UNIVERSITY OF CALIFORNIA SAN DIEGO

Vaginal *Tritrichomonas foetus* infection in mice as an *in vivo* model for drug development against *Trichomonas vaginalis*

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by

Noelle M. Nieskens

Committee in charge:

Professor Lars Eckmann, Chair
Professor Emily Troemel, Co-Chair
Professor James Golden

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The Thesis of Noelle M. Nieskens is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Vaginal *Tritrichomonas foetus* infection in mice as an *in vivo* model for drug development against *Trichomonas vaginalis*

by

Noelle M. Nieskens

Master of Science in Biology

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Professor Lars Eckmann, Chair
Professor Emily Troemel, Co-Chair

Trichomonas vaginalis is the causative agent of the common sexually transmitted disease, trichomoniasis, which affects more than a hundred million people worldwide. Metronidazole and tinidazole, agents belonging to the 5-nitroheterocyclic class of antimicrobials, are most often

used to treat infection, but increased resistance has been reported and adverse effects of the drug can be significant. Consequently, an urgent need exists for the development of novel drug entities against trichomoniasis. Critical for antimicrobial drug development is the demonstration of *in vivo* efficacy. Murine models of vaginal *T. vaginalis* infection are often not reliable, while infections with the related bovine pathogen, *Tritrichomonas foetus*, tend to be more robust, although susceptibility to different antimicrobials might differ from *T. vaginalis*. Here, we explored the utility of *T. foetus* infection as a surrogate model for drug development against *T. vaginalis*. Four different *T. foetus* strains caused robust vaginal infection in young mice, while none of four diverse *T. vaginalis* strains did. Comparison of drug susceptibility profiles revealed that *T. foetus* and *T. vaginalis* were similarly susceptible to a range of 5-nitroheterocyclic and gold(I) compounds. By comparison, proteasome inhibitors were 10- to 15-fold less active against *T. foetus* than *T. vaginalis*, although one of the proteasome inhibitors, bortezomib, had low micromolar activity or better against multiple strains of both trichomonads. Different strains of *T. foetus* were used to demonstrate the utility of the murine vaginal infection models for *in vivo* efficacy testing, including for bortezomib and a gold(I) compound. The differences in susceptibility to proteasome inhibitors may be partially explained by differences in the proteasome subunit sequences between the two trichomonads, although the functional relevance of the proteasome was similar in both organisms. These findings indicate that *T. foetus* can serve as a reliable surrogate model for *T. vaginalis in vitro* and in murine infections *in vivo*, but caution must be exercised for specific drug classes with targets, such as the proteasome, that may display genetic divergence between the trichomonads.

INTRODUCTION

Trichomoniasis, caused by infection with the protozoan parasite *Trichomonas vaginalis*, is the most common, non-viral sexually transmitted disease in the world with a global incidence of ~156 million and an incidence of 6.9 million in the U.S. alone (WHO, 2021). The parasite can adhere to a variety of genitourinary cell tissues, including the urethra, vagina, and cervix of women, and the prostate and urethra of men (Soper, 2004). Infected women can experience a broad range of symptoms, including abnormal vaginal discharge, odor, and itching, vulvar or vaginal erythema, and colpitis macularis ("strawberry cervix"). Many women remain infected for extended periods (Wølner-Hanssen et al., 1989). Infection in men can lead to mild to moderate urethral discharge and urethral inflammation, but symptoms tend to be less severe than in women (Krieger, 1995; WHO, 2021). The global prevalence of *T. vaginalis* infection is almost 10 times lower in men compared to women (WHO, 2021), which may reflect a lack of testing due to milder infection symptoms or could be related to an abbreviated course of infection. Nonetheless, lack of symptoms does not protect an individual from the complications associated with infection. For example, *T. vaginalis* infection can increase susceptibility to human immunodeficiency virus (HIV) infection by 50% (Mirmonsef et al., 2012). Furthermore, a positive correlation between HIV infection and trichomoniasis symptom severity was found in men (Price et al., 2004). In women, infection has also been linked to increased risk of cervical cancer, low infant birth weight, preterm birth, and stillbirths (Zhang et al., 1995; Cotch et al., 1997). In men, a decrease in sperm viability and motility was noted, suggesting a decrease in fertility (Gopalkrishnan et al., 1990). To avoid potential complications, it is important to treat infection upon diagnosis, particularly since infection can last for months to years (CDC, 2022).

Metronidazole and tinidazole are two antimicrobials currently FDA-approved for the treatment of trichomoniasis (Fung & Doan, 2005). They belong to the class of 5-nitroheterocyclic drugs. Metronidazole, first developed in 1959, is the primary drug for trichomoniasis treatment worldwide (Löfmark et al., 2010). Tinidazole, a structural derivative of metronidazole, was FDA-approved for trichomoniasis treatment in 2004 (Fung & Doan, 2005). They are prodrugs that must be reduced into a free radical inside microbial cells before they can covalently bind to multiple protein and nucleic acid targets. Adduct formation leads to inhibition of DNA synthesis and many vital protein functions, eventually killing the organism. Both drugs primarily target anaerobic microorganisms, including *T. vaginalis*, as they have a low enough negative redox potential to donate electrons to the molecule (Löfmark et al., 2010). For metronidazole, cure rates range from 86-97% (Lossick, 1980; Hager et al., 1980; Aubert & Sesta, 1982). Despite its general effectiveness, it is estimated that 2-10% of all clinical trichomoniasis cases are caused by strains that display resistance to metronidazole and other 5-nitroheterocyclic drugs (Kirkcaldy et al., 2012; Schmid et al., 2001; Schwebke & Barrientes, 2006). Beyond loss of efficacy due to resistance, metronidazole can have significant adverse effects that compromise its use and effectiveness. Thus, a large percentage of women treated with the drug have reported nausea, vomiting, unpleasant taste, diarrhea, headache, vaginal irritation, and yeast infections (Ayinde & Ross, 2023; Kissinger et al., 2018). Currently no other drug classes are FDA approved to treat trichomoniasis. With the threat of growing resistance, and significant adverse effects, development of alternative antimicrobials in other drug classes remains urgent for treatment of trichomoniasis.

Library screens of approved FDA drugs and drug candidates have been done to identify new activities against *T. vaginalis* but have not resulted in promising candidates (Goodhew &

Secor, 2013). *De novo* drug design against unexploited targets is currently the most realistic strategy for development of new antitrichomonal agents. One such strategy is exemplified by proteasome inhibitors (O'Donoghue et al., 2019). The proteasome is an enzymatic complex present in all eukaryotic and some prokaryotic cells, whose function is to degrade and recycle proteins that are misfolded or not needed (Coux et al., 1996). The enzyme complex consists of a barrel-like structure, consisting of two pairs of rings with seven α-subunits and seven β-subunits. Peptide substrates are fed through the center of the barrel where they cleaved into smaller fragments. Three of the 14 proteasome subunits are catalytically active: β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like). Each cleaves the substrate at its preferred site via hydrolysis (Kisselev et al., 1999). Proteasome inhibitors were first developed as anti-inflammatory agents and as novel agents for cancer treatment (Raedler, 2015). More recently, they have been found to be effective against *Plasmodium falciparum*, the protozoan responsible for malaria. The proteasome inhibitor bortezomib, which is FDA-approved for the treatment of multiple myeloma and mantle cell lymphoma, was found to have an inhibitory effect against *P. falciparum in vitro* (Reynolds et al., 2007). Importantly, several new proteasome inhibitors were shown to be selective for the parasite over human cells (Tschan et al., 2013). *T. vaginalis* also possesses a proteasome that can be targeted for the development of new agents against the parasite, since several proteasome inhibitors were active against the parasite *in vitro* and have modest selectivity over human cells (O'Donoghue et al., 2019).

Critical for antimicrobial drug development is the demonstration of *in vivo* efficacy. Ideally, animal models of infection can be used that employ the very same pathogens that infect humans. In the case of trichomoniasis, several reports have shown that mice can be infected vaginally with *T. vaginalis* (Cobo et al., 2011; McGrory & Garber, 1992; Abraham et al., 1996),

but infections tend to be tenuous and unreliable for poorly understood reasons. As an alternative, mouse infection models with surrogate trichomonad pathogens can be used if it can be shown that those infections are robust, and the pathogens respond to antimicrobial candidates in similar ways to the human target pathogens. For example, vaginal infection of mice with the bovine pathogen, *Tritrichomonas foetus*, have been shown to cause sustained infection (Cobo et al., 2011). *T. foetus* is a protozoan parasite that causes tritrichomoniasis in cattle, a similar disease to human trichomoniasis. The parasite is closely related to *T. vaginalis*, being in the same phylum (Iriarte et al., 2023). Despite its promise, the broader utility of this model for drug development against *T. vaginalis* remains to be established. Therefore, the aims of this study were to establish infectious characteristics in mice, similarities in *in vitro* drug responses, and structural target similarities of a set of *T. foetus* strains in relation to *T. vaginalis* and establish the overall utility and limits of the murine *T. foetus* model as a basis for current and future drug development against *T. vaginalis*.

MATERIALS AND METHODS

Trichomonad strains

T. foetus strains 003 (ATCC 30003), 166 (ATCC 30166), 232 (ATCC 30232), 924 (ATCC 30924), and *T. vaginalis* strain G3 (ATCC PRA-98) were obtained from the American Type Culture Collection (Manassas, VA). *T. vaginalis* strains R88, MSA1172, S520 and S1489 were kindly provided by Dr. William Secor (Centers for Disease Control and Prevention, GA). *T. foetus* strain D1 (Skirrow & BonDurant, 1990) and *T. vaginalis* strain F1623 (Brown et al., 1999) were obtained from prior studies. Trichomonads were grown in trypticase-yeast extract maltose (TYM) medium, supplemented with 10% horse serum (Omega), 0.74% ammonium iron (II) sulfate hexahydrate (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich), and adjusted to a pH of 6.2. Cultures were grown axenically at 37°C, without CO_2.

Antimicrobial compounds

The following 5-nitro heterocyclic and gold(I) compounds were purchased: metronidazole (Sigma-Aldrich), ronidazole (APExBIO), tinidazole (TCI America), nitazoxanide (AdipoGen), nithiamide (MedChemExpress), auranofin (Enzo Life Sciences), (Tri-*n*-ethylphosphine) gold(I) chloride (compound 4, CPD4), (Tri-*n*-methylphosphine) and gold(I) chloride (compound 10, CPD10) and myochrysine (Sigma-Aldrich). The gold(I) compounds (Tri-*n*-propylphosphine) gold(I) chloride (CPD11), (Triisopropylphosphine) gold(I) chloride (CPD12), Bis(triethylphosphine) gold(I) chloride (CPD14), and Bis(tri-*n*-propylphosphine) gold (I) chloride (CPD15) were synthesized and characterized as described previously (Miyamoto et al., 2021). Compound numbering follows prior descriptions for gold(I) compounds (Miyamoto et al., 2021). The proteasome inhibitors, bortezomib, ixazomib and carfilzomib were obtained from

MedChemExpress. The benzimidazole compounds, lansoprazole, omeprazole, albendazole and fenbendazole, were also purchased from MedChemExpress. Pantoprazole was obtained from APExBIO and disulfiram was purchased from TCI America.

Mice

BALB/cJ mice (obtained from The Jackson Laboratory) were bred in-house. Female mice were used within one week of weaning at 3-4 weeks of age. Groups of mice were housed in single cages with free access to feed and water. All animal studies were reviewed and approved by the University of California San Diego Institutional Animal Care and Use Committee under protocol number S00205.

Vaginal infections and treatment

Trichomonads were cultured overnight, centrifuged, and resuspended at a density of 2 x 10^8 trophozoites/mL. Five µL of this suspension (containing 10^6 trophozoites) were slowly administered intravaginally into mice with a 2-20 µL micropipette (Gilson). Mice were held briefly upside-down after agent administration to minimize leakage.

For some experiments, mice were pretreated one day prior to infection with one of the following: 100 µL intraperitoneal (i.p.) injection of 4 mg/mL 17β-estradiol (Sigma-Aldrich), 200 µL i.p. injection of 4 mg/mL 17β-estradiol, 100 µL i.p. injection of 10 mg/mL ammonium iron (III) citrate (Sigma-Aldrich), or 5 µL intravaginal administration of 10 mg/mL ammonium iron (III) citrate. The ammonium iron (III) citrate solution was made in PBS containing 1% hypromellose. Control mice received no pretreatment.

For treatments, 1% drug suspensions were prepared in 0.2% hypromellose in PBS. Control mice received only hypromellose solution. Five µL of the suspensions were administered intravaginally as outlined above. A total of five treatments were given over 2.5 days, starting one day after infection.

Infectious load was determined on day 4 after infection. Using 30 µL of TYM media, mice were lavaged intravaginally by slowly dispensing the liquid into the vagina, taking it up again, and repeating the process two more times. The washes were diluted to 50 µL in TYM media, and 10 µL of the diluted washes were placed into a hemocytometer for counting of motile trophozoites under a phase-contrast microscope. Infectious load is expressed as total number of trophozoites per wash per mouse.

***In vitro* drug testing**

Drug stocks were prepared as 10 mM solutions in DMSO. A 75 µM working dilution was made in PBS, followed by 1:3 serial dilutions in TYM media in 96-well plates. Parasite cultures grown overnight in a 15 mL tube were centrifuged and resuspended to a density of 7×10^5 - 2×10^6 trophozoites/mL. Ten µL of this suspension were added into each well, resulting in a final number of 7,000 - 20,000 trophozoites per well. Plates were incubated at 37°C for 24 hours under anaerobic conditions (AnaeroPack, Mitsubishi Gas Chemical). Viable cell numbers were determined with a luciferase-based ATP assay (Promega) (Sule et al., 2009). Luminescence was measured with the SpectraMax M2^e plate reader, using the SoftMax Pro program (version 7.0.3). To assay the minimum lethal concentration (MLC), drugs were added to 96-well plates, and serially 2-fold diluted in TYM media. Parasites were added to each well at a density of 7×10^5/mL (*T. foetus* D1) or 10^6 trophozoites/mL (*T. vaginalis* R88), resulting in a final number of

28,000 or 40,000 trophozoites per well, respectively. Plates were incubated at 37°C for 24 hours under anaerobic conditions. Cultures (200 µL) were then transferred to media-filled 15 mL tubes, a 1:75 dilution, and kept at 37°C for a maximum of five days, for observation of any cell growth indicative of survivors of the initial drug treatment.

Proteasome analysis

For sequence analysis of proteasome subunits, a phylogenetic tree was generated using MEGA software (version 11.0.13). Protein sequences were aligned with the MUSCLE algorithm and an unrooted tree was constructed using the Le Gascuel model, with gamma distribution and invariant sites. Gaps were partially deleted, so that sites with missing data were only removed when necessary. UniProt accession numbers were not available for all organisms, so GenBank accession numbers are shown when applicable in Fig. 6. Genome information for the following parasite strains were used for the analysis: *T. foetus* CC09-1, *T. vaginalis* G3, and *P. falciparum* NF54. Proteasome subunit designation followed the findings in Gomes, 2013.

For immunoblotting, *T. foetus* D1 and *T. vaginalis* R88 cultures were incubated at 37°C for 1 hour with 0.3 µM or 3 µM of bortezomib, or with DMSO as a control. Lysates were made with radioimmunoprecipitation (RIPA) buffer (Cell Signaling Technology), with added Halt protease inhibitor cocktail (Thermo Scientific). Proteins were separated on a 4-20% SDS-PAGE gel (Bio-Rad), transferred onto a PVDF membrane (Millipore) and blocked with 5% nonfat dry milk. The membrane was incubated with a primary rabbit anti-ubiquitin polyclonal antibody (Proteintech, Cat No. 10201-2-AP) at a 1:10,000 dilution and a secondary horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody (Cell Signaling Technology, Cat No. 7074S) at a 1:20,000 dilution. Proteins were detected with a chemiluminescent HRP substrate (Millipore).

For labeling of catalytically active proteasome subunits with an activity-based probe, total cell lysates of *T. foetus* D1 or *T. vaginalis* F1623 were incubated at 37°C for 1 hour with 0.1, 1, 2, 5, or 10 μM of bortezomib, or with DMSO as a control. Afterwards, 2 μM of the fluorescent proteasome probe, MV151 (R&D Systems, Cat No. I-190) (Verdoes et al., 2006), was added and lysates were further incubated at 37°C for 2 hours. Proteins were separated on a 4-20% SDS-PAGE gel, and bands were visualized by fluorescent imaging at excitation of 470 nm and emission of 530 nm.

Statistics

All analyses were performed with GraphPad Prism (version 10.0.0). For the compound activity screenings, luminescence values were normalized and plotted against the log drug concentration in each well to create a concentration-response curve for each drug. The half-maximal inhibitory concentration (IC_{50}), or concentration of drug needed to inhibit trophozoite growth and viability by 50%, and negative log10 of the IC_{50} (pIC_{50}) were calculated. The assay sensitivity for pIC_{50} was set at 4.7, equivalent to the highest drug concentration tested in the assays. Each experiment was repeated three times per compound and per trichomonad strain.

For *in vitro* data comparisons, parametric tests (ANOVA, t-tests) were used. For comparison of multiple groups, a one-way ANOVA was performed, and if a significant difference was found, Tukey's multiple comparisons test was applied to specific pairs. For *in vivo* data comparisons, a non-parametric Mann-Whitney test was used to account for a small sample size with skewed distribution.

Chapter 1 MURINE VAGINAL INFECTION MODEL WITH DIVERSE STRAINS OF *T. FOETUS* AND *T. VAGINALIS*

Previous studies had suggested that murine vaginal infections with *T. vaginalis* are characterized by low infectivity and/or short-lived infections, even with various manipulations of the host designed to increase susceptibility (Cobo et al., 2011). It is possible that marginal infectivity is related to the particular strains of *T. vaginalis* used for the infections, so we tested a range of four diverse strains of *T. vaginalis* for their ability to cause vaginal infection in young female mice. Three days after intravaginal inoculation, vaginal washes showed no detectable trophozoite counts for any of the tested strains (Fig. 1A). Furthermore, systemic (i.p.) or topical (vaginal) treatment with iron, which improves *T. vaginalis* growth in culture (Lehker & Alderete, 1992), or estrogen, which has been suggested to be improve susceptibility of mice to *T. vaginalis* infection (Meysick & Garber, 1992), did not promote infectivity (Fig. 1B). These experiments suggest that *T. vaginalis* cannot reliably, or perhaps not at all, infect mice, regardless of particular strains or conditioning regimens.

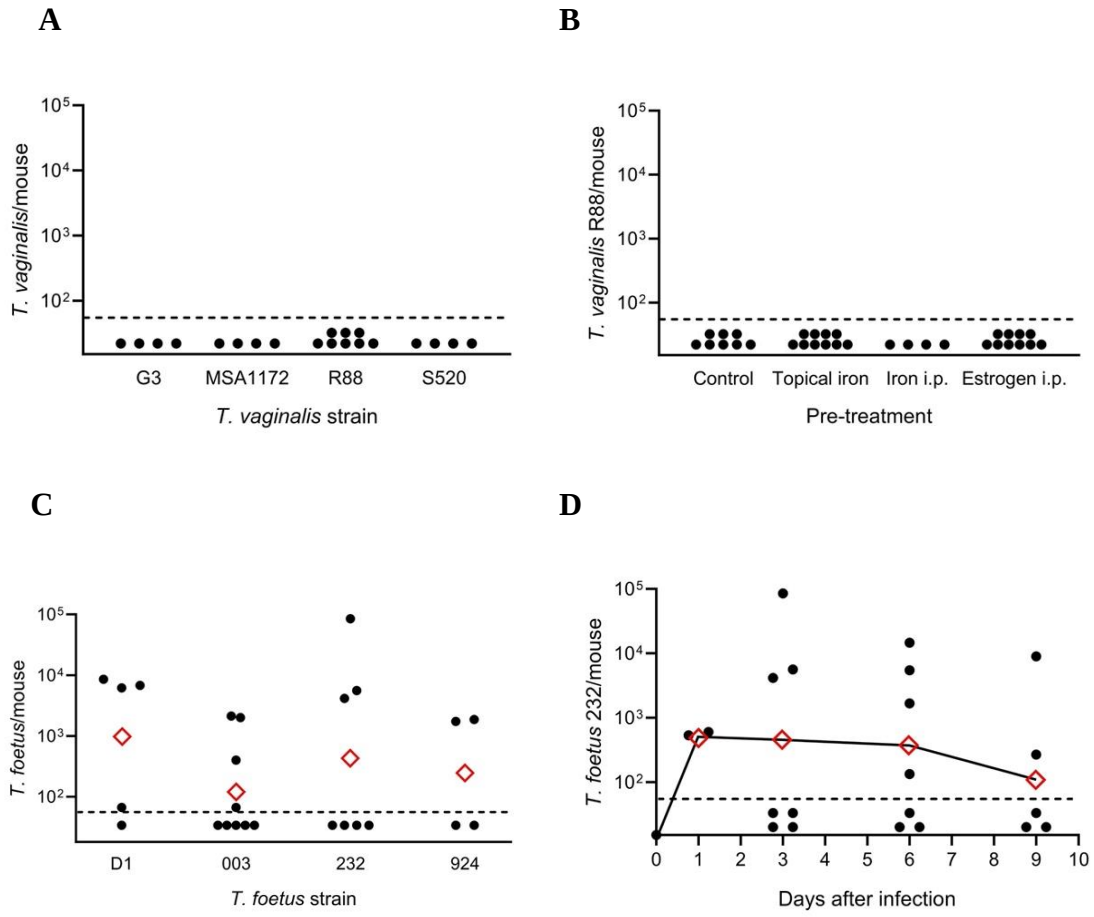

Figure 1: Murine susceptibility to vaginal *T. foetus* and *T. vaginalis* infection.

Young female BALB/cJ mice were intravaginally inoculated with the indicated strains of *T. vaginalis* (A, B) or *T. foetus* (C, D), vaginal lavages were taken on day 3 of infection (A-C) or on the indicated days (D), and motile trophozoites were counted in a hemocytometer. Data points represent individual mice, dotted lines indicate the detection threshold of the assays, and red diamonds show geometric means.

As an alternative to *T. vaginalis,* we explored the related trichomonad, *T. foetus,* which was originally derived from cows and had been used for mouse infections (Cobo et al., 2011, O'Donoghue et al., 2019, Natto et al., 2021). Four different *T. foetus* strains were obtained and tested for their vaginal infectivity in young female mice. All four strains were able to cause infection without any pre-treatment, albeit with some variation in infectivity between the strains, ranging from 43-80% of inoculated mice (Fig. 1C). To examine the time course of infection, we selected the *T. foetus* strain 232 and sampled mice every 2-3 days. The proportion of mice with detectable infection remained relatively constant and trophozoite loads remained similar over at least 9 days after infection, indicating a lack of spontaneous clearance during that period (Fig. 1D). These findings demonstrate that *T. foetus* constitutes a more reliable murine model of vaginal trichomonad infection than *T. vaginalis*.

Chapter 2 DIFFERENTIAL GROWTH CHARACTERISTICS OF *T. FOETUS* AND *T. VAGINALIS*

To explore potential reasons for the differential murine infectivity of *T. vaginalis* and *T. foetus*, we examined two parasite characteristics that may be plausibly involved, maximal growth rate and tolerance to different pH environments. The *T. vaginalis* strains had significantly slower growth rates in culture with longer doubling times compared to the *T. foetus* strains despite optimal growth conditions and media (Fig. 2A). Furthermore, *T. vaginalis* growth was inhibited by even modestly alkaline pH *in vitro*, while *T. foetus* was able to grow mostly unhindered under these conditions (Fig. 2B). These data indicate that intrinsic properties of the two trichomonad species exhibit significant differences in regard to growth and pH tolerance that may be, at least in part, responsible for the difference in the murine infectivity of *T. vaginalis* and *T. foetus*.

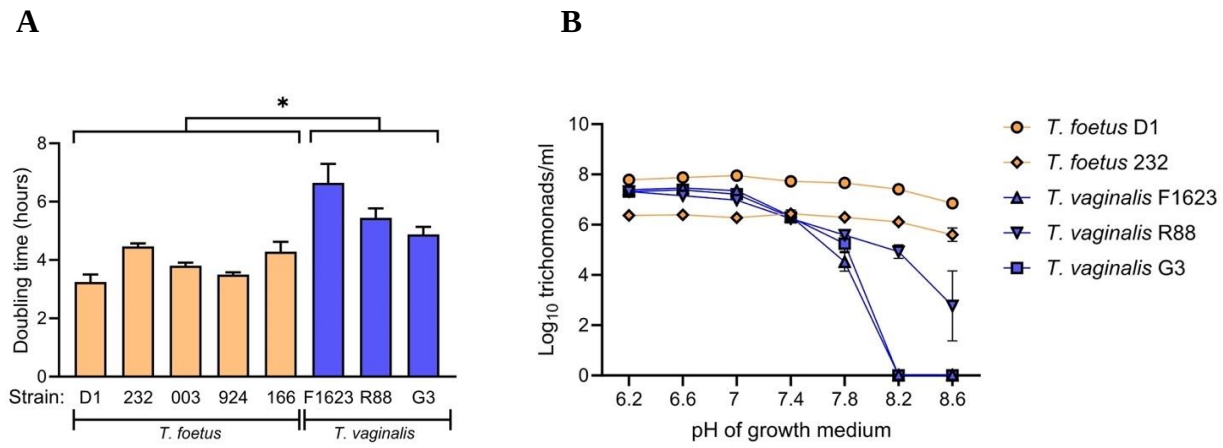

Figure 2: Growth characteristics of *T. foetus* and *T. vaginalis* strains.

Trophozoites of the indicated strains were cultured *in vitro* in trypticase-yeast extract-maltose (TYM) media. A. Cell numbers were counted before and after a 24-hour time incubation period and doubling times were calculated. Data are mean ± SE; * = $p < 0.05$ (unpaired t-test for all *T. vaginalis* strains vs. all *T. foetus* strains). B. Media were adjusted to the indicated pH and trophozoites were grown in each medium for 24 hours and counted. Data are mean ± SE.

Chapter 3 ACTIVITY PROFILES OF DIFFERENT ANTIMICROBIAL DRUG CLASSES AGAINST DIVERSE *T. FOETUS* AND *T. VAGINALIS* STRAINS

To compare susceptibility to antimicrobial drugs between the two trichomonad species, we screened different strains against four major drug classes that are clinically used in trichomoniasis or have been shown to have significant *in vitro* activity against *T. vaginalis*: 5-nitroheterocyclic drugs (Workowski & Levine, 2002), gold(I) compounds (Miyamoto et al., 2021), proteasome inhibitors (O'Donoghue et al., 2019), and benzimidazoles (Goodhew & Secor, 2013). The most consistently active compounds were 5-nitroheterocyclic compounds and gold(I) compounds (Table 1). By comparison, the tested proteasome inhibitors had excellent activity against the *T. vaginalis* strains but were significantly less potent against the *T. foetus* strains (Table 1). The benzimidazoles and another compound with reported *in vitro* activity against *T. vaginalis*, disulfiram (Goodhew & Secor, 2013), had only minimal or no activity against either *T. foetus* or *T. vaginalis* (Table 1).

Table 1: Activity of antimicrobial compounds against diverse *T. foetus* and *T. vaginalis* strains.

T. foetus and *T. vaginalis* cultures were incubated for 24 hours with a range of concentrations of the indicated drugs. ATP content was measured as a measure of cell growth and viability, and pIC_{50} values were calculated from the concentration-responses curves. Data are shown as mean ± SE of three or more independent experiments. IC_{50} values were derived from the mean pIC_{50}. Drugs that showed less than 50% inhibition at the highest tested concentration are shown as IC_{50} >20 µM and pIC_{50} <4.7.

		T. foetus strains								*T. vaginalis* strains					
		D1		232		924		166		F1623		R88		S1489	
Drug class	Drug name	pIC50 (mean ± SE)	IC50 (µM)	pIC50 (mean ± SE)	IC50 (µM)	pIC50 (mean ± SE)	IC50 (µM)	pIC50 (mean ± SE)	IC50 (µM)	pIC50 (mean ± SE)	IC50 (µM)	pIC50 (mean ± SE)	IC50 (µM)	pIC50 (mean ± SE)	IC50 (µM)
5-nitro heterocyclic compounds	Metronidazole	5.8 ± 0.15	1.5	5.6 ± 0.03	2.4	5.7 ± 0.09	1.9	5.8 ± 0.04	1.8	6.2 ± 0.06	0.62	6.0 ± 0.05	1.0	6.6 ± 0.13	0.26
	Ronidazole	6.0 ± 0.07	1.0	5.4 ± 0.32	3.9	5.6 ± 0.07	2.5	5.7 ± 0.18	1.8	6.1 ± 0.17	0.87	5.9 ± 0.03	1.3	6.3 ± 0.02	0.46
	Tinidazole	5.2 ± 0.19	6.0	5.1 ± 0.11	7.9	5.1 ± 0.11	7.6	5.3 ± 0.11	4.6	5.6 ± 0.06	2.3	5.6 ± 0.14	2.9	5.9 ± 0.09	1.2
	Nitaxozanide	5.5 ± 0.07	2.9	5.2 ± 0.14	6.0	5.6 ± 0.12	2.4	5.4 ± 0.09	4.1	5.8 ± 0.04	1.6	5.5 ± 0.04	3.5	6.0 ± 0.01	0.96
	Nithiamide	5.6 ± 0.07	2.4	5.3 ± 0.17	4.8	5.4 ± 0.07	3.9	5.4 ± 0.11	3.7	6.1 ± 0.06	0.89	5.7 ± 0.11	2.1	6.5 ± 0.04	0.36
Gold (I) compounds	Auranofin	6.6 ± 0.03	0.27	6.5 ± 0.07	0.30	6.1 ± 0.23	0.80	6.1 ± 0.15	0.88	6.5 ± 0.08	0.29	6.8 ± 0.19	0.17	7.0 ± 0.23	0.11
	CPD4	6.6 ± 0.10	0.25	6.3 ± 0.04	0.54	6.4 ± 0.04	0.42	6.2 ± 0.20	0.71	6.7 ± 0.20	0.18	6.8 ± 0.08	0.17	6.6 ± 0.30	0.26
	CPD10	5.2 ± 0.16	7.0	5.4 ± 0.20	4.1	5.3 ± 0.17	5.5	4.9 ± 0.06	12	5.9 ± 0.05	1.3	6.2 ± 0.12	0.69	6.2 ± 0.17	0.64
	CPD11	5.8 ± 0.12	1.6	5.5 ± 0.29	3.2	5.7 ± 0.16	1.8	5.3 ± 0.18	5.2	5.9 ± 0.11	1.2	5.9 ± 0.21	1.3	6.8 ± 0.29	0.17
	CPD12	6.2 ± 0.30	0.59	5.9 ± 0.12	1.4	6.0 ± 0.13	0.90	5.7 ± 0.23	1.9	6.1 ± 0.09	0.75	5.9 ± 0.26	1.2	6.7 ± 0.44	0.19
	CPD14	6.4 ± 0.04	0.39	6.0 ± 0.04	1.1	6.1 ± 0.06	0.72	5.8 ± 0.13	1.6	6.4 ± 0.04	0.40	6.3 ± 0.17	0.49	6.6 ± 0.08	0.26
	CPD15	6.5 ± 0.14	0.30	6.4 ± 0.05	0.39	6.3 ± 0.11	0.55	5.8 ± 0.13	1.6	6.5 ± 0.05	0.30	6.5 ± 0.20	0.30	6.8 ± 0.04	0.16
	Myochrysine	<4.7	>20	4.7 ± 0.03	19	<4.7	>20	4.7 ± 0.01	19	<4.7	>20	<4.7	>20	<4.7	>20
Proteasome inhibitors	Bortezomib	6.1 ± 0.15	0.74	5.9 ± 0.23	1.2	5.7 ± 0.06	2.2	6.0 ± 0.16	1.0	6.6 ± 0.08	0.24	7.0 ± 0.12	0.11	6.7 ± 0.15	0.19
	Ixazomib	5.5 ± 0.17	3.3	5.3 ± 0.27	4.8	5.0 ± 0.07	9.9	5.5 ± 0.18	3.5	6.4 ± 0.11	0.36	6.8 ± 0.11	0.16	6.8 ± 0.09	0.17
	Carfilzomib	4.8 ± 0.05	17	4.8 ± 0.10	16	4.8 ± 0.05	18	4.8 ± 0.04	16	5.2 ± 0.03	5.8	5.8 ± 0.63	1.7	7.1 ± 0.17	0.09
Benzimidazole compounds	Lansoprazole	4.7 ± 0.01	20	<4.7	>20	4.7 ± 0.02	19	<4.7	>20	4.8 ± 0.05	16	<4.7	>20	<4.7	>20
	Omeprazole	4.8 ± 0.05	18	<4.7	>20	4.7 ± 0.03	19	<4.7	>20	<4.7	>20	4.7 ± 0.02	19	<4.7	>20
	Pantoprazole	<4.7	>20	<4.7	>20	<4.7	>20	<4.7	>20	<4.7	>20	4.7 ± 0.01	19	<4.7	>20
	Albendazole	4.7 ± 0.01	20	4.7 ± 0.01	20	4.7 ± 0.03	18	<4.7	>20	<4.7	>20	<4.7	>20	<4.7	>20
	Fenbendazole	<4.7	>20	<4.7	>20	4.7 ± 0.03	18	<4.7	>20	<4.7	>20	<4.7	>20	4.7 ± 0.02	19
Miscellaneous	Disulfiram	5.5 ± 0.10	3.4	5.1 ± 0.33	7.6	5.4 ± 0.15	4.2	5.4 ± 0.35	4.3	5.8 ± 0.25	1.5	5.4 ± 0.58	4.4	5.9 ± 0.58	1.4

Chapter 4 COMPARISON OF DRUG SUSCEPTIBILITIES OF *T. FOETUS* AND *T. VAGINALIS*

Comparison of specific drugs within the three major drug classes with the greatest potencies revealed that all tested drugs were less active in *T. foetus*, although differences were observed between the drug classes (Fig. 3). 5-Nitroheterocyclic drugs and gold(I) drugs displayed, on average, a ~3-fold lower activity in the *T. foetus* strains compared to the *T. vaginalis* strains, but this difference did not reach statistical significance (Fig. 3). By comparison, the tested proteasome inhibitors were ~14-fold less active against *T. foetus* relative to *T. vaginalis*, which was statistically significant (Fig. 3).

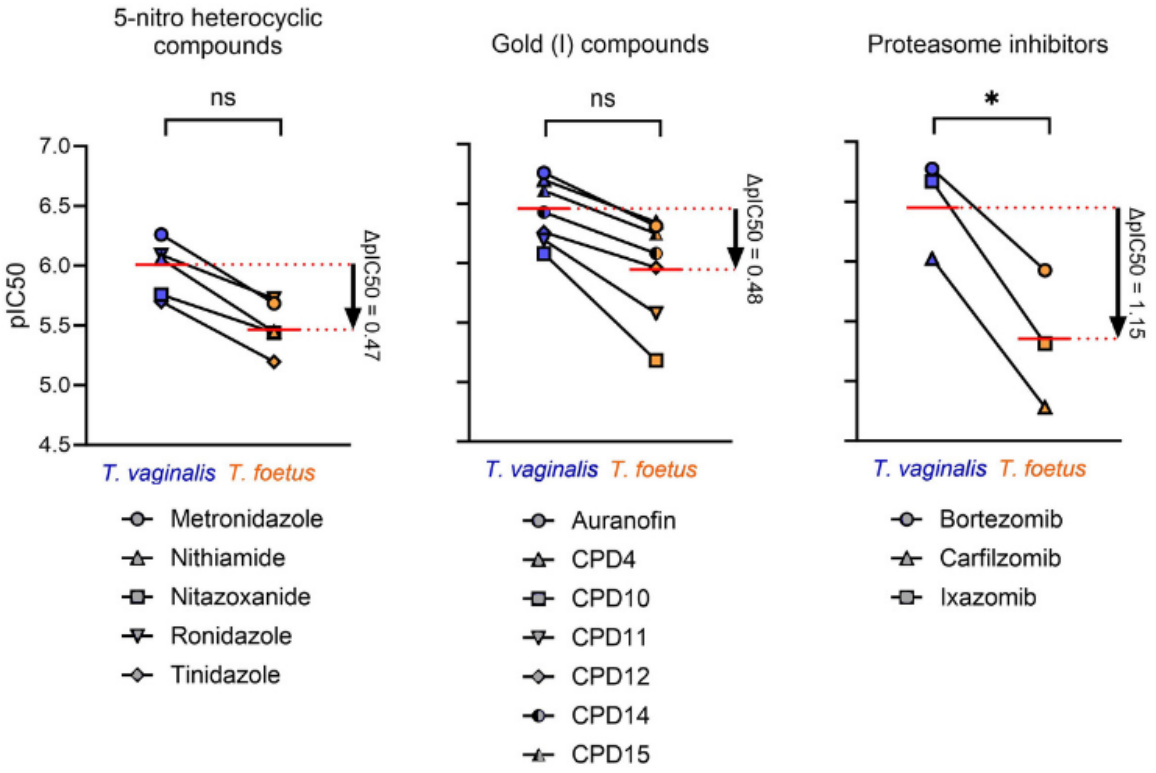

Figure 3: Comparison of *T. foetus* and *T. vaginalis* susceptibility to different antimicrobial drug classes.

Activity (pIC_{50}) of the indicated compounds from each of the three drug classes against multiple strains of *T. foetus* and *T. vaginalis* was determined by 24 h growth and survival assay (see Table 1). Each point represents the mean pIC_{50} of four (*T. foetus*) or three *(T. vaginalis*) different strains. ΔpIC_{50} was calculated for each drug class by subtracting the mean *T. foetus* pIC_{50}s from the mean *T. vaginalis* pIC_{50}s. Red lines represent geometric means. Significance was calculated with Tukey's multiple comparisons test (*p <0.01).

Pairwise comparisons of strains were conducted to discover whether any individual *T. foetus* strain had similar drug susceptibility profiles to any of the *T. vaginalis* strains. Strong positive correlations were seen among the different *T. foetus* strains for all three major drug classes (Table 2, example in Fig. 4A). In contrast, only modest average correlations were observed between strains of *T. foetus* vs. *T. vaginalis* (Table 2), which particularly affected the proteasome inhibitors and gold(I) compounds (Fig. 4B). This might be partially explained by the only modest average correlations of drug susceptibilities among the different *T. vaginalis* strains (Table 2). In particular, the *T. vaginalis* strain S1489 displayed a very different drug susceptibility profile than the other *T. vaginalis* strains (Table 2, Fig. 4C), thereby markedly skewing the correlation data for both *T. vaginalis* and *T. foetus* (Table 2).

Table 2: Comparisons of drug responses in different *T. foetus* and *T. vaginalis* strains.

Correlation plots were generated from the mean pIC_{50} values of the indicated pairs of trichomonad strains for the active drugs listed in Table 1. Linear regression was performed on each correlation to determine R^2 values and slopes of the curves of best fit. P-values state whether the slopes of the lines of best fit are significantly different from zero (i.e., no correlation); n.s., not significant. Mean R^2 values are shown for the different strain comparisons.

Comparisons	Strains	R^2	Slope	P-value
T. foetus vs. *T. foetus*	D1 vs. 166	0.85	0.63	< 0.01
	D1 vs. 232	0.87	0.85	< 0.01
	D1 vs. 924	0.89	0.80	< 0.01
	166 vs. 232	0.69	1.1	< 0.01
	166 vs. 924	0.65	0.99	< 0.01
	232 vs. 924	0.79	0.83	< 0.01
	Mean:	**0.79**		
T. foetus vs. *T. vaginalis*	D1 vs. F1623	0.73	0.63	< 0.01
	D1 vs. R88	0.34	0.55	n.s
	D1 vs. S1489	0.12	0.23	n.s
	166 vs. F1623	0.75	0.93	< 0.01
	166 vs. R88	0.36	0.82	n.s
	166 vs. S1489	0.06	0.24	n.s
	232 vs. F1623	0.73	0.69	< 0.01
	232 vs. R88	0.52	0.75	< 0.01
	232 vs. S1489	0.21	0.33	n.s
	924 vs. F1623	0.52	0.63	< 0.01
	924 vs. R88	0.17	0.45	n.s
	924 vs. S1489	0.05	0.18	n.s
	Mean:	**0.38**		
T. vaginalis vs. *T. vaginalis*	F1623 vs. R88	0.69	1.1	< 0.01
	F1623 vs. S1489	0.12	0.31	n.s
	R88 vs. S1489	0.38	0.44	n.s
	Mean:	**0.40**		

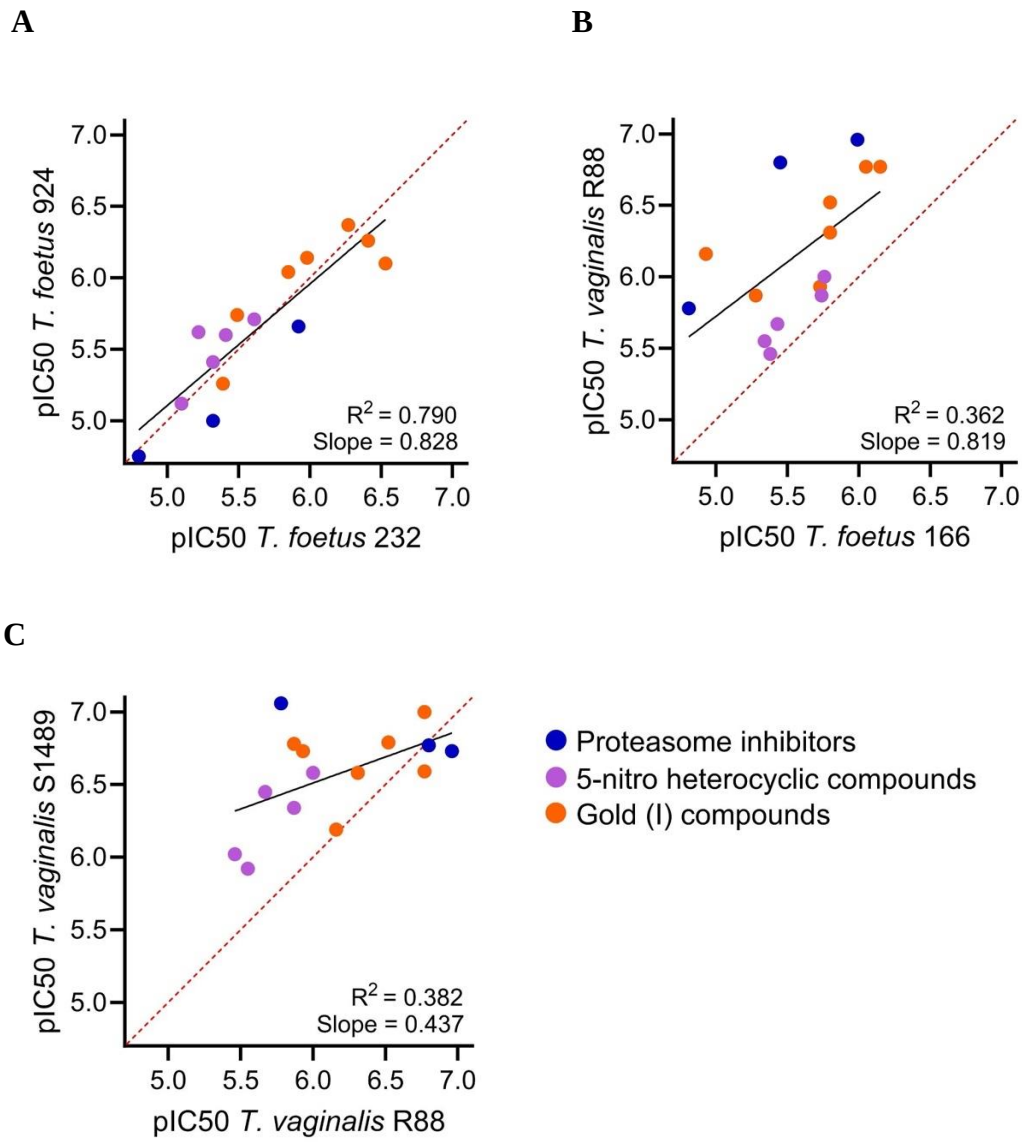

Figure 4: Correlation of drug susceptibility between different *T. foetus* and *T. vaginalis* strains. Graphs show correlations of the pIC_{50} values between the indicated pairs of trichomonad strains. Each point represents the pIC_{50} values of one drug and is color-coded by drug class. Plots are shown with a linear regression line (black) and line of identity (dotted red).

Together, these data indicate that *T. foetus* is generally less susceptible than *T. vaginalis* to all tested drugs, but this difference is particularly prominent and significant for the class of proteasome inhibitors.

Chapter 5 MLCS OF SELECTED DRUGS AGAINST *T. FOETUS* AND *T. VAGINALIS*

To further characterize antimicrobial drug activity in the two trichomonad species, we determined MLCs by culturing cells for 24 h in the presence of titrated drug concentrations, diluting the cultures 75-fold (to minimize the impact of residual drugs), and testing for survivors by outgrowth over the ensuing 5 days. It was found that having a minimum of 4% of culture being DMSO (corresponding to a concentration of 400 μM) was sufficient in killing the majority of trophozoites. Therefore, the experiment using 400 μM of carfilzomib was excluded. The MLCs of metronidazole as a representative 5-nitroheterocyclic compound, and auranofin as a representative gold(I) compound, were very similar for *T. foetus* or *T. vaginalis*, reflecting the trends of the respective IC_{50} findings (Table 3). As expected, MLCs were generally 3- to 10-fold higher than IC_{50}, consistent with the notion that complete parasite killing requires higher drug concentrations than growth inhibition by 50%. In contrast, MLC values could not be determined for the two tested proteasome inhibitors, even when tests were done with >100-fold higher drug concentrations than IC_{50} (Table 3). These results confirm that the antimicrobial activities of 5-nitroheterocyclic and gold(I) compounds are not significantly different between the two trichomonad species. The data also show that the tested proteasome inhibitors are not trichomonacidal for either *T. foetus* or *T. vaginalis*.

Table 3: MLC assays of *T. foetus* and *T. vaginalis*.

Cultures of the indicated trichomonad strains were incubated for 24 hours with the listed drugs at the indicated concentrations, diluted to minimize any residual drug impact, and further incubated for 5 days to detect any survivors (+, growth occurred indicating survivors; -, no growth occurred).

				Final concentration (μM)				
Compound	Strain	IC50 (μM)	Experiment	Survival (+/—)				
Metronidazole				**0.4**	**0.8**	**1.6**	**3.2**	**6.4**
	T. foetus D1	1.5	1	+	+	+	—	—
			2	+	+	+	+	—
			3	+	+	+	—	—
	T. vaginalis R88	1.0	1	+	+	+	—	—
			2	+	+	+	—	—
			3	+	+	+	—	—
Auranofin				**0.4**	**0.8**	**1.6**	**3.2**	**6.4**
	T. foetus D1	0.27	1	+	+	—	—	—
			2	+	+	+	—	—
			3	+	+	+	—	—
	T. vaginalis R88	0.17	1	+	+	+	—	—
			2	+	+	+	—	—
			3	+	+	+	—	—
Bortezomib				**12.5**	**25**	**50**	**100**	**200**
	T. foetus D1	0.74	1	+	+	+	+	+
			2	+	+	+	+	+
			3	+	+	+	+	+
	T. vaginalis R88	0.11	1	+	+	+	+	+
			2	+	+	+	+	+
			3	+	+	+	+	+
Carfilzomib					**25**	**50**	**100**	**200**
	T. foetus D1	17	1		+	+	+	+
			2		+	+	+	+
			3		+	+	+	+
	T. vaginalis R88	1.7	1		+	+	+	+
			2		+	+	+	+
			3		+	+	+	+

Chapter 6 *IN VIVO* EFFICACY TESTING OF DRUGS IN MURINE *T. FOETUS* INFECTION MODELS

To determine the utility of the new *T. foetus* infection models for efficacy testing, and to determine whether selected drugs are efficacious *in vivo*, we evaluated two representative compounds with excellent *in vitro* activity, the gold(I) compound, CPD4, and the proteasome inhibitor, bortezomib, using two different *T. foetus* strains. Mice infected with *T. foetus* 232 were topically treated five times over three days with CPD4 and infectious load was determined on day 4 (Fig. 5A). None of the CPD4-treated mice had countable trophozoites, while ~70% of control mice were infected with an average of ~10^3 trophozoites per mouse (Fig. 5B). Similarly, mice infected with the *T. foetus* strain D1 and treated with bortezomib showed no countable trophozoites, while all of the control mice had detectable infection (Fig. 5C). These results demonstrate that the murine vaginal infection models with different *T. foetus* strains are suitable for efficacy testing of new drug candidates against trichomonads.

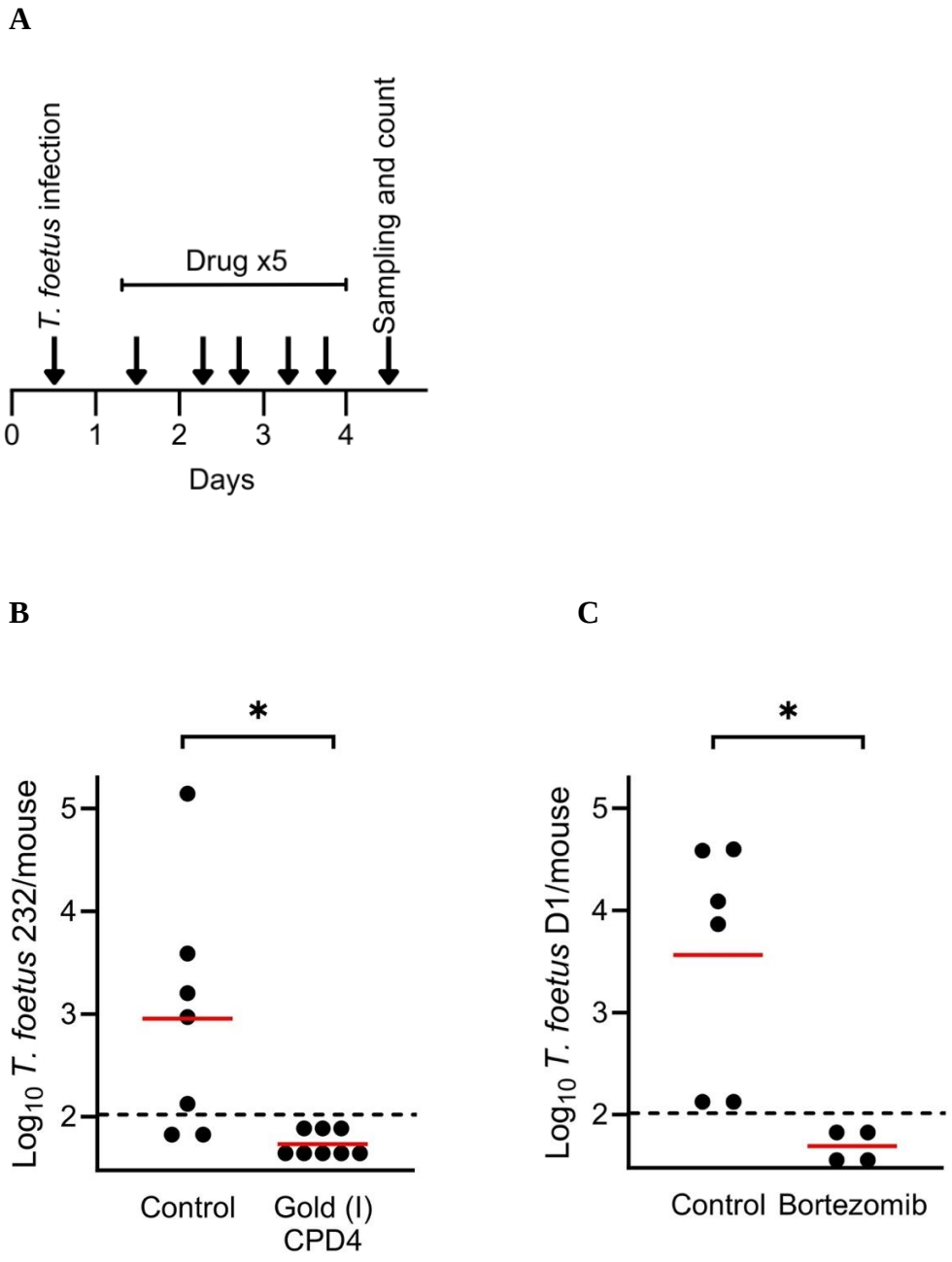

Figure 5: *In vivo* efficacy of select antimicrobial compounds against *T. foetus*.

Timeline of infection and treatment. B/C. Mice were infected with the indicated *T. foetus* strains and either treated topically with bortezomib (B) or the gold(I) compound CPD4 (C), or were left untreated as controls. After 4 days, trophozoite numbers were determined. Each point represents an individual mouse. Dashed black lines represent the detection threshold. Red lines represent geometric means. Significance was calculated by Mann-Whitney test (*p <0.01).

Chapter 7 CHARACTERIZATION OF *T. FOETUS* 20S PROTEASOME AS A DRUG TARGET

Bortezomib showed good *in vitro* and *in vivo* activity against *T. foetus*, yet it and the other proteasome inhibitors were markedly less potent against the different *T. foetus* strains than *T. vaginalis*. This discrepancy raised the question whether the *T. foetus* proteasome is substantially different from *T. vaginalis*. Analysis of the *T. foetus* genome identified all 14 proteasome subunits, seven α subunits (α1-7) and seven β subunits (β1-7), as expected for all eukaryotes (Fig. 6). Sequence comparison of the subunits showed good homology (50-87% amino acid identity) with *T. vaginalis*, but much less homology (31-57%) with the human and murine counterparts, or even the protozoan parasite *Plasmodium falciparum* (Fig. 6). By comparison, the human and murine proteasome subunits are more closely related to each other than the *T. foetus* subunits are to *T. vaginalis*. These data indicate greater structural differences exist between the *T. foetus* and *T. vaginalis* proteasome subunits than between the human and mouse proteasomes, potentially offering an explanation for the differential activity of the tested proteasome inhibitors against the two trichomonad species.

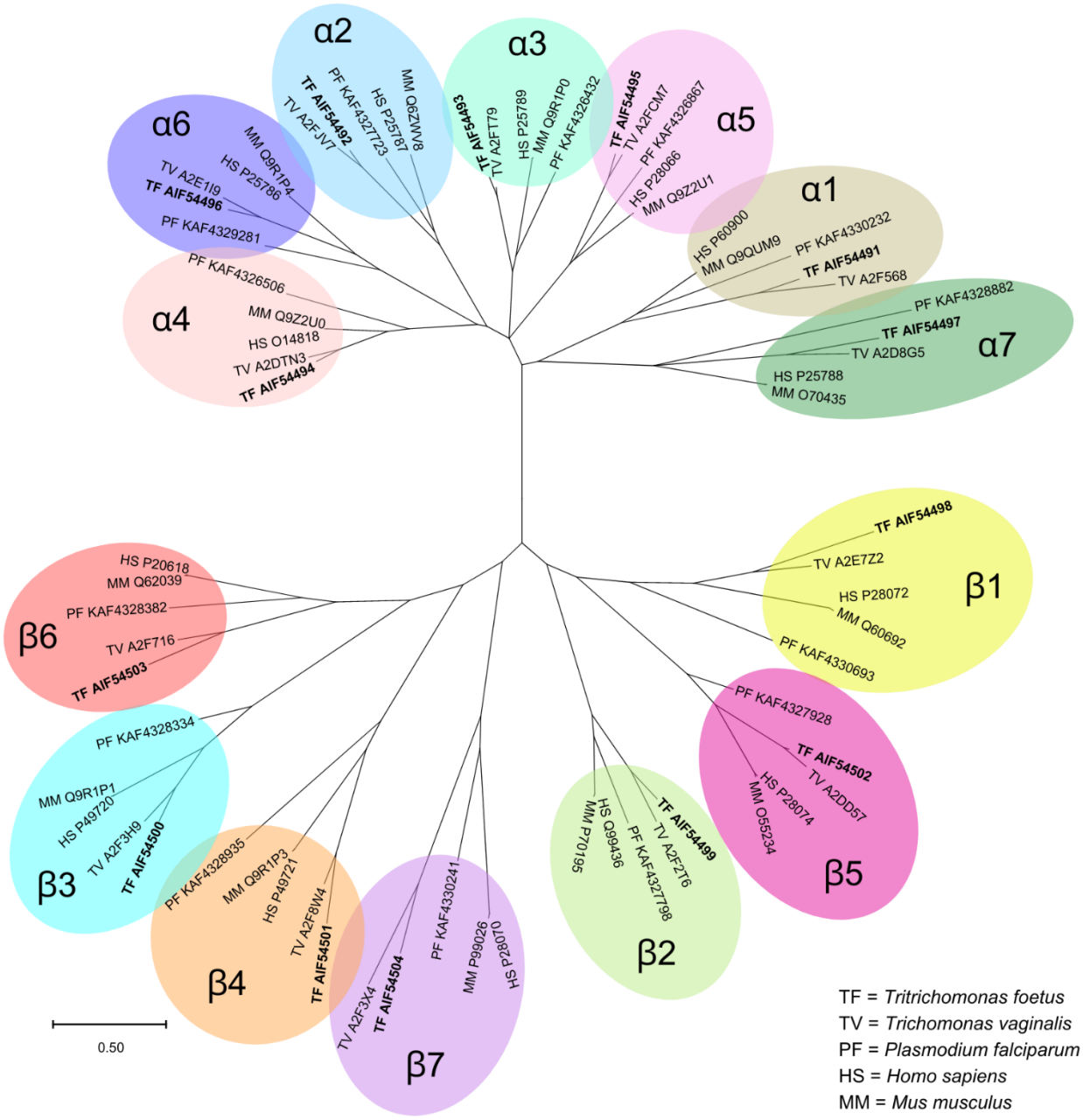

Figure 6: Phylogenetic analysis of trichomonad 20S proteasome subunits.

Phylogenetic analysis of 20S proteasome α- and β-subunits from *T. foetus* (TF, bolded), *T. vaginalis* (TV), *Plasmodium falciparum* (PF), *Mus musculus* (MM), and *Homo sapiens* (HS). Branches are labeled with UniProt accession numbers when available, or GenBank accession numbers. Subunit groups are highlighted with the same color. Distance scale is shown on bottom left.

To confirm that the *T. foetus* proteasome is functionally similar to *T. vaginalis,* we analyzed the accumulation of ubiquitin-labeled proteins after proteasome inhibition by western blotting. For both trichomonads, bortezomib caused concentration-dependent increases in the abundance of ubiquitin-labeled proteins (Fig. 7A). Furthermore, we used an activity-based probe, MV151 (Verdoes et al., 2006), for labeling of the catalytic proteasome subunits of the parasites. For *T. foetus*, labeling of β1 and β5 was strongly inhibited by bortezomib, while β2 labeling was only minimally affected at the highest concentrations (Fig. 7B). By comparison, for *T. vaginalis,* inhibition was most prominent for the β1 subunit followed by β5 and, to a lesser degree, β2 (Fig. 7B). Together, these findings show that bortezomib inhibits the proteasome in both *T. foetus* and *T. vaginalis,* most likely by targeting the same catalytic subunits.

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All chapters are currently being prepared for submission for publication of the material. Nieskens, Noelle M., Miyamoto, Yukiko, Hurysz, Brianna M., O'Donoghue, Anthony J. and Eckmann, Lars. The thesis author was the primary researcher and author of this material.

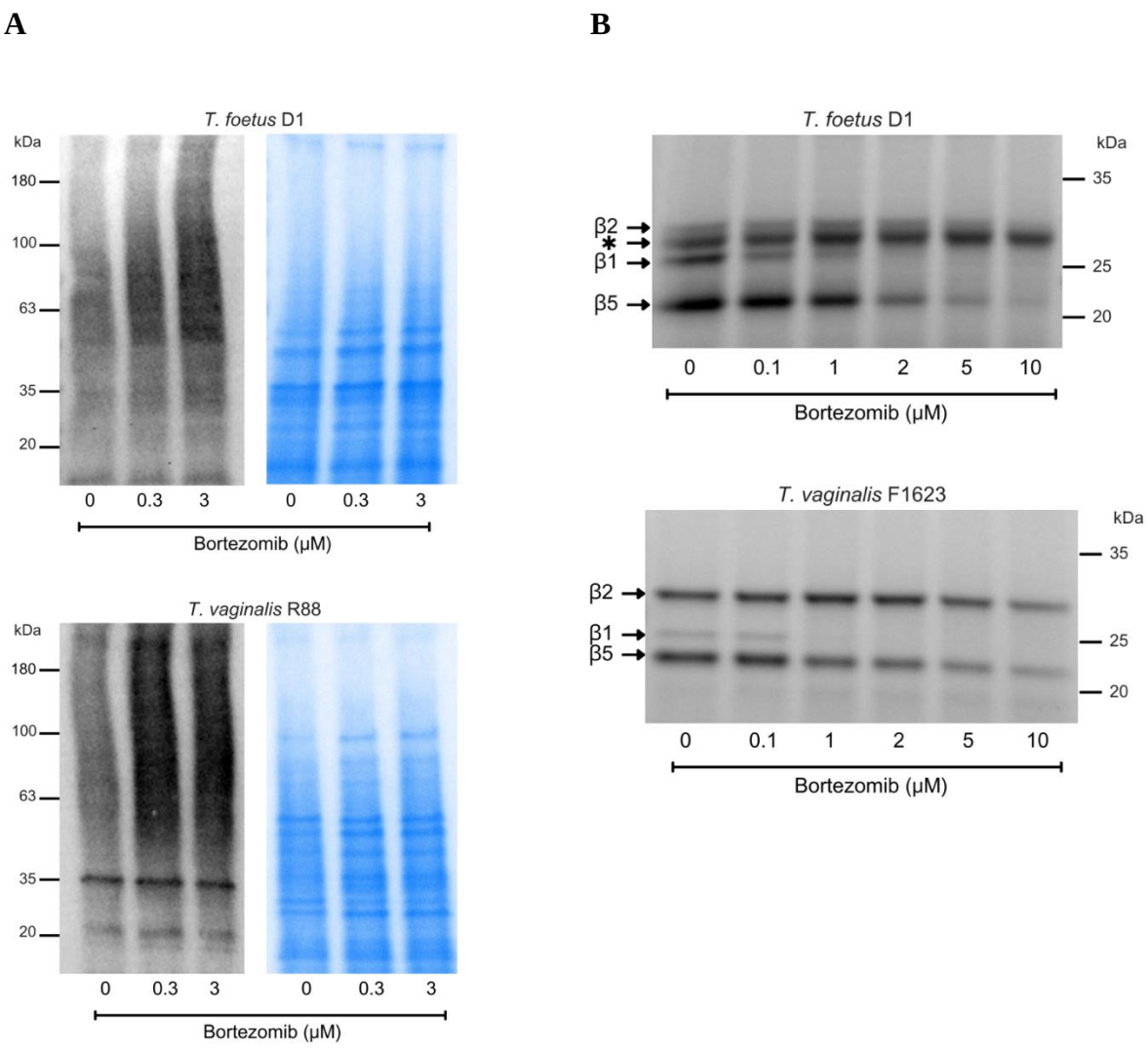

Figure 7: Functional analysis of trichomonad proteasomes.

A. The indicated trichomonad strains were incubated for 1 h with bortezomib or left without inhibitor (0 μM). Total protein extracts were prepared and analyzed by western blotting with an anti-ubiquitin antibody (left panels). Total protein was visualized by Coomassie Blue staining (right panels). B. Total cell lysates of *T. foetus* and *T. vaginalis* were incubated for 1 h with the indicated concentrations of bortezomib and subsequently labeled for 2 h with the fluorescent activity-based proteasome probe, MV151. Lysates were fractionated by SDS-PAGE and labeled proteins were visualized by fluorescent imaging. Band labeled with a * corresponds to a non-specific target of MV151 that could not be competed for with any available proteasome inhibitor and is thus unlikely to be a proteasome subunit.

DISCUSSION

Development of new antimicrobial drugs depends heavily on the use of suitable animal models for *in vivo* efficacy testing (McGrory & Garber, 1992). Robust infections with the relevant pathogens for a sufficiently long duration are key features of a good model. For trichomoniasis, infection of amenable animals, particularly rodents such as mice, with the causative pathogen, *T. vaginalis*, would be desirable, but we found here that murine vaginal infections with any of four *T. vaginalis* strains did not lead to detectable infection. In contrast, different strains of another trichomonad, *T. foetus*, were found to reliably infect mice for at least one week. The striking difference in infectivity is unlikely to be related to particular strains of the respective pathogen, since the findings were consistent for four diverse strains of each species. General characteristics of the two trichomonad species are probably more important. For example, we found a difference in doubling time and pH tolerance between the two organisms. Faster growth rates of *T. foetus* might promote infectivity by counteracting inevitable vaginal losses, while the slower growth of *T. vaginalis* may not be sufficient to make up for such losses. Consistent with this notion, highly infectious organisms tend to have faster doubling times due to optimization of codon usage bias and increased copies of rRNA and tRNA (Vieira-Silva & Rocha, 2010). Another potential explanation for differential infectivity could be differences in pH tolerance. The murine vagina, unlike the normally acidic environment of the human vagina, has a pH close to neutral or even slightly alkaline (Ganesan & Kadalmani, 2016). The ability of *T. foetus* to grow under alkaline pH conditions may promote vaginal infection, while *in vitro* growth of *T. vaginalis* was strongly inhibited by alkaline pH and may thus also be compromised *in vivo* in mice. Other possible explanations are conceivable for differential infectivity. For example, the estrus cycle of mice is characterized by periodic influx of leukocytes (McLean et

al., 2012). *T. foetus* may be more resistant to killing by polymorphonuclear leukocytes compared to *T. vaginalis* (Cobo et al, 2011). While the presence of vaginal leukocytes has no apparent impact on infectivity for *T. foetus,* it has been shown to have a negative impact on *T. vaginalis* infection (Cobo et al, 2011). The relative importance of these and perhaps other possible mechanisms for differential infectivity between *T. foetus* and *T. vaginalis* remains to be established, but our data clearly demonstrate that vaginal *T. foetus* infections are robust murine models suitable for *in vivo* drug testing.

For a microorganism to be a useful surrogate model for drug development against a related but different infectious agent, both must have similar responses to the drugs of interest. We observed such congruency in the drug responses of *T. foetus* and *T. vaginalis* for 5-nitroheterocyclic drugs and gold(I) compounds. Both drug classes are thought to act on a broad range of targets (Lamp et al., 1999; Saei et al., 2020). In the case of 5-nitroheterocyclic drugs, the prodrug forms are first reductively activated, then form covalent adducts on multiple target molecules, including different proteins as well as nucleic acids (Schmid & Schmid, 2002). Which of these molecular targets are critical for the ultimate cytocidal activity of 5-nitroheterocyclic drugs has not been defined, but it is likely that inactivation of multiple targets has synergistic effects that irreversibly damage the targeted cells (Lamp et al., 1999). The mechanism of action of gold(I) compounds is not well understood but also involves adduct formation and likely inactivates multiple targets (Saei et al., 2020). The multiplicity of cellular targets for both drug classes can provide an explanation for their similar activities against *T. foetus* and *T. vaginalis,* since even differences in the sequence or structure of any particular target are unlikely to interfere with the overall effectiveness of these drugs. It must be noted, however, that while not statistically significant, *T. foetus* had generally lower pIC_{50}s for each

compound of the two drug classes in the *in vitro* screening assays, which was also observed in prior studies with fewer compounds (O'Donoghue et al., 2019; Natto et al., 2021). Interestingly, we found no activity differences in the MLC assays, perhaps suggesting that the IC_{50} growth assays and MLC survival assays test slightly different drug properties or are maybe differentially impacted by parasite characteristics not directly related to the drug action. For example, the IC_{50} assays could be affected by the faster growth rate of *T. foetus* over *T. vaginalis*. Drugs such as 5-nitroheterocyclic compounds that form covalent adducts may be consumed during the culture period, so a faster growing organism may deplete drugs more rapidly, thereby requiring apparently higher IC_{50} values for achieving 50% maximal growth inhibition. By comparison, MLC assays measure the ability of a drug to kill all target organisms, which typically requires higher drug levels, so the impact of drug consumption would be less apparent. Nonetheless, both IC_{50} and MLC provide valuable information for comparative assessment of the drug responses of *T. foetus* and *T. vaginalis*, and clearly show that the drug susceptibility of *T. foetus* closely resembles *T. vaginalis* in regard to 5-nitroheterocyclic and gold(I) compounds. Consequently, our demonstration of *in vivo* efficacy for one of the gold(I) compounds is promising for its ultimate *in vivo* efficacy against *T. vaginalis*.

Contrary to the 5-nitroheterocyclic and gold(I) compounds, we observed marked differences in the activity of proteasome inhibitors against *T. foetus* and *T. vaginalis*. Thus, *T. foetus* had markedly lower pIC_{50}s for all tested drugs in this class compared to *T. vaginalis.* The target of these compounds is well defined and involves at most the three catalytic subunits of the proteasome and perhaps only one or two of them (Khalesi et al., 2021; Kisselev, 2021). Differences in the sequence or fine structure of the relevant proteasome subunits between the two trichomonad species, which clearly exist based on our phylogenetic analysis, can probably

impact the binding of specific inhibitors. In this context, it is important to note that the tested inhibitors were originally developed against the human proteasome (Dimopoulos et al., 2022), but not specifically selected for activity against either *T. vaginalis* or *T. foetus*. Screening of large proteasome inhibitor libraries may well identify compounds that are equally active against both trichomonads. It is therefore possible, that inhibitors could be found that are more active against *T. foetus* than *T. vaginalis* and even human cells, which could represent a promising starting point for new veterinary drugs against bovine tritrichomoniasis (Michi et al., 2015). Nevertheless, based on the phylogenetic and functional analyses, we were able to conclude that no fundamental differences in the 20S proteasome exist between *T. foetus* and *T. vaginalis*. We could also demonstrate that one proteasome inhibitor, bortezomib, has low micromolar or better activity against both *T. foetus* and *T. vaginalis* and can eliminate *T. foetus* infection *in vivo*, underlining that drug candidates with adequate activity against *T. foetus* can be active *in vivo* even if their relative potency in *T. foetus* does not closely mimic the findings in *T. vaginalis.*

In summary, the *T. foetus* murine infection models described here have excellent utility as surrogate *in vivo* models for a drug development campaign for new drugs against *T. vaginalis*, particularly if the predicted targets are multiple. Caution should be exercised, however, for compounds with singular targets if those targets differ significantly between the two trichomonad species. In such situations, it would be advisable to conduct careful *in vitro* compound testing beforehand to confirm that drug activity is not significantly different between the two organisms or at last remains sufficiently high in *T. foetus* that *in vivo* efficacy testing is not likely to yield false negative results.

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