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Ammonium salts of carbamodithioic acid as potent vaginal trichomonacides and fungicides

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ABSTRACT

Chemical attenuation of the reactive oxygen species (ROS)-sensitive anaerobes *Trichomonas vaginalis*, which is the most prevalent non-viral sexually transmitted infection, and two often coexisting vaginal infections, namely *Candida albicans* and *Staphylococcus aureus*, which are opportunistic reproductive tract infections, was attempted with novel ammonium salts of carbamodithioic acid through inhibition of free thiols. In vitro and in vivo efficacies of the designed compounds were evaluated as topical vaginal microbicides. Five compounds showed exceptional activity against drug-resistant and -susceptible strains with negligible toxicity to host (HeLa) cells in vitro in comparison with the standard vaginal microbicide nonoxynol-9 (N-9), without disturbing the normal vaginal flora (i.e. *Lactobacillus*). The compounds significantly inhibited the cytopathic effects of *Trichomonas* on HeLa cells in vitro with efficacies comparable with metronidazole (MTZ); however, their efficacy to rescue host cells from co-infection (protozoal and fungal) was greater than that of MTZ. The compounds inhibited β-haemolysis of red blood cells caused by *Trichomonas* and were found to be active in vivo in the mouse subcutaneous abscess assay. Some compounds rapidly immobilized human sperm. A mechanism involving inhibition of free thiols and consequently the cysteine proteases of *T. vaginalis* by the new compounds has been proposed. Thus, a unique scaffold of antimicrobial agents has been discovered that warrants further investigation for development as contraceptive vaginal microbicides.

1. Introduction

Worldwide, *Trichomonas vaginalis* is the most prevalent non-viral sexually transmitted infection (STI) [1] that promotes the acquisition and transmission of viral STIs such as human immunodeficiency virus (HIV), human papillomavirus (HPV) and herpes simplex virus (HSV) [2,3]. Trichomoniasis in women is also associated with vaginitis, endometritis, adnexitis, pyosalpinx, infertility, preterm delivery, bacterial vaginosis and risk of cervical cancer [4], whilst in men it has been implicated in the pathogenesis of prostate cancer [5]. Trichomoniasis is often asymptomatic (especially in males) and therefore goes unreported, resulting in persistent spread of *T. vaginalis* through heterosexual contact [6]. Hence, physical and/or chemical shielding of the genital tract during sexual intercourse becomes desirable. Unfortunately, condom use is grossly limited by the users' personal preferences, and the US Food and Drug Administration (FDA)-approved metronidazole (MTZ) has limited efficacy when used vaginally. On the other hand, 5-nitroimidazole class compounds are prone to drug resistance. The first case of drug resistance was reported with MTZ within 2 years of its introduction [7], and more than 100 cases were reported by 2003 [8]. Similarly, vulvovaginal candidiasis, caused by *Candida albicans*, has a very high prevalence in sexually active women, with a significant number of drug-resistant cases [9]. This infection often co-exists with *T. vaginalis*, resulting in increased morbidity [10]. Since women disproportionately bear the long-term consequences of STIs [11], highly effectual molecules (preferably non-nitroimidazoles) against *T. vaginalis* and associated yeast infections need to be discovered for potential use as 'women controlled' topical microbicides.

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We previously reported a unique molecular series that acted by its exceptional affinity towards thiols present on *Trichomonas* [6], suggesting thiols as a vulnerable target for directing topical antitrichomonal agents for prophylaxis during unprotected sex. Since chiefly anaerobic cells are susceptible to agents depleting thiols, which are meant for reactive oxygen species (ROS) buffering, the new compounds were also tested against some pathogenic bacteria and fungi that infect the female vaginal tract. Here we report some novel molecules with much improved scaffolds for better efficacy and safety over the previous drug designs. An attempt has been made to mechanistically substantiate the potential of these molecules as antitrichomonal agents suitable for topical use.

2. Materials and methods

2.1. Chemical synthesis

All of the new compounds were designed and synthesized in-house by the medicinal chemist authors (DM, VB and VLS). The synthesis schemes and chemical characterization details are available as Supplementary data.

2.2. Trichomonas vaginalis *cultures and trichomonacidal assays*

Trichomonads were cultured axenically in trypticase–yeast extract–maltose (TYM) (pH 6.8) (Sigma–Aldrich, St Louis, MO) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco™; Life Technologies–Thermo Fisher Scientific, Waltham, MA), 2% vitamin mixture (Sigma–Aldrich), 100 U of penicillin/mL and 100 μg/mL streptomycin at 37 °C under partial anaerobic conditions (i.e. with ca. 0.5 mL of air trapped above the medium). Drug susceptibility assays were conducted as detailed previously [6]. Briefly, the parasites were incubated under partial anaerobic conditions at 37 °C in culture medium containing serially diluted test compounds or MTZ (Sigma–Aldrich) in 48-well Corning culture plates (Corning Inc., Corning, NY). Dimethyl sulphoxide (DMSO) 0.05% (Sigma–Aldrich) in culture medium (the highest concentration of DMSO in test wells) was used as vehicle in the control wells. Cell viability was checked after 48 h by trypan blue exclusion assay (Sigma–Aldrich) and the minimum concentration of test agent at which all cells were found dead was considered its minimum inhibitory concentration (MIC). 100% eradication was confirmed by transferring 100 μL of the suspension to a 15 mL tube with fresh medium and recording the growth at 37 °C. MTZ, which is the most widely used drug against *T. vaginalis*, was used as the reference standard. All experiments were repeated three times.

2.3. Microbial cultures, and antifungal and antibacterial assays

The fungi *C. albicans* and *Candida glabrata* were maintained in RPMI 1640 (Sigma–Aldrich) buffered with MOPS [3-(*N*-morpholino)propanesulfonic acid] (Sigma–Aldrich) and were incubated at 35 °C. *Staphylococcus aureus* ATCC 25923 was grown in Mueller–Hinton broth (Difco™; BD, Franklin Lakes, NJ) and was incubated at 37 °C. Susceptibility assays were performed by the standard broth microdilution method as per Clinical and Laboratory Standards Institute (CLSI) guidelines. The maximum concentration tested was 50 mg/L and the inoculum load in each test well was in the range of $1–5 \times 10^3$ cells. Plates were incubated for 24–48 h for yeasts and 24 h for bacteria at 37 °C for determination of the MIC [12].

2.4. Cervical epithelial (HeLa) and fibroblast (L929) cell cultures and cytotoxicity assays

HeLa and L929 cells procured from the National Centre for Cell Science (NCCS, Pune, India) and the American Type Culture Collection (ATCC, Manassas, VA), respectively, were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma–Aldrich) supplemented with FBS (10%) and antibiotics (penicillin/streptomycin mixture, 100 U/mL) (Sigma–Aldrich) [6]. The cytotoxicity of compounds was tested by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma–Aldrich) [13]. L929 cells were also fixed and stained with Giemsa for 30 min at 37 °C for light microscopy.

2.5. Compatibility of commensal vaginal flora (Lactobacillus) with new compounds

Lactobacillus jensenii ATCC 25258 and a cocktail of *Lactobacillus* spp. (*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus fermentum*; Lactogut™, Medispan Ltd., Chennai, India) were grown in 6% Rogosa SL broth medium (HiMedia, Mumbai, India) containing 0.132% acetic acid at 37 °C. The effect of test compounds on *L. jensenii* was determined as described previously [6].

2.6. Spermicidal assay

Freshly ejaculated human semen samples received from healthy volunteers with their prior informed consent was analyzed for sperm count and motility. Samples with >65 million/mL sperm count, >70% motility and normal sperm morphology were used to determine the spermicidal minimum effective concentration (MEC) of the test compounds in vitro [6]. This study was approved by the Institutional Animal Ethics Committee of CSIR – Central Drug Research Institute (Lucknow, India).

2.7. Flow cytometric analysis of Trichomonas vaginalis *viability*

Trichomonas vaginalis incubated with test compounds at their MICs for 24 h were pelleted, washed and re-suspended in medium containing 25 μg/mL propidium iodide (Sigma–Aldrich) for 10 min and the viability was evaluated on a flow cytometer (FACSCalibur™; BD Biosciences, Singapore) equipped with an argon laser (488 nm) for excitation [14].

2.8. Inhibition of cytopathic effects of Trichomonas vaginalis *and* Candida albicans

HeLa and L929 cells were grown separately on eight-chambered slides (BD) for 24 h at 37 °C, were washed and were then infected with pathogen(s) in interaction medium DMEM:TYM (2:1) containing test compounds at trichomonacidal MICs in co-cultures of HeLa and *T. vaginalis*, and at anticandidal MICs in co-cultures of L929, *T. vaginalis* and *C. albicans*. Following incubation, slides were fixed with methanol. The cytopathic effects of parasites on the host cells were observed by Giemsa staining under a microscope.

2.9. Inhibition of the β-haemolytic activity of Trichomonas vaginalis *by the test compounds*

An equal number of freshly washed rat erythrocytes were mixed with 2×10^6 trophozoites in 2.5 mL of Hank's Balanced Salt Solution (Sigma–Aldrich) with or without the test compounds