In vitro effects of cysteine protease inhibitors on *Trichomonas foetus*–induced cytopathic changes in porcine intestinal epithelial cells

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OBJECTIVE
To investigate the effects of specific cysteine protease (CP) inhibitors on cytopathic changes to porcine intestinal epithelial cells induced by *Trichomonas foetus* isolated from naturally infected cats.

SAMPLE
*T. foetus* isolates from 4 naturally infected cats and nontransformed porcine intestinal epithelial cells.

PROCEDURES
*T. foetus* isolates were treated with or without 0.1 to 1.0mM of the CP inhibitors antipain, cystatin, leupeptin, and chymostatin and the vinyl sulfone inhibitors WRR-483 and K11777. In-gel gelatin zymography was performed to evaluate the effects of these inhibitors on CP activity of *T. foetus* isolates. Each treated or untreated isolate was also cocultured with monolayers of porcine intestinal epithelial cells for 24 hours, and cytopathic effects of *T. foetus* were evaluated by light microscopy and crystal violet spectrophotometry.

RESULTS
Results of in-gel gelatin zymography suggested an ability of WRR-483, K11777, and cystatin to target specific zones of CP activity of the *T. foetus* isolates. These inhibitors had no effect on *T. foetus* growth, and the cytopathic changes to the intestinal epithelium induced by all 4 *T. foetus* isolates were significantly inhibited.

CONCLUSIONS AND CLINICAL RELEVANCE
This study revealed that certain protease inhibitors were capable of inhibiting regions of CP activity (which has been suggested to cause intestinal cell damage in cats) in *T. foetus* organisms and of ameliorating *T. foetus*–induced cytopathic changes to porcine intestinal epithelium in vitro. Although additional research is needed, these inhibitors might be useful in the treatment of cats with trichomonosis. (Am J Vet Res 2016;77:890–897)

*Trichomonas foetus*, a flagellated enteric-dwelling protozoan, is the causative agent of trichomonosis in cats. Infection with the organism causes a waxing and waning diarrhea that can endure for life and is one of the major causes of infectious diarrhea in cats. The reported prevalence of *T. foetus* infection is as high as 30% in dense housing environments, and the infection is recognized worldwide.1

Despite periods of clinical remission of diarrhea, cats can remain subclinical carriers of *T. foetus* for life.2 Ronidazole, which is a 5-nitroimidazole, is the only drug recommended to treat *T. foetus* infection and is associated with an unacceptable risk of adverse effects, including neurotoxic effects.3 Moreover, rapidly developing drug resistance dictates the need for new treatments.4 Thus, identification of safe and effective treatments remains a subject of considerable interest.

Cellular proteases are important virulence factors that directly mediate the cytopathic effects caused by the venereal trichomonads *Trichomonas vaginalis* and *T. foetus* in humans and cattle, respectively.5,6 We previously demonstrated that adhesion and elaboration of CPs produced by *T. foetus* in cats are also important events in the induction of intestinal cytopathic effects.7 Therefore, targeted inhibition of *T. foetus* CPs may represent a novel approach to the treatment of trichomonosis in cats. However, cats, like all mammals, also produce CPs that are part of life-critical systems (eg, cellular protein turnover or programmed cell death),8 and broad inhibition of CPs would inevitably be damaging to cats.

To reduce the possibility of unnecessary adverse effects to cats, protease inhibitors need to be identified that selectively inhibit the pathogen proteases responsible for the cytopathic effects of *T. foetus* or those that have similar mechanisms of production or

**ABBREVIATIONS**
CP Cysteine protease
DPBS Dulbecco phosphate-buffered saline solution
function to those of the feline host. The development of protease inhibitors that target specific families of CPs, rather than broadly inhibiting all CPs, has paved the way for the safe and uniquely tailored treatments for many protozoal diseases. Such drugs may represent a promising new direction to explore for treatment of cats with trichomonosis. The purpose of the study reported here was to investigate whether currently available protease inhibitors would be capable of inhibiting CP activity of feline isolates of T. foetus and ameliorating T. foetus-induced cytopathic effects within the intestinal epithelium.

Materials and Methods

Porcine jejunal epithelial cells

For assessment of cytopathic effects, the porcine jejunal epithelial cell line IPEC-J2 was used. This cell line represents a nontransformed primary cell line originally isolated from the jejunum of a neonatal piglet. The IPEC-J2 cells were grown in coculture medium that included advanced Dulbecco minimal essential medium (including nutrient mixture F-12) supplemented with insulin, transferrin, and selenium (5 µg/mL for each); epidermal growth factor (5 ng/mL); penicillin (50,000 U/mL); streptomycin (50,000 mg/mL); and 5% fetal bovine serum and incubated at 37°C in 5% CO₂. For evaluation of the cytopathic effects of feline T. foetus isolates, IPEC-J2 cells were seeded onto 24-well polystyrene plates and grown to confluence. The IPEC-J2 cells were used at passages 40 to 55.

T. foetus isolates

Isolation and culture of feline T. foetus isolates were performed as described elsewhere. Each isolate was cultured in modified Diamond medium supplemented with antimicrobials (penicillin, amphotericin B, and streptomycin) and incubated at 37°C. Four T. foetus isolates (A, F, J, and S) recovered from 4 naturally infected cats were used for comparative tests of the effects of protease inhibitor treatment on in-gel gelatin zymography patterns and cytopathic effects.

In-gel gelatin zymography

Effects of currently available CP inhibitors (0.01 to 1mM E-64, antipain, cystatin [from chicken egg white], leupeptin, chymostatin, K11777 [N-methyl-piperazinyl-phenylalanyl-homophenylalanylvinylsulfone-phenyl], and WRR-483 [an arginine variant of K11777]) on targeting regions of feline T. foetus CP activity were determined by pretreatment of whole cell trichomonads with protease inhibitors immediately prior to protein extraction for in-gel gelatin zymography. The A isolate was used as a sentinel isolate against which all CP inhibitors were tested. Trichomonads (2 X 10⁸/mL) were incubated as described elsewhere with protease inhibitors for 20 minutes at 25°C. E-64, which is known to eliminate all feline T. foetus CP activity, was used as a positive control substance. As negative control substances, CP inhibitor diluents were incubated with trichomonads in a manner identical to that of the inhibitors of interest.

Following incubation, trichomonads (1.2 X 10⁸) were washed twice in DPBS, lysed in radioimmuno- precipitation assay buffer (1X PBS solution, 1% octylphenoxypolyethoxyethanol, 0.5% sodium deoxycholate, and 0.1% SDS), sonicated twice, and incubated for 30 minutes at 4°C. Samples were centrifuged at 15,800 X g for 10 minutes at 4°C, and supernatants were collected. Protein lysate concentrations were determined.
by bicinchoninic acid assay, and bovine serum albumin was used as a standard. Lysates were diluted in lithium dodecyl sulfate buffer without a reducing agent and immediately used for substrate-gel electrophoresis or were stored as single-use samples of approximately 400 µg at -80°C for no more than 72 hours.

Feline T. foetus protein lysates were separated and analyzed in nondenaturing and nonreducing conditions as described elsewhere in 10% tris-glycine gels containing 0.1% gelatin as the protein substrate. Protein samples were electrophoretically separated at 125 V for 90 minutes. Following electrophoresis, gels were immersed in a renaturing buffer for 30 minutes at 25°C to stimulate protease activation. Gels were equilibrated in a developing buffer for 30 minutes at 25°C, followed by overnight incubation in fresh developing buffer at 37°C. After overnight incubation, gels were washed 3 times for 5 minutes each in deionized water, stained for 8 hours with Coomassie blue dye, and incubated overnight in deionized water to reduce background staining. Protease activity was made visible as clearing bands against a stained background.

**Cytopathic effect assays**

Quantitative and qualitative analysis of the ameliorating effects of protease inhibitors on T. foetus-induced epithelial cytotoxic effects was performed by crystal violet spectrophotometry and light microscopy, respectively. The IPEC-J2 cells were grown to confluence on 24-well polystyrene plates and infected or not infected with 10 X 10^6 T. foetus at 37°C to yield a multiplicity of infection of 50:1, as described elsewhere. Trichomonads were treated with 0.01 to 0.1mM each of antipain, cystatin, WRR-483, and K11777 for 20 minutes at 25°C prior to coculture. Trichomonads were then cocultured with the IPEC-J2 monolayers for 24 hours in serum-deficient coculture medium to prevent them from replicating or regaining the ability to newly synthesize the previously inhibited CPs.

At the completion of the 24-hour incubation period, the motility of trichomonads contained within the medium was examined by light microscopy. The IPEC-J2 monolayers were gently washed with DPBS, fixed with 2% paraformaldehyde in PBS solution for 15 minutes at room temperature (approx 25°C), washed with DPBS, and stained with 100 µL of 0.13% crystal violet solution dissolved in 5:2 (vol/vol) ethanol-paraformaldehyde solution. The IPEC-J2 monolayers were gently washed twice with deionized water and allowed to air dry. Monolayers were either immediately inspected by light microscopy with an inverted light phase-contrast microscope or were solubilized in 100 µL of 1% SDS in 50% ethanol for spectrophotometric analysis. Solubilized cells were transferred to 96-well plates. Intensity of staining was quantified by use of a spectrophotometer at a wavelength of 570 nm.
Each treatment was represented by 7 to 16 replicates.

Figure 4—Photograph of results of in-gel gelatin zymography showing the inhibitory effect of cystatin at concentrations of 10µM (lane 2) and 100µM (lane 3), compared with that of no inhibitor (DPBS; lane 1), on CP activity of T foetus (A isolate). A specific region of CP activity appears to be inhibited with 100µM cystatin.

Statistical analysis
Data were analyzed for normality of distribution (Shapiro-Wilk test) and variance (Levene test) by use of statistical software and tested for significant differences among cell treatment groups via 1-way ANOVA (parametric data) or Kruskal-Wallis 1-way ANOVA on ranks (nonparametric data). When a significant treatment effect was identified, the post hoc Holm-Sidak (1-way ANOVA) or Tukey (1-way ANOVA on ranks) test was performed to determine the pairs of groups that differed significantly from each other. Results are reported as mean ± SD. For all analyses, a value of P ≤ 0.05 was considered significant.

Results
Effect of protease inhibitors on feline T foetus CP activity
Treatment of feline T foetus isolates with 0.01 to 1.0mM each of antipain, cystatin, WRR-483, and K11777 inhibited a narrow band of proteolytic ac-

Figure 5—Mean absorbance values (arbitrary units) for crystal violet staining of monolayers of porcine intestinal epithelial (IPEC-J2) cells cocultured (infected) or not cocultured (uninfected) for 24 hours with feline T foetus A isolate following pre-treatment of the organism with vehicle (DMSO) or 100µM antipain (A), WRR-483 (B), K11777 (C), or cystatin (D). Each value represents 8 to 11 replicates. Bars represent SD. *Value differs significantly (P < 0.001) from that for uninfected cells. †Value differs significantly (P < 0.001) from that for vehicle-treated, T foetus–infected cells.
Effect of protease inhibitors on feline *T. foetus*–induced epithelial cytotoxic effects

The protease inhibitors identified as inhibiting *T. foetus* CP activity during in-gel gelatin zymography tests (antipain, cystatin, WRR-483, and K11777) were used for cytotoxicity assays at the lowest concentrations at which inhibition had been achieved. The A isolate was used as a sentinel isolate against which CP inhibitors were initially tested in coculture tests. Inhibitors that were effective against the A isolate were then tested against the 3 other feline *T. foetus* isolates (F, J, and S). Although differences in the response of individual isolates to CP inhibition were observed, WRR-483 and K11777 were effective against the cytopathic effects of most *T. foetus* isolates at concentrations as low as 10µM. Because the 10µM concentration did not completely eliminate the band representing CP activity detected via in-gel gelatin zymography, coculture tests were also performed with a concentration of 100µM. Cystatin was not effective in eliminating CP activity at 10µM but was effective in eliminating a region of *T. foetus* CP activity at 100µM (Figure 4).

Quantitative examination of destruction of porcine intestinal epithelial cells in monolayers following pretreatment of *T. foetus* with cystatin, WRR-483, and K11777 revealed a significantly lesser extent of cell loss, compared with cell loss in monolayers infected with vehicle-treated *T. foetus*. Cystatin had the most pronounced effect (Figure 5). This reduction in intestinal epithelial cell loss was dose dependent (Figure 6). Light microscopy examination of IPEC-J2 cells after infection with *T. foetus* that had been pretreated with cystatin, WRR-483, or K11777 revealed prevention of monolayer destruction (Figure 7). With these 3 specific CP inhibitors, a reduction in both *T. foetus* CP activity and epithelial-induced cytopathic effects was observed for all 4 isolates tested (Figures 3 and 8). Treatment of *T. foetus* with antipain at concentrations found to be effective in targeting CP activity by in-gel gelatin zymography had no sparing effect on *T. foetus*–induced cytopathic effects, compared with the effects on monolayers infected with vehicle-treated *T. foetus*. Pretreatment with these protease inhibitors at concentrations used to inhibit CP activity had no adverse effect on motility of the trichomonads, compared with motility when pretreated with vehicle alone for periods of up to 24 hours.

**Discussion**

Cellular proteases play various roles in the life cycle and pathogenicity of many protozoan parasites, including replication, adhesion, nutrient acquisition, and evasion of host immune defenses. For this rea-
son, oral administration of protease inhibitors has been beneficial in eliminating disease or reducing morbidity for individuals with toxoplasmosis, cryptosporidiosis, and experimentally induced leishmaniasis, malaria, trypanosomiasis, and venereal trichomonosis. Analysis of the feline *T. foetus* transcriptome has revealed transcripts encoding at least 8 CPs. Consequently, CP inhibitors represent attractive options to explore for treatment of intestinal trichomonosis.

In previous research, we established that feline *T. foetus* isolates produce CPs that promote adhesion-dependent cytotoxic effects within the intestinal epithelium. Inhibition of feline *T. foetus* CPs with the broad-spectrum E-64 considerably ameliorates the intestinal epithelial cytopathic effects induced by *T. foetus*. Given the fact that cats also produce CPs that are part of life-critical systems, targeting selected CPs or regions of CPs that are important to *T. foetu*s may be necessary to circumvent the probable adverse effects that would result from administration of broad-spectrum CP inhibitors to cats.

In the present study, we evaluated the ability of currently available CP inhibitors to selectively inhibit zones of *T. foetus* CP activity and to ameliorate cytopathic effects of *T. foetus* to the intestinal epithelium. The cellular CP inhibitors used were specifically chosen because they had yielded promising results in other in vitro and in vivo experimental models of infectious disease, including venereal trichomonosis. In-gel gelatin zymography is commonly used for the detection of previously unidentified enzymes and for the study of enzyme inhibitors. In addition to its application in basic scientific research, the method is also used in the clinical setting, aiding in analysis of proteases involved in cardiovascular, inflammatory, and infectious disease.

Using in-gel gelatin zymography, we demonstrated that 4 of the tested CP inhibitors targeted regions of CP activity in feline *T. foetus* isolates. We could not discount a possible in vivo effect of the other protease inhibitors tested. Identification of the specific CPs inhibited by the protease inhibitors via chromogenic or fluorogenic peptide substrate cleavage assays was beyond the scope of the study. Rather, in-gel gelatin zymography was used as a screening tool to select the inhibitors most likely to have a protective effect against the cytopathic effects of *T. foetus* toward intestinal epithelial cells. Additional investigation is warranted into the potential usefulness of these CP inhibitors for treatment of cats with trichomonosis.

In addition, studies are needed to determine the specific families and identities of CPs inhibited by these inhibitors.

The coculture model used in the present study allowed demonstration that 3 of the 4 CP inhibitors shown to be effective by in-gel zymography were capable of reducing the *T. foetus*-induced cytopathic effects to porcine intestinal epithelium.

Figure 8—Mean absorbance values (arbitrary units) for crystal violet staining of monolayers of porcine intestinal epithelial (IPEC-J2) cells cocultured (infected) or not cocultured (uninfected) for 24 hours with feline *T. foetus* isolates (A, S, J, and F) following pretreatment of the organisms with vehicle (DMSO) or 10 or 100 µM WRR-483 (top row), K11777 (middle row), or cystatin (bottom row). Each value represents 8 to 16 replicates. See Figure 5 for remainder of key.
Both the vinyl sulfone inhibitors WRR-483 and K11777 and cystatin had long-lasting activity in the in vitro tests, resulting in an inhibitory effect of a minimum of 24 hours following a single treatment. The vinyl sulfone inhibitors are particularly promising chemical protease inhibitors that reduce genital colonization with bovine T. foetus isolates in mice.\textsuperscript{18,23} Moreover, K11777, a clan CA-specific inhibitor that has been FDA approved for a phase I clinical trial for treatment of Trypanosoma cruzi infection, has acceptable bioavailability when administered orally and a low degree of toxic effects in dogs and mice.\textsuperscript{24,25} The drug WRR-483, which is an analog of K11777, is also efficacious in preventing disease in mice with experimentally induced T. cruzi infection and in targeting CP1 in Entamoeba bistolytica.\textsuperscript{26}

Chicken egg white cystatin, a type II cystatin belonging to the superfamily of cystatins, was the most efficacious inhibitor evaluated for reduction of T. foetus-induced epithelial cytopathic changes for all isolates tested in the coculture portion of the present study. Cystatins result in tight yet reversible inhibitory bonds within the catalytic cleft of papain proteases.\textsuperscript{27} Although the role of papain proteases in the pathogenesis of trichomonosis in cats has yet to be determined, papain and papain-like proteases have been identified as critical for cellular mechanisms and cytopathogenicity in a wide range of parasites.\textsuperscript{27}

To the authors’ knowledge, the present study represents the first to reveal the efficacy of cystatin against trichomonad-induced cytopathic effects. This particular inhibitor has potential for the treatment of cats with trichomoniasis and may have comparative implications for treatment of humans and cattle with venereal trichomoniasis. Additional in vivo and in vitro studies are needed to explore the safety and efficacy of cystatin, K11777, and WR-483 for the treatment of cats with T. foetus infection and to identify the specific CPs of T. foetus that induce cytopathic effects. Additional studies are also needed to characterize the specific proteases inhibited by these CP inhibitors.

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Footnotes

\textsuperscript{a}. Provided by Dr. Helen Berschneider, North Carolina State University, Raleigh, NC.

\textsuperscript{b}. Corning Inc, Lowell, Mass.

\textsuperscript{c}. Trans-eqoxysuccinyl-l-leucylamido(4-guanidino)butane, chymostatin N-(\textsuperscript{N}α-carbonyl)-capreomycinide-X-phe-al)-phe, Sigma Aldrich Corp, St Louis, Mo.

\textsuperscript{d}. Sigma Aldrich Corp, St Louis, Mo.

\textsuperscript{e}. Provided by Professor William Roush, Department of Chemistry, Scripps Florida, Jupiter, Fla.

\textsuperscript{f}. Thermo Fisher Scientific, Rockford, Ill.

\textsuperscript{g}. Life Technologies Corp, Carlsbad, Calif.

\textsuperscript{h}. Novex zymogram renaturing buffer, Life Technologies Corp, Carlsbad, Calif.

\textsuperscript{i}. Novex zymogram developing buffer, Life Technologies Corp, Carlsbad, Calif.

\textsuperscript{j}. Nikon, Tokyo, Japan.

\textsuperscript{k}. SigmaPlot, Systat Software Inc, San Jose, Calif.

References


Correction: Effects of repetition within trials and frequency of trial sessions on quantitative parameters of vertical force peak in horses with naturally occurring lameness

In the report “Effects of repetition within trials and frequency of trial sessions on quantitative parameters of vertical force peak in horses with naturally occurring lameness” (*Am J Vet Res* 2016;77:756–765), the first sentence under the Objective section of the abstract was incorrect. The correct sentence should read, “To analyze the effects of vertical force peak (VFP) repetition within trials and between trial sessions in horses with naturally occurring appendicular lameness.”