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Short communication

Effect of vinyl sulfone inhibitors of cysteine proteinases on *Tritrichomonas foetus* infection

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ABSTRACT

Tritrichomonas foetus is a sexually transmitted protozoon that causes genital inflammation and adverse pregnancy outcomes in cattle. Cysteine proteinases (CPs) released by *T. foetus* degrade immunoglobulin G (IgG) antibodies, complement component 3 and matrix proteins as well as inducing apoptosis of bovine genital epithelial cells. In this study, the efficacies of the vinyl sulfone CP inhibitors K11777 and WRR-483 were tested against CPs of *T. foetus*. The activity of secreted *T. foetus* CPs in culture supernatants was decreased in the presence of vinyl sulfone inhibitors. Inhibitor K11777 reduced the in vitro cytopathogenic effects of *T. foetus* in bovine foetal trophoblast cells, which are relevant target cells since this pathogen interferes with pregnancy. Pre-treatment of *T. foetus* prior to intravaginal inoculation diminished genital infection in a murine model. Therefore, vinyl sulfone CP inhibitors reduce several effects of *T. foetus*-secreted CPs, including cytotoxicity on relevant target host cells and genital infection in a murine model. These inhibitors have potential as chemotherapeutic agents against bovine trichomoniasis. Generalisation to human trichomoniasis requires further study.

1. Introduction

Tritrichomonas foetus is a sexually transmitted protozoan parasite that causes vaginitis, cervicitis, endometritis and reproductive failure [1]. It closely resembles the human pathogen *Trichomonas vaginalis*, which causes genital inflammation and adverse pregnancy outcomes in women (e.g. preterm birth, premature membrane rupture and low birth weight) [2]. We and others have shown that cysteine proteinases (CPs) released by *T. foetus* are virulence factors in the bovine genital tract. They degrade bovine immunoglobulin G2 (IgG2) and IgG1, complement component 3, fibronectin and fibrinogen [3] as well as inducing apoptosis of bovine cultured vaginal and uterine epithelial cells [4,5]. The major extracellular *T. foetus* proteinase has been identified as CP8 [6], also called CP30 [4], which has a strong preference for basic C-terminal residues, mainly arginine and phenylalanine in the P_2 position [5,7]. The vinyl sulfone CP inhibitor K11777 and its derivative WRR-483 were chosen for study because they have phenylalanine and arginine, respectively, at the P_2 position. Thus, they should be good candidates for inhibition of *T. foetus* CP8/CP30, with preference for basic C-terminal residues. Moreover, K11777 and WRR-483 have been shown to block CPs from *Trypanosoma cruzi*, *Leishmania tropica*, *Entamoeba histolytica* and *Schistosoma mansoni* [8–11]. Both K11777 and WRR-483 have acceptable pharmacokinetics with no adverse effects on mammalian cells [8,9]. The aim of this study was to assess the ability of vinyl sulfone CP inhibitors to decrease secreted CP activity in *T. foetus* pathogenesis. Both K11777 and WRR-483 reduced *T. foetus* cytotoxicity for target cells of the natural host as well as genital infection in a murine model.

2. Materials and methods

2.1. *Tritrichomonas foetus* and extracellular proteinase production

Trophozoites of *T. foetus* strain D1, originally isolated from a cow with *T. foetus* pyometra, were grown at 37 °C in trypsin–yeast–iron (TYI) medium supplemented with Diamond vitamins and 15% adult bovine serum (pH 7.0). Extracellular proteinases in *T. foetus* culture supernatant (SUP) were collected by centrifugation periodically following inoculation of 10^7 *T. foetus*/mL. Concentrated extracellular proteinases of *T. foetus* [trichomonad conditioned buffer (TCB)] were obtained by incubation of 1×10^8 *T. foetus*/mL for 3.5 h in Dulbecco's phosphate-buffered saline (PBS) (Invitrogen, Grand Island, NY) with HEPES (10 mM), L-cysteine (0.1%) and ascorbic acid (0.02%) (pH 7.2) as previously described [7].

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2.2. Cysteine proteinase inhibitors

Vinyl sulfone CP inhibitors K11777 (*N*-methylpiperazine-urea-phenylalanyl-homophenylalanyl-vinylsulfone-benzene) and its derivative WRR-483 (with arginine substituted for phenylalanine at the P_2 position) were obtained from Dr James McKerrow (University of California–San Francisco, San Francisco, CA) [9]. Epoxide E-64 (*N*-[*N*-{L-3-trans-carboxirane-2-carbonyl}-L-leucyl]-agmatine) (Roche, Indianapolis, IN), an irreversible wide-range CP inhibitor, was included as a positive control. Stock compounds were diluted in dimethyl sulphoxide (DMSO) (0.4 mM).

2.3. Proteinase activity of Tritrichomonas foetus

The activity of *T. foetus* SUP and TCB following incubation with inhibitors was determined by release of the fluorescent leaving group, 4-amino-7-methylcoumarin (AMC), from synthetic peptide substrates. Aliquots of SUP and TCB (25 μL) were combined with synthetic peptide substrate Z-Arg-Arg-AMC (25 mM) (Bachem, Torrance, CA) diluted in 75 μL of Tris (0.5 M), NaCl (0.15 M), ethylene diamine tetra-acetic acid (EDTA) (0.5 mM), dithiothreitol (DTT) (1 mM) and Triton X-100 (0.003%) (pH 7.5). Relative fluorescent units (RFU) were measured in a microplate fluorometer (Thermo Labsystem Fluoroskan Ascent; Labotal Scientific Equipment, Abu-Gosh, Israel). The 50% effective concentration (EC_{50}) was determined as the concentration of inhibitor that inhibited 50% of the initial activity.

The effect of the specific CP inhibitors on extracellular proteinase activity during *T. foetus* culture (SUP) was determined by incubating 10^7 *T. foetus*/mL with or without proteinase inhibitors in the culture media for up to 48 h. This was a large inoculum so is not measuring release during growth but rather during maintenance in culture medium. Inhibition of CP activity in TCB was determined by incubating with or without CP inhibitors for 25 min at room temperature (RT).

2.4. Co-culture of trophoblast cells and Tritrichomonas foetus

Bovine trophoblast cells (strain 87.3), originally isolated by Dr Linda Munson (University of California–Davis, Davis, CA) were maintained in Dulbecco's Modified Eagle Medium/F12 (1:1) (Gibco®; Invitrogen) supplemented with foetal bovine serum (FBS) (10%) (Benchmark Gemini, Sacramento, CA), insulin (5.8 μg/mL), transferrin (5.3 μg/mL), selenite (5.8 ng/mL) (Sigma, St Louis, MO), epidermal growth factor (10 ng/mL) (Becton Dickinson & Co., Sparks, MD) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Thermo Scientific HyClone, South Logan, UT) in a humidified 37 °C, 95% air/5% CO_2 environment. Trophoblast cells were grown in 96-well flat-bottom tissue culture plates (Costar, Corning, NY) by inoculating 75 μL containing 1.8×10^4 cells/well for 24–48 h until confluent (at which time the cell count equalled 2.4×10^4 cells/well). To harvest cells, 0.05% trypsin/EDTA (Gibco; Invitrogen) (25 μL/well) was added and incubated for 10–15 min at 37 °C before dilution with medium (75 μL/well). After two washes with PBS (100 μL/well), confluent trophoblast cells were incubated for up to 36 h with 10 multiplicity of infection (MOI) of *T. foetus* pre-incubated or not with inhibitors (20–100 μM) for 25 min at RT (2.4×10^5 trophozoites/well, diluted in 75 μL of medium without serum). Trichomonads were removed by three washes with PBS and then PBS was added (75 μL/well) before storage at −70 °C for 1–4 days. Viability of trophoblast cells was determined by measurement of ATP. Frozen 96-well plates containing treated trophoblast cells were equilibrated at RT for 20 min before treatment at RT with 75 μL/well of lysis buffer (BacTiter-Glo™; Promega, Madison, WI) for 5 min. The total volume (150 μL) was transferred to solid white 96-well plates (MaxiSorp® surface; Nunc, Rochester, NY) and end-point luminescence was recorded in a microplate reader (SpectraMax M-5; Molecular Devices, Sunnyvale, CA). Cytotoxicity for trophoblast cells [relative ATP luminescence units (RLU)] was expressed as percent ATP from control trophoblast cells with same treatment but without *T. foetus* [(RLU from trophoblast cells exposed to *T. foetus* × 100)/RLU from trophoblast cells identically treated but not exposed to *T. foetus*]. Samples were done in triplicate.

2.5. Effect of cysteine proteinase inhibitors on Tritrichomonas foetus murine genital infection

Six-week-old, female, BALB/c mice (Charles River Laboratories, Hollister, CA) maintained under specific pathogen-free conditions were infected intravaginally with *T. foetus* as described previously [12] with modifications. Trophozoites of *T. foetus* were harvested by centrifugation and washed with PBS. The final pellet was suspended in TYI medium with 0.32% agar (Bacto™ Agar; Becton, Dickinson & Co.) to contain 10^4 trophozoites/mL and each mouse was inoculated in the fornix of the vagina with 10 μL of this suspension (10^2 *T. foetus*/mouse). The infective dose of 10^2 *T. foetus* was previously shown to persistently infect non-oestrogenised mice [13]. Vaginal samples were collected by flushing with PBS (50 μL) before inoculation and thereafter. Approximately 40 μL of each vaginal sample was cultured in TYI medium (500 μL) containing penicillin (200 U/mL)/streptomycin (200 μg/mL) (Gibco; Invitrogen), amphotericin B (0.5 μg/mL) (CellGro®; Mediatech, Herndon, VA), kanamycin sulphate (200 μg/mL) (AllStar, Sunnyvale, CA) and gentamicin sulphate (10 μg/mL) (Fisher Scientific, Fair Lawn, NJ). The remaining sample (10 μL) was used for counting trichomonads in a haematocytometer chamber to determine the number in 50 μL. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California–San Diego (San Diego, CA).

2.6. Statistical analysis

Differences between groups were compared by unpaired Student's *t*-test or analysis of variance (ANOVA) with Bonferroni post-tests (GraphPad Prism 4.0; GraphPad Software Inc., La Jolla, CA). Data are given as the mean ± standard error of the mean of one of two to three independent experiments. The level of significance was set at $P < 0.05$.

3. Results

3.1. Cysteine proteinase inhibitors decreased extracellular proteinase activity of Tritrichomonas foetus but did not kill trophozoites

It was first shown that *T. foetus* extracellular proteinase activity in TCB is blocked by vinyl sulfone CP inhibitors or E-64. In this experiment, EC_{50} values were 0.001 μM for WRR-483, 0.01 μM for E-64 and 6.5 μM for K11777 against TCB. Next, whether incubation of *T. foetus* with inhibitors during culture affected CP activity was investigated. None of the inhibitors killed *T. foetus* as the numbers of viable trophozoites were similar to untreated trophozoites over 48 h (Fig. 1A). However, addition of one dose of WRR-483 (20 μM) and E-64 (100 μM) inhibitors at time 0 to *T. foetus* cultures decreased the activity of SUP on Z-Arg-Arg-AMC for up to 48 h, whilst incubation with one dose of K11777 (100 μM) reduced this activity for 24 h ($P < 0.05$) (Fig. 1B). Similar results were obtained with trophozoites cultured in minimal essential M199 medium without serum, although the number of trophozoites decreased over time and CP production was lower than in TYI with serum (maximum enzymatic activity at 24–48 h in untreated *T. foetus*

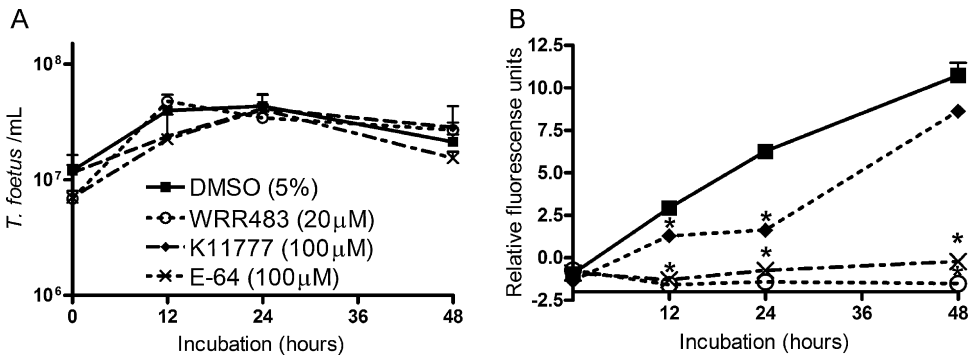

Fig. 1. (A) Survival of *Tritrichomonas foetus* and (B) production of released proteases after treatment with cysteine proteinase inhibitors. Trophozoites of *T. foetus* (10^7/mL) were incubated with inhibitors (added once at the beginning) at 37 °C in trypsin–yeast–iron (TYI) medium with serum. Viable trophozoites of *T. foetus* were counted by haematocytometer (A) and protease activity in culture supernatant was measured against Z-Arg-Arg-AMC (pH 7.5) (B). All three inhibitors (WRR-483, E-64 and K11777) blocked protease activity compared with control untreated cells in dimethyl sulphoxide (DMSO) (*$P < 0.05$), although inhibitors did not kill *T. foetus*.

of 190 RFU in TYI and 1.5 RFU in M199). Thus, vinyl sulfone CP inhibitors decrease the activity of *T. foetus* extracellular CP for 24–48 h during culture.

3.2. Cytotoxicity for bovine target cells and murine genital colonisation of Tritrichomonas foetus are decreased by vinyl sulfone cysteine proteinase inhibitors

Bovine trophoblast cells were chosen for study because these cells, which line the placenta, are natural host target cells for reproductive failure caused by *T. foetus*. Pre-treatment of *T. foetus* with CP inhibitor K11777 (20 μM) decreased cytotoxicity for trophoblast target cells by ca. 50% throughout the 48 h experiment ($P < 0.05$) (Fig. 2). No significant inhibition was detected with WRR-483 (20 μM) and E-64 (20 μM) (Fig. 2). Inhibitors did not affect the viability of trophoblastic cells.

Next, the effect of CP inhibitors on genital colonisation of *T. foetus* was evaluated in a murine model. Mice challenged intravaginally with 10^2 *T. foetus* pre-incubated with WRR-483 (100 μM) or K11777 (100 μM) showed a significant reduction in the vaginal load of trophozoites at 3 days after challenge ($P < 0.05$) (Fig. 3). By 6 days post inoculation of trichomonads pre-incubated with inhibitors, no significant difference between groups of mice was detected (Fig. 3).

4. Discussion

This study demonstrates that vinyl sulfone CP inhibitors decrease cytotoxicity for bovine trophoblast target cells and temporarily decrease numbers of trichomonads in a mouse model of genital infection. This was not due to killing of trichomonads because treatment of *T. foetus* in vitro did not decrease *T. foetus* numbers, even though activity of extracellular CP was impaired. The cytotoxicity of the predominant extracellular proteinase of *T. foetus*, CP8 or CP30, for bovine uterine and vaginal epithelial cells was demonstrated previously [5,6], so K11777 and WRR-483 likely inhibit cytotoxicity to several target cells associated with bovine reproductive failure. We have previously shown that *T. foetus* CP8 is expressed by *T. foetus* in the bovine uterus during infection [14]. This indicates that inhibition of *T. foetus* extracellular CP8 proteinase-induced cytotoxicity would be relevant in the uterus during infection since uterine and placental trophoblast epithelial cells would be the critical target cells. It is possible that the first step in cytotoxicity due to *T. foetus* extracellular proteinase is adherence of the parasite to epithelial cells. This hypothesis is based on studies showing that *T. vaginalis* CPs mediate attachment to human vaginal epithelial cells or HeLa cells, and abrogation of adherence by treatment of *T. vaginalis* with TLCK, a broad-spectrum inhibitor of CP, prevented cytotoxicity as well as adherence [15]. Thus, part of the mechanism for increased *T. foetus* clearance in mice may be decreased adherence to genital tissues in the presence of CP inhibitors. We previously showed that immunisation of cattle with a lipophosphoglycan–protein conjugate (TF 1.17) of *T. foetus* accelerates parasite clearance from the genital tract [16]. Perhaps prophylactic or therapeutic vaccination with TF 1.17 antigen followed by treatment with CP inhibitors would completely

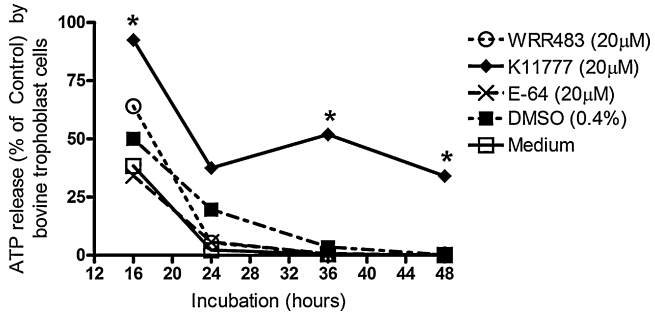

Fig. 2. Cytotoxicity of *Tritrichomonas foetus* for bovine trophoblast cells in the presence or absence of cysteine proteinase inhibitors. Trophoblast cells were incubated with *T. foetus* (10 multiplicity of infection) pre-incubated with proteinase inhibitors or control dimethyl sulphoxide (DMSO). Cytotoxicity was determined in an ATP assay [relative luminescence units (RLU)] and was expressed as percent release of ATP by control trophoblast cells with same treatment but without *T. foetus*. The inhibitor K11777 significantly decreased the cytotoxicity effect of *T. foetus* compared with the DMSO vehicle control. *$P < 0.05$.

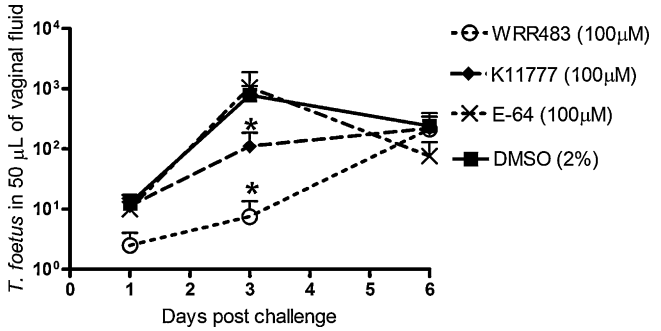

Fig. 3. Inhibition of murine genital colonisation by *Tritrichomonas foetus* pre-treated with cysteine proteinase inhibitors. Five mice per group were infected intravaginally with 10^2 *T. foetus* pre-incubated with proteinase inhibitors and then trophozoites were quantified in vaginal secretions. The inhibitors WRR-483 and K11777 reduced the number of trichomonads in vaginal secretions for up to 3 days post challenge compared with the control mice treated with the dimethyl sulphoxide (DMSO) vehicle. *$P < 0.05$.

eliminate *T. foetus* as well as the accompanying tissue damage due to CPs.

Since the in vitro data suggested that a single dose of the vinyl sulfones inhibited CP activity in culture for 24–48 h, repeat dosing would likely be required in vivo. This is suggested by the observation that decreased worm and egg burdens of *S. mansoni* and reduced organ pathology were achieved after treatment with K11777 for 1–5 weeks intraperitoneally [8]. Likewise, K11777 reduced footpad thickness in mice infected with *L. tropica* when given intraperitoneally daily for 28 days [10]. Therefore, multiple doses of CP inhibitors may be necessary to control *T. foetus* infection as well. It will be interesting to test whether CP inhibitors and nitroimidazoles might be synergistic because of their different mechanisms of action. If so, combination therapy may overcome drug resistance. In conclusion, this study shows that the vinyl sulfone CP inhibitors WRR-483 and K11777 reduce some of the pathogenic effects of trichomonad extracellular CPs and may represent a therapeutic strategy against *T. foetus* as well as the closely related human pathogen, *T. vaginalis*.

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Ethical approval: Not required.

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