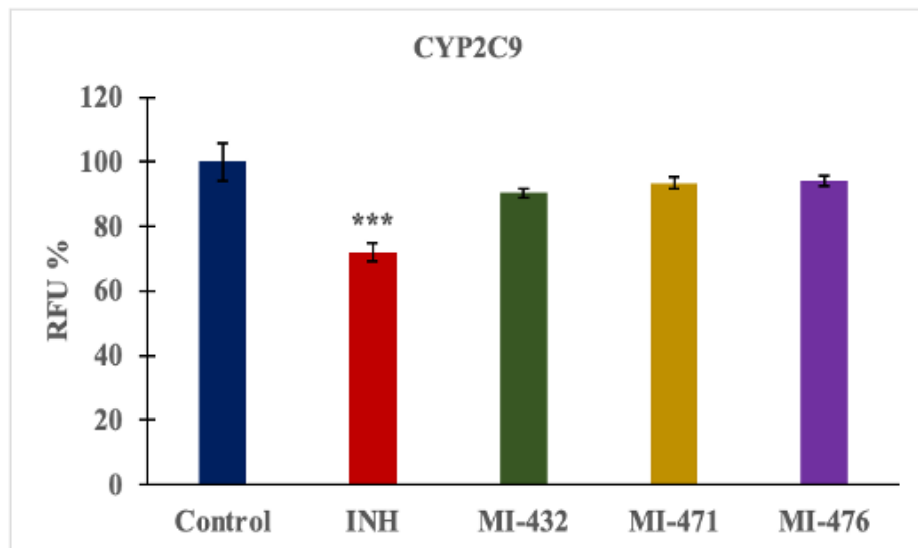
Figure 2. The influence of the protease inhibitors on human hepatic microsomal CYP3A4 isoenzyme function at concentrations of 10 μ M, 25 μ M, and 50 μ M. The microsomal preparations were exposed to the inhibitors for at 37 $^{\circ}$ C for 15 min. Ketoconazole (INH) was used as a reference inhibitor at a concentration of 5 μ M and significantly reduced human CYP3A4 activity (***) $p < 0.001$). The expressed data are the mean relative fluorescence intensities (RFUs) shown as a percentage of the untreated control fluorescence values $\pm$ SD ($n = 3$); *** signifies $p < 0.001$ compared to controls.

In **Figure 3.** similar results are demonstrated where the same inhibitors were administered (50 μ M) to human microsomal preparations for 15 min where quinidine, α -naphthoflavone, N-3-benzylrivanol and tienilic acid served as reference inhibitors for measurements with CYP1A2, 2C9, 2C19 and 2D9 respectively. The addition of these inhibitors did not reduce the activity of the four CYP isoenzymes ($p > 0.05$).

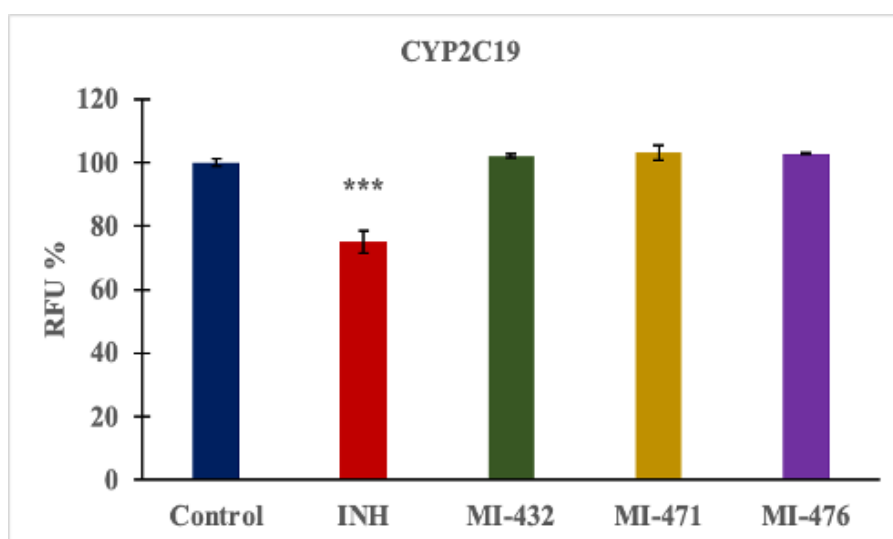
A



B



C



D

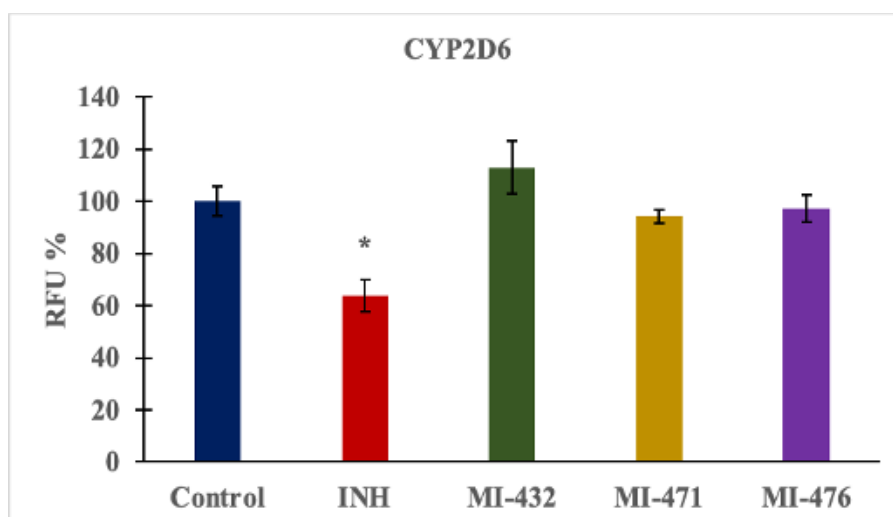
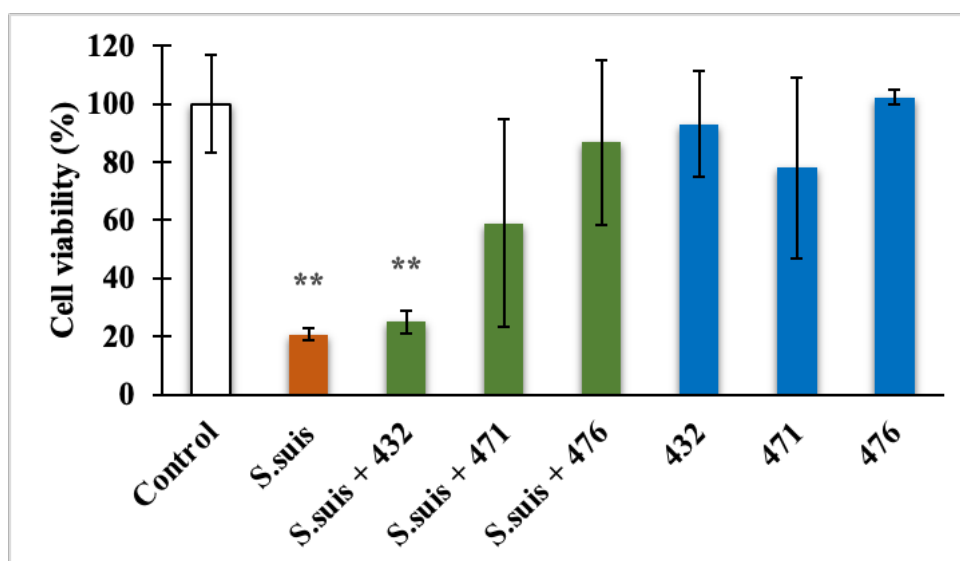


Figure 3. The impact of the protease inhibitors (50 μ M) on human hepatic microsomal CYP1A2 (A), 2C9 (B), 2C19 (C), and 2D6 (D) isoenzyme activities. The inhibitors were added to the microsomal preparations at 37 $^{\circ}$ C for 15 min. α -Naphthoflavone (reference inhibitor) was used at a concentration of 6 μ M significantly reduced human CYP1A2 activities (** $p < 0.001$); however, CYP1A2 activity was not altered by either of the protease inhibitors at a concentration of 50 μ M. The reference inhibitor, quinidine significantly reduced CYP2D6 activities at a concentration of 3 μ M (* $p < 0.05$). Moreover, the reference inhibitors (+)-N-3 benzylnirvanol at 30 μ M and tienilic acid at 60 μ M significantly mitigated CYP2C9 and CYP2C19 function (** $p < 0.001$). The presented data are the mean relative fluorescence intensities (RFUs) shown as a percentage of the fluorescence values measured in the untreated control $\pm$ SD ($n = 3-4$); * indicates $p < 0.05$, and *** indicates $p < 0.001$ compared to controls.

6.3 Cell viability assay

The viability of IPEC-J2 cells treated with *S. suis* for 24 h in the presence or absence of inhibitors was measured prior to 2 h of incubation with Neutral red dye (**Figure 4A**). *S. suis* (10^4 CFU/mL) alone increased cell death rate ($p = 0.00248$), and together with inhibitor MI-432 at concentration of 50 μ M a minor reduction in cell death can be seen ($p = 0.00436$). However, no deteriorating effects on cell viability were seen when treated with *S. suis* together with inhibitors MI-471 and MI-476 at 50 μ M after 24 h treatment ($p > 0.05$). Moreover, in **Figure 4B**, *S. suis* significantly reduced cell viability in primary porcine hepatocytes ($p < 0.001$) alone and in combination with inhibitor MI-432 ($p < 0.001$). Contrarily, *S. suis* together with inhibitors MI-471 and MI-476 at 50 μ M had no toxic effects on primary porcine hepatocytes ($p > 0.05$). All three inhibitors used alone at 50 μ M for 24 h had no negative effects on porcine hepatocytes ($p > 0.05$).

A



B

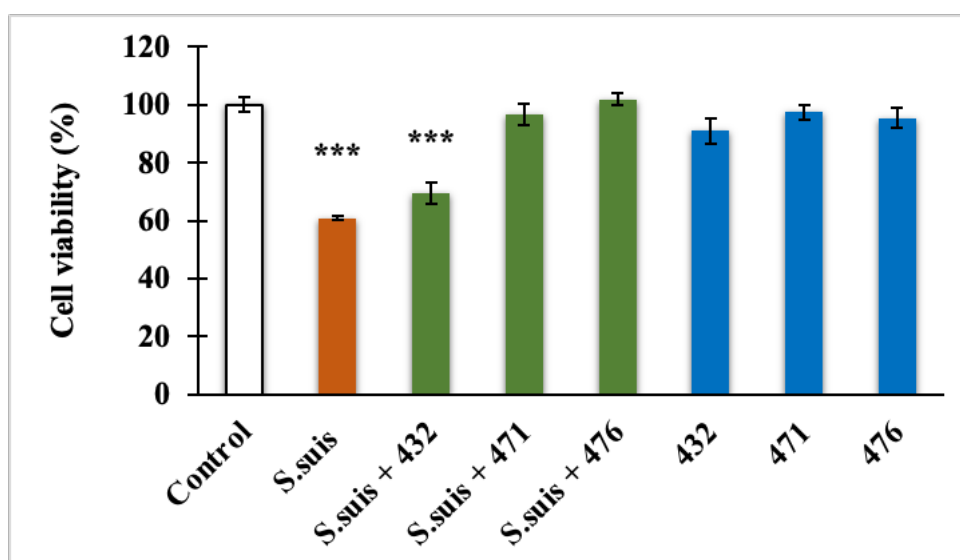


Figure 4. Evaluation of cell viability in IPEC-J2 cells (A) and porcine hepatocytes (B). Alterations in cell viability evaluated by absorbance measurements after 24 h treatment of *S. suis* (10^4 CFU/mL), MI-432, MI-471, and MI-476 at 50 μ M alone and in their combinations. Data are represented as cell viability % expressed in control % with standard errors of mean (SEM), n = 3–4 samples per group. ** indicates $p < 0.01$ and *** signifies $p < 0.001$ compared to controls.

6.4 Determination of EC ROS status

In **Figure 5A**, a significant elevation is shown in EC H₂O₂ secretion in IPEC-J2 cells treated with *S. suis* (10⁴ CFU/mL) alone or together with inhibitor MI-471 (*** $p < 0.001$). However, IPEC-J2 cells exposed to *S. suis* with addition of the inhibitors MI-432 and MI-476 at concentration 50 μ M after 24 h treatment had no effect on H₂O₂ levels ($p > 0.05$), similarly to inhibitors MI-432, MI-471, or MI-476 alone ($p > 0.05$). As shown in **Figure 5B**, hepatocytes exposed to *S. suis* (10⁴ CFU/mL) alone or together with all three inhibitors significantly increased EC H₂O₂ production (** $p < 0.01$ for each treatment). Nonetheless, hepatocytes exposed to the inhibitors alone demonstrated no alterations in redox balance ($p > 0.05$).

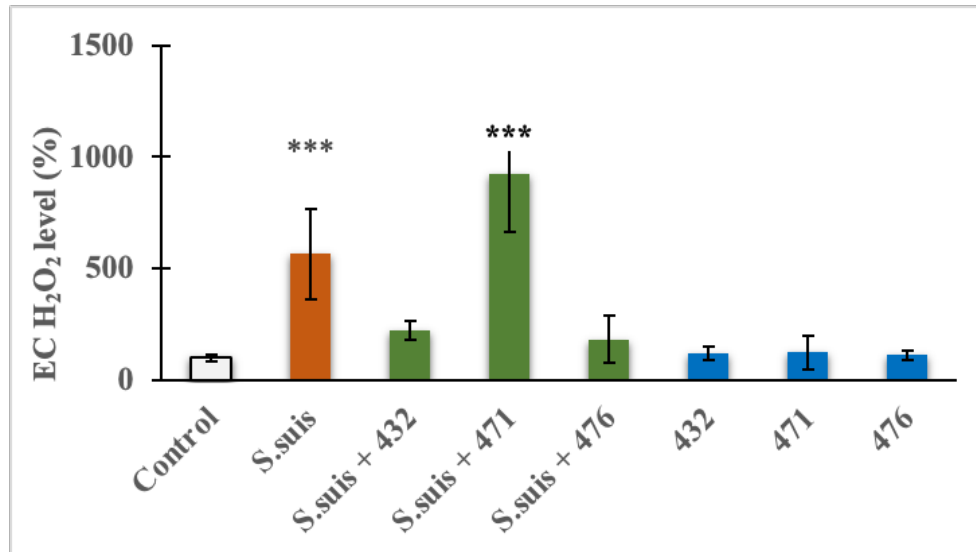
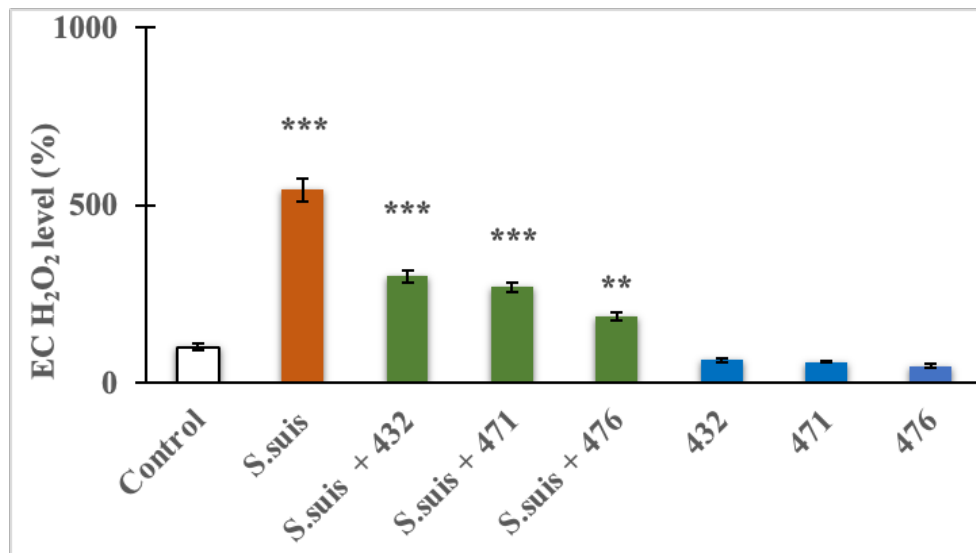
A**B**

Figure 5. Evaluation of EC H₂O₂ levels following exposure to *S. suis* (10⁴ CFU/mL) alone or together with the demonstrated inhibitors MI-432, MI-471, and MI-476 in IPEC-J2 cells (A) and in hepatocytes (B) at a concentration of 50 μ M using the Amplex red method. The EC H₂O₂ production was evaluated by measurements of the relative fluorescence intensities compared to the control (100%) with SEM, n = 3 samples per group. ** indicates p < 0.01, and *** indicates p < 0.001 compared to controls.

6.5 Evaluation of IC ROS status

A significant increase in IC ROS production in IPEC-J2 cells exposed to *S. suis* (10^4 CFU/mL) alone (***) $p < 0.001$) can be seen in **Figure 6**. However, IPEC-J2 cells treated with *S. suis* together with inhibitors MI-432, MI-471 and MI-476 had no significant effect on IC ROS production ($p > 0.05$). Additionally, IPEC-J2 cells treated with these three inhibitors respectively showed no significant alterations to IC ROS production ($p > 0.05$).

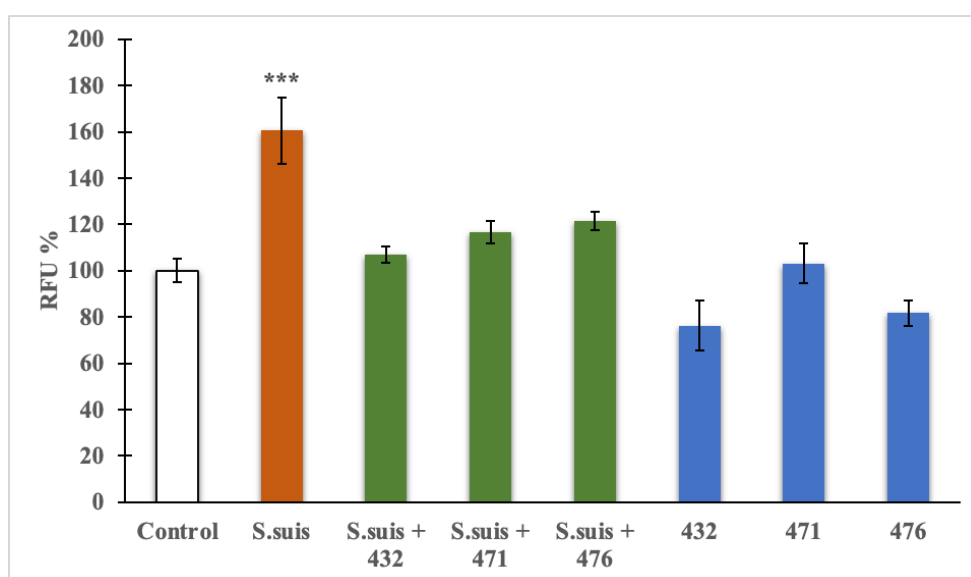


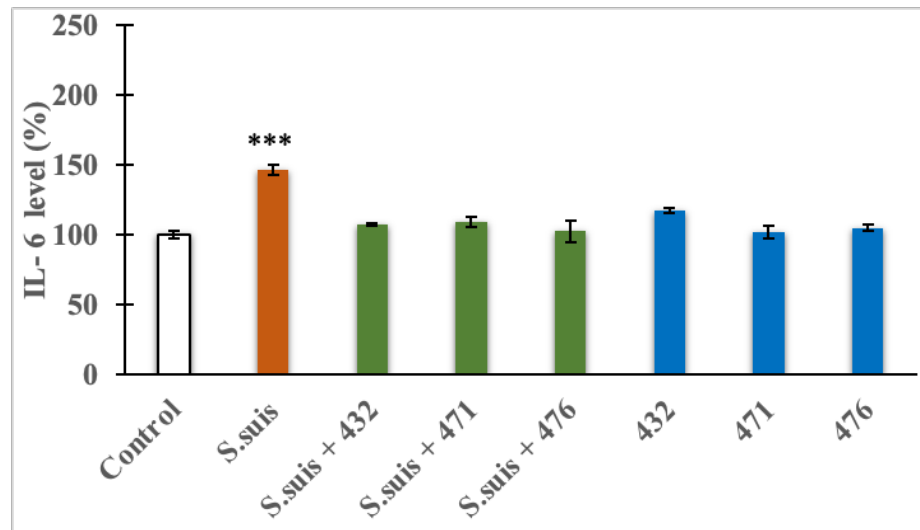
Figure 6. Determination of IC ROS production after 24 h exposure to *S. suis* (10^4 CFU/mL) alone or together with the demonstrated inhibitors MI-432, MI-471, and MI-476 IPEC-J2 cells at a concentration of 50 μ M in IPEC-J2 cells. The IC ROS production was evaluated by measurements of the relative fluorescence intensities compared to the control (100%) with SEM, $n = 3$ samples per group. ** indicates $p < 0.01$, and *** indicates $p < 0.001$ compared to controls.

6.6 Changes in proinflammatory cytokine IL-6 expression

IL-6 production increased in cell-free supernatants of IPEC-J2 cells treated with *S. suis* (10^4 CFU/mL) alone (***) $p < 0.001$) as shown in **Figure 7A**. All inhibitors alone and together with *S. suis* for 24 h did not significantly elevate IL-6 concentrations ($p > 0.05$). Similarly, as shown in **Figure 7B**, after treatment with *S. suis* alone, IL-6 levels were elevated to a larger degree in

hepatocytes (* $p < 0.05$). Contrarily, IL-6 production was not increased in hepatocytes after treatment with *S. suis* together with all three inhibitors ($p > 0.05$) or with the inhibitors alone ($p > 0.05$).

A



B

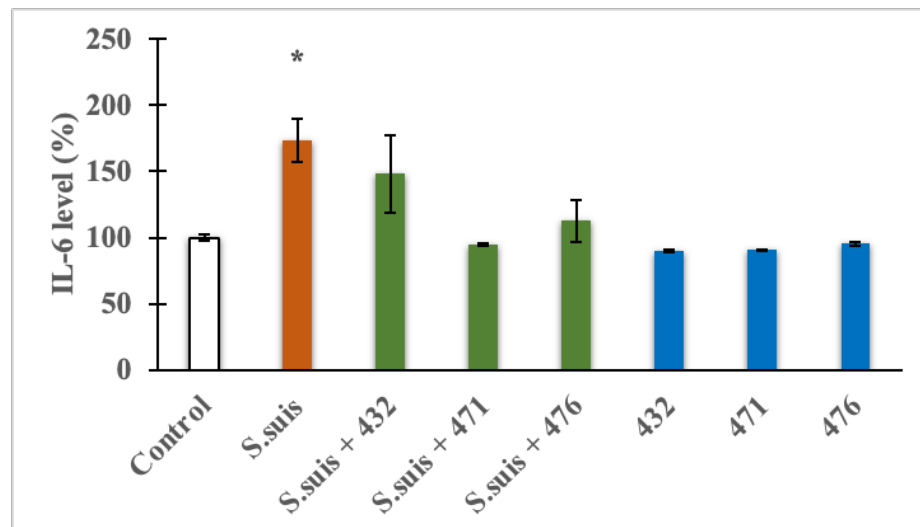


Figure 7. Determination of interleukin-6 levels after 24 h exposure to *S. suis* (10^4 CFU/mL) alone or together with the demonstrated inhibitors MI-432, MI-471, and MI-476 or with the inhibitors alone in IPEC-J2 cells (**A**) and in hepatocytes (**B**). Data are represented as IL-6 production % expressed in control with SEM, $n = 3-4$ samples per group. * Signifies $p < 0.05$ and *** indicates $p < 0.001$ compared to controls.

7. Discussion and conclusion

S. suis is a globally recognised pathogen and continues to represent a major public health risk in several countries against which β -lactams are used primarily [3,38-39].

However, there is concern about the increasing emergence of strains resistant to florfenicol and enrofloxacin, which are AMEG (antimicrobial advice ad hoc expert group) B category antibiotics, due to inappropriate use [3,24-25] in some countries. Among the screened bacterial isolates, *P. aeruginosa*, *S. enterica*, *E. coli*, and *E. faecalis* could not be suppressed by the test substances, even at very high, nearly millimolar concentrations. Regarding *B. cepacia*, a slight antibacterial activity was only shown at high concentrations (400 μ M) of the test substances MI-471, MI-476, and MI-1904, and against *S. aureus* and *P. multocida*, some inhibitory activity was observed for several test substances at lower concentrations (50 μ M). Six test compounds used against *S. suis* demonstrated the most prominent suppression which showed antibacterial activity even at concentration of 50 μ M or less. In the presence of inhibitors MI-432, MI-471 and MI-476, MIC values were the lowest. However, to evaluate the antibacterial effect in animal models, additional *in vivo* studies are required.

All compounds that are currently examined can be characterised as sulfonated tertiary amides of Phe(3-Am). The N terminus of the Phe(3-Am) is grouped with a 2',4' disubstituted (either dichloro- or dimethoxy) 3-sulphonyl-biphenyl moiety, while its carboxyl group is amidated with various piperidine derivatives containing either an ureido group or a terminal free amino. The side chain of Phe(3-Am) consists of a benzamidine moiety that is strongly basic, and the best antibacterial effects were observed for dibasic compounds, which contain a piperidine moiety together with an additional amino group. Contrastingly, inhibitor MI-490 demonstrated a negligible antibacterial effect, despite being a dibasic inhibitor with an additional second basic group in a different position at the N-terminal biphenyl moiety.

Moreover, all the most effective compounds (MIC values against *S. suis*: MI-432, <1.5625 μ M; MI-471, 3.125 μ M; MI-476, 6.25 μ M) contain an N-terminal 2',4' disubstituted biphenyl moiety. The stronger antibacterial effect was observed by the 2',4'-dichloro-substituted compounds when compared to the other structurally similar 2',4'-dimethoxy-substituted derivatives. The latter compounds were used at significantly higher concentrations. Additionally, no significant antibacterial activity was shown for the monobasic compound MI-463, which features a bulky cyclohexyl-substituted C-terminal ureido group, against any of the tested strains.

Human liver microsomes were used to determine the inhibitory effects on CYP activities in order to evaluate the potential drug interactions of the added inhibitors. The inhibitors MI-432, MI-471 and MI-476 had no significant inhibitory effects on CYP1A2, CYP2C9, CYP2C19, and CYP2D6 activities. Contrastingly, a previous study has demonstrated a significant inhibition on CYP1A2 of monkey origin after the addition of inhibitor MI-432 [40], which suggests species-specific variations in the CYP-modulating function of the inhibitors. Similar to previous studies, CYP3A4 activity was significantly decreased by inhibitors MI-432, MI-471, and MI-476, whereas other Phe(3-Am) derivatives have shown similar inhibitory effects on CYP3A4 function [41].

IPEC-J2 cells have demonstrated to be a suitable model for determining intestinal functions due to their sufficient characteristics for bacterial adhesion and cellular entrance [42]. In addition to these features, the advantages of IPEC-cells in this study compared to typically used intestinal cell lines such as Caco-2, HT-29 or T84 can also be justified based on their carcinogenicity. These IPEC-J2 cells, which are obtained from different carcinoma types found in the colon, may have non-physiological properties. The IPEC-J2 cells are favoured over the use of cancerous cell types due to their relatively reduced responsiveness to cytokines, along with the adapted glycosylation pattern or deviating protein expression [43].

The primary porcine hepatocytes utilized in this study more accurately resemble the physiological *in vivo* conditions as they are not sourced from a hepatoma, which is observed with regularly used cell lines such as HepG2.

Despite the benefits of human liver cancer cells, like an extended lifespan and a stable phenotype, they are not dependable for majority drug-metabolising enzymes and hepatotoxicity studies due to their minimal expression [44]. The importance of evaluating the effect of *S. suis* on IPEC-J2 cells provides insight, not only in the veterinary field by improving pig gut health for higher production rates but also because of the shared characteristics to the human epithelium and therefore the human gut [19]. Interactions between multiple pathogens and the jejunum have been demonstrated through microbiological experiments on porcine IPEC-J2 cells. Recent studies have shown infection of intestinal epithelial cells with Enterobacteriaceae such as *S. enterica* and pathogenic *E. coli* [45-46]. Inflammatory cytokine IL-8 and tumour necrosis factor (TNF)- α levels of porcine enterocytes administered with *Lawsonia intracellularis* alone or together with *S. enterica* serovar *Typhimurium* were significantly higher, and synergistic effects were seen in case of co-infection [47].

In our studies IPEC-J2 and primary pig hepatocytes were utilized as cell-based models to evaluate the (sub)cellular changes triggered by *S. suis* infection and to assess the possible beneficial effects of the repurposed antiviral drug candidates. To the best of our knowledge, this study was the first to evaluate the effect of this type of matriptase/TMPRSS2 inhibitor on the redox status and cell viability of IPEC-J2 cells and primary porcine hepatocytes treated with *S. suis*. It was demonstrated that *S. suis* administered at a concentration of 10^4 CFU/mL for 24 h negatively affected IPEC-J2 cell viability and hepatocytes. In the cell viability assay, both IPEC-J2 cells and hepatocytes had shown fewer viable cells after treatment with *S. suis* together with inhibitor MI-432. Contrastingly, the cytotoxic impact of *S. suis* was significantly suppressed through the simultaneous treatment of compounds MI-471 and MI-476 in both cell models.

Previous studies have proven deviations in certain interleukin levels after supplementing with Enterobacteriaceae. However, inflammatory processes can be indicated by all proinflammatory cytokines, if increased above their physiological values. The emphasis of this study was on the evaluation of IL-6 levels, which are primarily responsible for triggering acute phase proteins [48]. IPEC-J2 cells and primary pig hepatocytes are both able to stimulate cytokine production [42,45,48], and have demonstrated significant elevation in IL-6 production after treatment with *S. suis* for 24 hrs. Interestingly, IL-6 levels were not elevated after simultaneous treatment with *S. suis* and the inhibitors, which is preferred because higher IL-6 serum levels can be indicative of serious hepatic diseases, such as hepatocellular carcinoma [48]. In summary, significant anti-inflammatory effects were observed in cell-based models treated with *S. suis* after adding MI-432, MI-471, and MI-476.

The two cell lines used in this study are also suitable for evaluating oxidative stress levels since they are both exposed to high levels of ROS under physiological conditions. Problems arise when the ROS surpasses antioxidant capacity, namely oxidative stress, resulting in lipid peroxidation or protein damage [49-50]. The IPEC-J2 experiment demonstrated a significant increase in H₂O₂ production when *S. suis* was used alone and when bacterial exposure was combined with MI-471. Further investigation is required for this phenomenon. Contrastingly, inhibitors MI-432 and MI-476 in *S. suis* infections could impose antioxidant effects. The hepatocytes demonstrated significantly elevated H₂O₂ levels during *S. suis* infection. Yet together with inhibitors H₂O₂ levels were not significantly reduced. The inhibitors alone indicated to be safe when used alone as they had no effect on H₂O₂ production.

The idea of drug repurposing is a unique area of research that aims to create drugs specifically for new variations of diseases in a short period of time. A reduced development time and lowering costs for repositioned drugs is also enabled by the presence of fundamental knowledge about the potential adverse effects of these compounds. Other examples include drugs that have not yet entered the market for their original purpose but have demonstrated additional purposes that facilitate their use against other targets.

In this study, the antibacterial effects of three inhibitors against matriptase/TMPRSS2 were confirmed against *S. suis* at low micromolar concentrations. A drug repurposing of already existing serine protease inhibitors may offer a suitable strategy to accelerate the process of supplying sufficient treatment options against resistant bacterial strains, reducing the overuse critically important antibiotics.

8. Summary

Piglets from industrial pig farming are most susceptible to environmental pathogens during the post weaning period as a result of their weakened immune system. *Streptococcus (S.) suis* serves as one of these pathogens which is often treated with antimicrobial agents. The increasing occurrence of antimicrobial resistance against zoonotic bacteria such as *S. suis* proves the need for new therapeutical strategies, including the repositioning of drugs. In this study, susceptibilities of bacterial isolates were tested toward ten different 3-amidinophenylalanine (Phe(3-Am)) derivatives via the determination of minimum inhibitory concentration values. The protease inhibitors, such as the compounds MI-432, MI-471, and MI-476, showed remarkable antibacterial effects against *S. suis*. Their drug interaction potential was evaluated by using human liver microsomal cytochrome P450 (CYP450) measurements. In our work, non-tumorigenic IPEC-J2 cells and primary porcine hepatocytes were infected with *S. suis* for 24 h in the presence or absence of inhibitors and the putative beneficial impact of these inhibitors was investigated on cell viability (Neutral red assay), on interleukin (IL)-6 levels (ELISA technique), and on IC and EC redox status (DCFH-DA and Amplex red method). All the protease inhibitors (except MI-432) prevented *S. suis*-induced cell death and reduced proinflammatory IL-6 levels. It was also found that MI-432 and MI-476 had extracellular antioxidant effects in an intestinal cell model upon *S. suis* infection. Whereas all three inhibitors had intracellular antioxidant effects in the same model upon *S. suis* infection. Concentration-dependent suppression of CYP3A4 function was proven via application of all three inhibitors at concentrations 10, 25 and 50 μ M to human microsomal preparations. In conclusion, our study indicates that the potential antiviral Phe(3-Am) derivatives with 2',4' dichloro-biphenyl moieties exert beneficial effects on IC and EC ROS status as well as IL-6 cytokine production *in vitro*. This suggests that these drugs are effective candidates against *S. suis* infection due to their antibacterial effects.

9. Summary in Hungarian

A nagyüzemi sertéstartásból származó malacok az elválasztás utáni időszakban a legfogékonyabbak a környezeti patogénekre az immunrendszerük legyengülése miatt. A *Streptococcus (S.) suis* elleni antimikrobiális rezisztencia növekvő előfordulása bizonyítja, hogy új terápiás stratégiákra van szükség beleértve a gyógyszerek repozicionálását a fertőzések elleni hatékony küzdelemben.

Vizsgálatunkban baktériumtörzsek érzékenységét tíz különböző 3-amidino-fenilalanin (Phe(3-Am)) származékkal szemben teszteltük a minimális gátló koncentráció érték meghatározásával. A proteáz inhibitorok, így az MI-432, MI-471 és MI-476 vegyületek jelentős antibakteriális hatást mutattak *S. suis* ellen. A vegyületek gyógyszerkölsönhatási potenciálját humán máj mikroszomális citokróm P450 (CYP450) mérésekkel határoztuk meg. Munkánk során nem tumoros IPEC-J2 sejteket és primer sertés májsejteket fertőztünk *S. suis* kezeléssel 24 órán keresztül inhibitorok jelenlétében vagy hiányában, és megvizsgáltuk ezen inhibitorok feltételezett jótékony hatását a sejtek életképességére (Neutral red assay). interleukin (IL)-6 szint változásra (ELISA technika), valamint az intra- és extracelluláris redox státuszra (DCFH-DA és Amplex red módszer).

Az inhibitorok (az MI-432 kivételével) megakadályozták a *S. suis* által kiváltott sejthalált, és csökkentették a proinflammatorikus IL-6 szintet. Azt is megállapítottuk, hogy mindhárom inhibitor antioxidáns hatást fejtett ki *S. suis* fertőzés esetén. A CYP3A4 aktivitást a vegyületek koncentrációfüggő módon gátolták humán mikroszomális preparátumokon.

Összefoglalva az eddigi eredményeket, a 2',4'-diklór-bifenil csoportot tartalmazó potenciális antivirális Phe(3-Am) származékok *in vitro* jótékony hatásúak a sejtek redox állapotára, valamint az IL-6 citokin termelésre. Ez arra utal, hogy ezek a vegyületek hatékony gyógyszerjelöltek a *S. suis* fertőzés ellen antibakteriális hatásuk miatt.

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