AAC Accepts, published online ahead of print on 27 June 2011
Antimicrob. Agents Chemother. doi:10.1128/AAC.00199-11

Novel trichomonacidal spermicides

Ashish Jain[1], Nand Lal[2], Lokesh Kumar[1], Vikas Verma[1], Rajeev Kumar[1], Lalit Kumar[2], Vishal Singh[1], Raghav K. Mishra[1], Amit Sarswat[2], SK Jain[3], JP Maikhuri[1], VL Sharma[2] and Gopal Gupta[1]*

[1]Division of Endocrinology, Central Drug Research Institute (CSIR), Lucknow 226 001, India; [2]Division of Medicinal & Process Chemistry, Central Drug Research Institute (CSIR), Lucknow 226 001, India; [3]Department of Biotechnology, Jamia Hamdard University, New Delhi, India.

Running Title: ***Microbicidal spermicides for prophylactic contraception***

***Corresponding Author. Mailing address: Division of Endocrinology, Central Drug Research Institute, Lucknow 226 001, India. Tel: +91-522-2612411; Fax: +91-522-2623405; email: g_gupta@cdri.res.in**

Abstract

Metronidazole, the FDA approved drug against trichomoniasis, is non-spermicidal and hence cannot offer pregnancy protection when used vaginally. Furthermore, increasing resistance of *Trichomonas vaginalis* to 5-nitro imidazoles is a cause of serious concern. On the other hand, the vaginal spermicide nonoxynol-9 (N-9) does not protect against STDs and HIV in clinical situations but may in fact increase their incidence due to its non-specific, surfactant action. We therefore designed dually active, non-surfactant molecules that were capable of killing *Trichomonas vaginalis* (metronidazole susceptible and resistant strains) and irreversibly inactivating 100% human sperm at doses that were noncytotoxic to human cervical epithelial (HeLa) cells and vaginal microflora (Lactobacilli) *in vitro*. Anaerobic energy metabolism, cell motility and defense against ROS, which are key to survival of both sperm and *Trichomonas* in the host after intravaginal inoculation, depend crucially on availability of free thiols. Consequently, molecules were designed with carbodithioic acid moiety as the major pharmacophore and chemical variations were incorporated to provide high excess of reactive thiols for interacting with accessible thiols on sperm and *Trichomonas*. We report here the *in vitro* activities, structure activity relationships and safety profiles of these spermicidal anti-trichomonas agents, the most promising of which was more effective than N-9 (the OTC spermicide) in inactivating human sperm and more efficacious than metronidazole in killing *Trichomonas vaginalis* (including metronidazole-resistant strain). It also significantly reduced the available free thiols on human sperm and inhibited cyto-adherence of *Trichomonas* on *HeLa*. Experimentally *in vitro*, the new compounds appeared safer than N-9 for vaginal use.

Introduction

Sexually transmitted infections (STIs) and unintended pregnancies are the leading causes of morbidity and mortality among women of child bearing age (8). An estimated 340 million cases of curable STIs occur annually worldwide with trichomoniasis having the highest incidence of ~51.2% (43). On the other hand, out of 208 million pregnancies that occurred globally in 2008, ~41% were unintended (33). It has been seen that the primary responsibility of pregnancy and STD protection lies with the female partner during most of the heterosexual contacts, including the "most vulnerable" contacts amongst adolescents and promiscuous adults (4). Often under such circumstances condom (the only dually protective contraceptive) is either inaccessible or its use is practically not negotiable. Consequently, woman-controlled, dually protective contraceptives that are safe, effective and virtually imperceptible are highly desirable to curb the STD/HIV epidemic.

Trichomoniasis, the most-prevalent non-viral STD, predisposes women to viral STDs including HIV (23). On the other hand, HIV infected men with symptomatic trichomonal urethritis have nearly six-fold higher HIV concentration in semen as compared to men without urethritis. Therefore, it would be quite rational to conclude that controlling trichomoniasis alone can significantly reduce the incidence of new HIV infections. Moreover, trichomoniasis in women is also associated with vaginitis, endometritis, adnexitis, pyosalpinx, infertility, preterm delivery, low birth weight, bacterial vaginosis, and increased risk of cervical cancer (10). Anti-trichomonas agents offering simultaneous pregnancy protection would have the advantage of providing the required impetus for use-compliance as women prefer dual protection contraceptives over pure microbicides (28). Metronidazole, the FDA approved drug against trichomoniasis (5), is non-spermicidal, and hence does not offer contraceptive protection when used vaginally. On the other hand, dually-active molecules like nonoxynol-9 (N-9) and cellulose sulfate failed to provide twin protection in clinical situations (13, 29) plausibly due to their general, non-specific toxicity against sperm and cervico-vaginal cells. Hence there is a need for designed synthesis of potent, more specifically acting spermicidal microbicides for prophylactic contraception. Our enduring attempts to target human sperm through differently guided molecules against various functional moieties yielded some very promising structures (7, 12, 14-20, 22) but the two most promising spermicides (14, 16) were weakly microbicidal against Trichomonas vaginalis (15).

We therefore took up designing of novel, dually capable molecules for killing human spermatozoa almost immediately after ejaculation in the vagina along with *Trichomonas vaginalis*, the causative agent of trichomoniasis and a prominent HIV risk factor. Trichomoniasis, though curable, is often asymptomatic (especially in males) and therefore goes unreported, resulting in its persistent spread through heterosexual contacts. On the other hand, growing resistance of *T. vaginalis* to 5-nitroimidazole drugs (45) like metronidazole is another serious concern and calls for identification of newer anti-trichomonal agents. Recently some non-imidazoles have shown exceptional intra-vaginal efficacy against *Trichomonas* infection, including the firmly metronidazole-resistant strains (36), but these are devoid of contraceptive activity.

Free thiol groups play an important role in the survival of predominantly anaerobic cells like spermatozoa (40) and *Trichomonas vaginalis* (11). The strongly nucleophilic character of the sulfur atom and the unique redox properties of the thiol group make it a key residue for enzyme catalysis, protein folding, and redox signaling and regulation (42), which are important for cellular energy metabolism, motility and subsistence of sperm and *Trichomonas*. We hypothesized that a topical agent capable of targeting free thiols would arrest both sperm and *Trichomonas* in semen quite specifically since free thiols are unlikely to be available elsewhere in normal vaginal environment due to the low pH. Though agents independently targeting sperm and *Trichomonas* are available, in this major study we have attempted to combine the two intense capabilities together through rational drug design. We report here the *in vitro* activity and safety profile of some very potent spermicidal microbicides, the most promising of which were more effective than N-9 (the OTC spermicide) in killing sperm and more efficacious than metronidazole in killing susceptible and resistant *Trichomonas vaginalis*. Experimentally *in vitro*, these compounds appeared safer than N-9 for intravaginal use.

Materials and methods

Chemicals

All the chemicals, culture media and other reagents were procured from Sigma-Aldrich, St. Louis, MO, USA, unless stated otherwise. Nonoxynol-9 was purchased from Spectrum Chemical Manufacturing Corporation, New Brunswick, NJ, USA. The 15 new compounds were synthesized according to the synthetic schemes provided as supporting information. The complete chemistry details of new compounds including characterization, purity and instrumentation is provided as supporting information.

Trichomonas vaginalis cultures

Clinical isolates of metronidazole susceptible *T. vaginalis* collected at Post Graduate Institute of Medical Research and Education, Chandigarah, were obtained from the laboratory of Dr. Divya Singh (37) and metronidazole-resistant strain of *Trichomonas vaginalis* [CDC 085 ATCC 50143]

was procured from ATCC, USA. Both the strains were cultured under partial anaerobic condition in TYM medium [0.1% K_2HPO_4, 0.06% KH_2PO_4, 0.5% NaCl, 0.5% glucose, 2.0% yeast extract, 0.2% L-Lysine, 0.15% tryptone, pH 6.8] supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2% vitamin mixture, 100 U/ml penicillin and 100 µg/ml streptomycin solution in 15 ml screw capped sterile tubes, and incubated at 37 °C (±0.5). Vitamin mixture contained niacin (41.6 µg/ml), 4-aminobenzoic acid (83.3 µg/ml), pyridoxine hydrochloride (41.6 µg/ml), thymine hydrochloride (16.6 µg/ml), D-pentothenic acid (16.6 µg/ml), myo-inositol (83.3 µg/ml), riboflavin (16.6 µg/ml), niacinamide (41.6 µg/ml), D-biotin (20.0 µg/ml), folic acid (20.0 µg/ml), calciferol (40 µg/ml), retinyl palmitate (40 µg/ml), vitamin K (menadione sodium bisulfite, 8.0 µg/ml) and α-tocopherol acetate (16.6 µg/ml) in Milli-Q water. Organisms in the logarithmic phase of growth and exhibiting motility and normal morphology were harvested, centrifuged and resuspended in fresh TYM medium for the experiments.

Human Sperm

Freshly ejaculated human semen samples were obtained by masturbation from healthy and fertile volunteers, collected directly into sterile plastic tubes and transported immediately into the laboratory. The samples were allowed to liquefy at 37°C for 45 min. Semen samples were analysed as per World Health Organization guidelines (44). Sperm count and motility was analysed manually as well as in a Computer Automated Semen Analyzer (CASA, Hamilton Thorne, USA) using a small drop of liquefied semen placed on a "Makler" counting chamber (Sefý´ Medica, Hafia, Israel) pre-warmed to 37°C. Semen samples with >65 million per ml sperm count, >70% motility and normal sperm morphology were used. This study was approved by the Institutional Ethics Committee.

Drug susceptibility assay

Trichomonas vaginalis parasites to be used in drug susceptibility assays were grown in TYM medium for one day following regular sub culturing and were in the log phase of growth. *In vitro* drug susceptibility assays were carried out using the standard procedure (38) and metronidazole susceptibility criteria of Sobel et al., (34) was used to determine the resistance of *T. vaginalis* strains to metronidazole. Accordingly, the clinical isolate was categorized as susceptible and

ATCC strain as firmly resistant strains. Stock solutions (100 mM) of test compounds were prepared in DMSO. These stock solutions were diluted with TYM medium to obtain a concentration of 400 µM and then further serially diluted with the same medium to 1.0 µM in a 48-well plate. 0.05% DMSO in TYM was used as vehicle in control wells. Parasites ($5x10^3$ trophozoites/well) were added to these wells and incubated anaerobically at 37°C. Trophozoite growth and viability in drug containing wells was monitored by trypan blue staining and cell number score on a daily basis, in comparison to control. Assay results were clearly defined after 48 hours in terms of minimum inhibitory concentration (MIC, lowest concentration of compound at which all trophozoites were found non-viable). Using this MIC value as a reference, finer dilutions of the active compounds were prepared and the drug susceptibility assay was repeated to obtain the final MIC precisely through a concentration versus viable-trophozoite-number curve. Viability was determined by trypan blue exclusion and 100% eradication was confirmed by transferring 100 µL of the suspension to a 15 ml tube with fresh medium and recording growth at 37°C for 14 days (1). Metronidazole (1-40 µM susceptible strain, 40 – 400 µM, resistant strain; Sigma-Aldrich) was used as reference standard. Three separate experiments were performed for each strain to confirm the MIC.

Spermicidal Assay

Minimum effective (spermicidal) concentration (MEC) was determined by the modified Sander–Cramer assay as detailed earlier (12, 15). Briefly, the test compounds were dissolved in a minimum volume of DMSO and diluted with physiological saline (0.85% NaCl in distilled water) to make a 10.0 mM solution and serially diluted to 0.125 mM with saline. A spermicidal test was performed with each compound solution starting from 10 mM until the minimum effective concentration (MEC) was arrived at. For this purpose 0.05 ml of liquefied human semen was added to 0.25 ml of test solution and vortexed for 10 sec at low speed. A drop of the mixture was immediately placed on a microscope slide, covered with a cover glass and immediately examined under a phase contrast microscope in five fields of vision. The results were scored positive if 100% spermatozoa became immotile in ~20 seconds and remained immotile even after dilution with 1.0 ml of Kreb Ringer bicarbonate buffer for another 30 min at 37°C. The MEC was determined in three individual semen samples from different donors. The

minimum concentration of compound capable of killing 100% sperm in ~30 sec in all the three semen samples was denoted as minimum effective concentration (MEC).

Cytotoxicity of compounds towards human cervical (*HeLa*) cells

We used *HeLa* cell monolayers as an *in vitro* model of cervicovaginal epithelium (31) for tesing cytotoxicity of new compounds. *HeLa* cells procured from National Centre for Cell Sciences (NCCS), Pune, India, were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with fetal bovine serum (10%), and antibiotics (100 U/mL penicillin/streptomycin mixture). Cells at 80–90% confluence were split by trypsin (0.25% in phosphate-buffered saline (PBS), pH 7.4) and medium was changed at every 24 h interval. Cultures were maintained in a CO_2 incubator at 37^0C in 5% CO_2/95% air atmosphere. The MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide) based colorimetric assay for evaluation of cyto-toxicity of drug formulations against human cervical cell line (***HeLa***) was adopted (6). Cells seeded at a density of 5.0 X 10^4 per well in 96 well plates were incubated in culture medium (DMEM with 10% FCS) for 24 hours at 37°C in 5%CO_2/95% air atmosphere. After 24 hours, culture medium was replaced with fresh medium containing dilutions of test compounds in experimental wells and 0.05% DMSO in culture medium in control wells. After an incubation for another 24 hours, 5 µl of MTT solution (5 mg/ml in PBS, pH 7.4) was added to each well. The formazan crystals formed inside the viable cells were solubilized in DMSO and the OD was recorded at 540 nm in a microplate reader (Microquant, Bio-Tek, USA).

Compatibility of compounds with *Lactobacillus*

Spores of *Lactobacillus jensenii* [ATCC 25258, *strain 62G*] were procured from ATCC, USA and grown in 6% Rogosa SL broth medium (Hi Media, India) containing 0.132% acetic acid, at 37^0C. The effect of test compounds on *Lactobacillus jensenii* was determined by the method published earlier with slight modification (15). Briefly, Rogosa SL broth medium was prepared in Milli-Q water, boiled for 2-3 min and distributed in 48 well plate (500µL/well). Serial dilutions of test compounds were added to experimental wells and vehicle to control wells, in triplicate. ~1000 *cfu* of *Lactobacillus jensenii* were inoculated in each well. The plates were

incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. At the end of the experiment, the cultures were mixed thoroughly and 100µl volume from each well was transferred to the corresponding well of a 96 well plate and number of Lactobacilli were estimated by measuring the turbidity (OD) at 610 nm in a microplate reader.

Inhibition of cytoadherence of trophozoites to host cells

Effect of the most promising compound-2 on *T. vaginalis* cytoadherence to human cervical (*HeLa*) cells was tested in an *in vitro* model of *Trichomonas* infection, and was adapted from a published procedure (2). Briefly, the parasites were labeled with tritium by incubating overnight in 1.0 µCi [^{3}H]-thymidine/ml of complete TYM medium. After incubation, the parasites were thoroughly washed with normal TYM medium to remove unincorporated [^{3}H]-thymidine and were resuspended in interaction medium [TYM:DMEM (1:2)]. For evaluation of cytoadherence, $1X10^6$ [^{3}H]-thymidine labeled trichomonads were allowed to interact with the monolayer of HeLa cells in the ratio of 5:1, for 3h at 37^0C, in the presence of different concentrations (10, 25, 50 and 100 µg/ml) of compound-2. [^{3}H] labeled trophozoites treated with 0.05% DMSO served as control. After incubation for a period of 3 h, cells were thoroughly washed with DMEM-TYM (2:1) for removal of non-adherent *Trichomonas vaginalis*. *HeLa* cells were finally trypsinized (0.25% trypsin + 1mM EDTA) and pelleted along with adherent *Trichomonas* and suspended in the aqueous scintillation counting fluid (ACS II; Amersham, USA). Radioactivity was measured on an LS Analyzer 6500 (Beckman Instruments Inc., USA).

Inhibition of free thiols on human sperm by new compound

Free thiols remaining on human sperm after treatment with new compound were measured using the colorimetric method utilizing 5,5′-Dithiobis(2-nitrobenzoic acid) [DTNB] (9) by making suitable adaptations. Briefly, 1.0 ml of human semen was treated with 5.0 ml of the most promising, dually active compound (No. 2, pyrrolidinium pyrrolidine-1-carbodithioate) at spermicidal MEC (stock solution diluted in saline) for 10 min. In parallel, 1.0 ml semen from the same sample was treated with 5.0 ml of 0.15% DMSO in saline and used as control. Thereafter, 1.0 ml of 100 mM ß-mercaptoethanol was added to experimental and control tubes and incubated at 4°C for 2 hrs to regenerate all the unreacted thiols. After incubation, the sperm samples were pelleted in a refrigerated centrifuge and washed repeatedly with phosphate buffered saline (4 – 5

times) to completely remove mercaptothanol and finally suspended in 1.0 ml PBS. To various aliquots of this sample (finally measuring 120 µl), 5 µl DTNB (100 mM) and 25 µl Tris buffer (1.0 M, pH 8.0) was added, mixed and incubated for 15 min. Thereafter, the multiwall plates were centrifuged and 80 µl of supernatant was carefully aspirated and transferred into the corresponding well of a new 96-well plate. The OD of supernatant was measured at 412 nm in a microplate reader (Microquant, Bio-Tek, USA) and corrected for 1.0 cm light path using KC Junior software (Bio-Tek, USA). A molar extinction coefficient of 14150 cm^{-1} M^{-1} of DTNB (30) was used to calculate the number of free thiols.

Activity in simulated vaginal fluid (pH 4.2) and aerobic growth condition of *Trichomonas*

Since the new molecules are intended for intravaginal use that normally has a low pH, alternatively stock solution of the most promising compound (**No. 2**, 100 mM in DMSO) was serially diluted in a simulated vaginal fluid, pH 4.2 (27) up to 1.0 mM. After 60 min incubation of this solution at 37°C, it was further diluted with TYM or PBS and its anti-trichomonal and spermicidal activities were determined. Given that the drug resistance is better evident under aerobic growth conditions of Trichomonas, the most active compound (No. 2) was also tested against trophozoites grown under aerobic conditions.

Data analysis

Each experiment was performed in triplicates and repeated three times. Semen sample from a different donor was used in every experiment. The data were analyzed by one-way analysis of variance and P values less than 0.05 were considered as significant. IC_{50} values of cytotoxicity towards *HeLa* and *Lactobacillus* were calculated by computer-based curve fitting using the 'CompuSyn' software.

Results

Anti-trichomonal and spermicidal activities

In comparison with standard drugs, ten new molecules [**1**-**3**, **7**-**13**] out of fifteen compounds synthesized exhibited dual activity and nine of these (except **7**) were more effective than metronidazole (MET) in inhibiting the growth of MET-resistant *Trichomonas* strain (Table 1). Five compounds were found to be more active than nonoxynol-9 in spermicidal potential with activity hierarchy of **6** > **2** = **9** > **3** > **1** > **N-9**, while two compounds exceeded metronidazole in anti-trichomonal activity (**2** > **3** > **MET**, susceptible strain). Three compounds appeared promising due to their superior dual activity as compared to N-9 (**2** > **3** > **1**) but the most promising compound (pyrrolidinium pyrrolidine-1-carbodithioate, **2**) was ~1.6 times more potent than N-9 as a spermicide and killed MET-susceptible and -resistant strains of *Trichomonas vaginalis* ~15 and 6.5 times more efficiently than N-9, and ~2 and 29 times more effectively than metronidazole, respectively. Compound-2, after preincubation in simulated vaginal fluid, pH 4.2, became marginally more active against *Trichomonas* (susceptible and resistant strains), while its spermicidal activity remained practically unchanged (Table 2). On the other hand, *Trichomonas* grown under aerobic conditions were moderately more resistant to both metronidazole and compound-2 (Table -2).

Structure Activity Relationships

The 15 new compounds synthesized [**1-15**, Table-1] included secondary amine salts of dialkyldithiocarbamic acid [**1-11**], tertiary amine salts of dialkyldithiocarbamic acid [**12** and **13**] and tertiary amine salts of cycloalkyldithiocarbamic acid [**14** and **15**]. Twelve compounds [**1**-**5**, **7**-**13**] showed appreciable anti-trichomonal activity against metronidazole (MET) susceptible strain with MIC ranging from 4.8 - 243.3 µM (MET = 10.7 µM). Among the compounds having identical amine residue in both the dithiocarbamic acid and ammonium portions [**1-11**], pyrrolidinium pyrrolidine-1-carbodithioate [**2**] and piperidinium piperidine-1-carbodithioate [**3**] were respectively 2.2 and 1.3 times more effective against susceptible *Trichomonas* than MET. Even as open chain amine residues [**1**] showed a marginal decrease in activity, a successive enlargement of cyclic ring of amine by one methylene group [**2**, **3**, **9**] also exhibited a decreasing trend in activity. On the other hand, substitution of methyl group [**4**-**6**] in piperidine ring from position-4 to position-2 showed a remarkable enhancement in activity (MIC >400 – 30.41 µM) whereas a hydroxyl [**7**] or a 1, 4-dioxospiro group [**8**] at position-4 of the piperidine ring retained

the anti-trichomonal activity at 37.47 and 20.14 µM, respectively. An additional hetero atom in the amine [**10, 11**] resulted in compounds with activity comparable with MET. Alternatively, in compounds with different amine residues in dithiocarbamic acid and ammonium parts [**12**-**15**], a secondary-amine/ tertiary-amine combination [**12, 13**] gave compounds with activity comparable to MET while a primary-amine/tertiary-amine arrangement [**14, 15**] resulted in total loss of activity. It seemed that an unsubstituted five or six membered secondary amine salts of dithiocarbamic acid were the desirable scaffolds for anti-trichomonal activity.

Evaluation of all the 15 new compounds against MET-resistant *Trichomonas* identified eleven compounds [**1**-**5** and **8**-**13**] that exhibited very potent to moderate activity with MIC ranging from 11.94 – 332.13 µM. (MET=340.8 µM). However, when matched against N-9 four compounds [**2** (6.3 fold), **8** (3.2 fold), **11** (1.8 fold) and **13** (1.9 fold)] were more potent while three compounds [**1**, **3**, **12]** showed comparable efficacy. Among compounds with same amine residue in dithiocarbamic acid and ammonium portions, compound-**2** was most effective while compounds with ring enlargement [**3**, **9**], acyclic residue [**1**] or methyl substitution at position-2 or 3 in piperidine ring [**4**, **5**] were marginally lesser active. A methyl [**6**] or a hydroxyl [**7**] group at position-4 of piperidine ring resulted in loss of activity whereas a dioxospiro group [**8**] increased activity by 14.8 and 3.28 fold over MET and N-9, respectively. Among the compounds with dissimilar amine residue in both the portions [**12**-**15**], results suggested the same pattern of SAR as with susceptible strain.

The spermicidal activity of new compounds [**1-15**, Table 1], was evaluated in comparison to MET and N-9. Eleven compounds [**1-3, 6-13**] irreversibly immobilized 100% human sperm at concentrations 121.65 – 8393.3 µM in ~30 s. MET showed no effect even at 50 mM and N-9 exhibited a spermicidal MEC of 243.18 µM. Among secondary amine salts of dialkyldithiocarbamic acid [**1**-**11**], five compounds were found to be more potent spermicide than nonoxynol-9 [compounds **6** (2 fold), **2** and **9** (1.6 fold), **3** (1.4 fold) and **1** (1.3 fold)]. Introduction of methyl substituent at position-4 [**6**] of piperidine ring increased the spermicidal activity in comparison to piperidine compound [**3**], while that at position-2 [**4**] or position-3 [**5**] resulted in loss of activity. On the other hand, substituting hydroxyl group [**7**], 1,4-dioxaspiro group [**8**], oxygen [**11**] or N-methyl group [**10**] at position-4 in piperidine exhibited mild

spermicidal activity, suggesting that the presence of methyl substituent at position-4 of piperidine ring [**6**] was more important for spermicidal activity. Nevertheless, spermicidal activity was retained either by increasing [**9]** or decreasing [**2**] the ring size, or by incorporating open chain analogue [**1**]. Substitution of secondary amine either in ammonium ion [**12-15**] or in dithiocarbamate ion [**14, 15**] by tertiary and primary amines reduced the activity. The overall results exhibited that a secondary amine residue with methyl group at position-4 of piperidine ring [**6**] was the most desirable for spermicidal activity.

Safety of new molecules

Based on safety of the new compounds [**1**-**15**] towards HeLa cells, we have compounds with IC_{50} >4000 µM [**10**, **14**, **15**], and compounds with IC_{50} in the range of ~1500 - 2650 µM [**1**-**9**, **11**-**13**]. Amongst the safest structures, only compound **10** exhibited appreciable anti-trichomonal and spermicidal activities, which could be attributed to its better tendency (than compounds **14** & **15**) to release thiocarbamic acid for interactions with sulfhydryl groups. Among the rest, the secondary amine salts of dialkyldithiocarbamic acid [**1**-**9**, **11**] exhibited enhanced safety towards HeLa cells by cyclization of carbon chain [**1**, **2**]. Moreover, an increment in the ring size further improved safety [**2**, **3**, **9**]. On the other hand, a substitution in six membered cyclic framework compromised the safety [**3**, **4**, **6**-**8**] whereas methyl group at position-3 of piperidine ring did not affect the safety profile [**3**, **5**]. Among tertiary amine salts of morpholino substituted dithiocarbamic acid, enhancement in carbon chain of ammonium ion marginally forfeited safety [**12**, **13**].

However, all the compounds [**1**-**15**] exhibited greater safety towards HeLa cells (IC_{50}: 1447 - >4000 µM) and much better compatibility with *Lactobacillus* (IC_{50}: 591.2 - >4000 µM) than N-9 (IC_{50}: 53.8µM and 52.7 µM, respectively), and therefore appeared apparently much safer for vaginal use.

Functional inactivation of Trichomonas and thiol inhibition of sperm by the most active compound

The most promising compound [**2**] significantly inhibited the cytoadherence of *Trichomonas vaginalis* on *HeLa* monolayer at 10 -100 µg/ml concentration during a 3-hour incubation period (Figure 1). No significant change in viability of Trichomonas was observed at 10 – 50 µg/ml concentrations of compound-**2** during this treatment period. On the other hand, when incubated with human sperm at spermicidal MEC for 10 minutes, compound-**2** significantly ($P<0.001$) reduced the number of available free thiols on sperm cell (Figure 2).

Discussion

The new, non-surfactant molecules exerted a more potent and specific dual action as compared to the general non-specific effect of N-9, which killed both sperm and *Trichomonas* by its universal ability to destabilize cell membranes through surfactant action. The non-specificity of N-9 action was also evident by its potential to disrupt cervical epithelial (HeLa) cells and eliminate the useful microflora (*Lactobacillus*) at its spermicidal/microbicidal concentrations. On the contrary, all the new molecules, though designed to provide reactive chemical groups for inactivating vital thiols on target cells, did not universally inhibit both sperm and *Trichomonas*. They were very active against either sperm or *Trichomonas* or both, while a few were negligibly active against either cell type. Only three structures exhibited potent dual activity. This indicates that the promising new structures not only provide reactive groups for incapacitating thiols but also present unique molecular design(s) capable of gaining access and targeting crucial thiols on both the cell types. Both sperm and *Trichomonas* possess very well developed mechanisms for survival in the 'host', which include cell motility, anaerobic energy metabolism and defense against reactive oxygen species (ROS). Free thiol groups on cell surface play an important role in the functional survival of human spermatozoa (40) and *Trichomonas vaginalis* (11). Both *Trichomonas* (39) and human sperm (25) are essentially anaerobes and depend on glycolysis for energy, which is also required for motility. Inhibition of glycolytic enzymes in *Trichomonas vaginalis* by thiol disruption is known to result in severe depletion of its intracellular ATP (35). On the other hand, ROS pose a serious threat to the survival of both sperm and *Trichomonas* wherein thiols play an essential role in protection against the oxidative stress (3, 26). Though these cells do not essentially require oxygen for energy metabolism, yet they are exposed to

oxygen in their natural environment inside the host. *Trichomonas vaginalis* lacks glutathione (the intracellular redox buffer), glutathione dependent peroxidase and catalase, and therefore it relies heavily on cysteine for protection against oxidative stress, which constitutes >70% of cell's total thiol pool (42). The thioredoxin/thioredoxin-reductase system maintains the thiol status of *Trichomonas* for deactivating ROS (26). Thiol inactivating agents are thus capable of seriously disrupting multiple fundamental processes in *Trichomonas* as well as sperm. A very important feature of the new molecules is their extreme potency against metronidazole-resistant strain of *Trichomonas*. Metronidazole, which itself is non-trichomonacidal, gets activated in the hydrogenosomes of *T. vaginalis* by the anaerobic reduction of its nitro group, resulting in formation of cytotoxic nitro radical-ion intermediates. This activation step is non-operational in resistant pathogen (21). On the other hand, the most promising new molecule (compound-**2**) killed metronidazole-resistant *Trichomonas* almost 30 times more efficiently than metronidazole. This may indicate that a mechanism different than metronidazole is operational in case of new compounds, which makes them almost equally effective against both metronidazole-susceptible and resistant strains of the parasite.

Surface thiols are equally important for virulence of *Trichomonas vaginalis*, which requires the activity of surface cyteine proteinases to adhere to the host cell during infection (2, 24). Cysteine proteases, also known as thiol proteinases, have nucleophilic thiols in their catalytic domain for imparting proteolytic activity, which is involved in cytotoxicity, hemolysis and immune evasion (24) of *T. vaginalis*. Adherence of *T. vaginalis* to the epithelial cells of the urogenital tract is an essential step in pathogenesis. Since the new molecules were designed to suppress thiols' action on cell surface, we expected a marked inhibition of the virulence of *T. vaginalis* before cell-death. In the *in vitro* model for *Trichomonas* infection of *HeLa* cell monolayer, the cytoadherence capability of *Trichomonas* to host cell surface was significantly inhibited by the most promising compound (pyrrolidinium pyrrolidine-1-carbodithioate, **2**) in the first 3 hour incubation period, during which the viability of protozoa did not change significantly. This indicates that the new compounds are capable of seriously impairing the pathogenic potential of *Trichomonas* before killing the parasite.

The normal human vagina is naturally protected against STDs by its low pH, which is growth inhibitory for several pathogenic organisms. At this pH, free thiols are liable to be protonated to sulfenic acid (41) and therefore the likelihood of available free functional thiols in the normal vaginal environment is quite negligible. However, it has been seen that infections normally occur when the vaginal pH is disturbed, especially during the deposition of alkaline semen containing sperm and STD pathogen(s). The capability of new compounds to target thiols on sperm and *Trichomonas* at seminal pH may add considerably to their activity and safety.

All the new compounds synthesized were apparently much safer than N-9 towards human cervical epithelial (*HeLa*) cells and vaginal microflora (*Lactobacillus*). Since N-9 is a mixture of oligomers (32), its activity and toxicity depends on the ratio of these oligomers in the final preparation (46). The standard N-9 sourced from a chemical company for the present study exhibited better activity and lower toxicity profile than the gift chemical used earlier (7); yet its lowest active concentration against either of the cell type (*Trichomonas*/sperm) was toxic enough to completely annihilate *HeLa* cells and *Lactobacilli in vitro*. On the other hand the most promising new compound [**2**] killed sperm and *Trichomonas* at concentrations that were about 15 and 450 times lower than its IC_{50} against *HeLa*, and about 8.5 and 280 times lower than its IC_{50} against *Lactobacillus*. This clearly indicates a highly specific action and much better safety index of the new compound in comparison with N-9, and warrants further investigation for development of a safe microbicidal contraceptive.

Acknowledgement

This study was supported by a grant from the Ministry of Health and Family Welfare, Government of India. Authors are thankful to Mrs. Tara Rawat and Mr. Chhatrapal for their technical assistance. AJ, VV, RK, VS are grateful to the Indian Council of Medical Research, New Delhi; NL, LK, LK to University Grants Commission, New Delhi, and RKM, AS to the Council of Scientific and Industrial Research, New Delhi, for the grant of research fellowships. Authors acknowledge the SAIF Division of CDRI for the spectral data.

References

1. **Adagu, I. S., D. Nolder, D. C. Warhurst, and J. F. Rossignol.** 2002. In vitro activity of nitazoxanide and related compounds against isolates of Giardia intestinalis, Entamoeba histolytica and Trichomonas vaginalis. J. Antimicrob. Chemother. **49**:103-11.
2. **Arroyo, R., and J. F. Alderete.** 1989. Trichomonas vaginalis surface proteinase activity is necessary for parasite adherence to epithelial cells. Infect. Immun. **57**:2991-7.
3. **Bansal, A.K., and A. R. Kaur.** 2009**.** Cooperative functions of manganese and thiol redox system against oxidative stress in human spermatozoa. J. Hum. Reprod. Sci. **2**:76-80.
4. **Chopra, M., L. Townsend, L. Johnston, C. Mathews, M. Tomlinson. H. O'bra, and C. Kendall**. 2007. Estimating HIV prevalence and risk behaviors among high-risk heterosexual men with multiple sex partners: use of respondent-driven sampling. J. Acquir. Immune Defic. Syndr. **51**:72-77.
5. **Cudmore, S. L., K. L. Delgaty, S. F. Hayward-McClelland, D. P. Petrin, and G.E. Garber.** 2004. Treatment of infections caused by metronidazole-resistant Trichomonas vaginalis. Clin. Microbiol. Rev. **17**:783-93.
6. **D'Cruz, O.J., and F. M. Uckun**. 1999. Novel derivatives of phenethyl-5-bromopyridylthiourea and dihydroalkoxybenzyloxopyrimidine are dual-function spermicides with potent anti-HIV activity. Biol. Reprod. **60**:1419–1428.
7. **Dwivedi, A. K., V. L. Sharma, N. Kumaria, S.T. Kiran Kumar, P. K. Srivastava, A. H. Ansari, J. P. Maikhuri, G. Gupta, J. D. Dhar, R. Roy, B. S. Joshi, P. K. Shukla, M. Kumar, and S. Singh**. 2007. Synthesis of disulfide esters of dialkylaminocarbothioic acid as potent, non-detergent spermicidal agents. Bioorg. Med. Chem. **15**:6642-8.
8. **Ebrahim, S. H., M. T. McKenna, J. S. Marks.** 2005 Sexual behaviour: related adverse health burden in the United States. Sex Transm Infect. **81**:38-40.
9. **Ellman, G. L.** 1958. A colorimetric method for determining low concentrations of mercatans. Arch. Biochem. Biophys. **74**:443-450.

10. **Fichorova, R. N.** 2009. Impact of T. vaginalis infection on innate immune responses and reproductive outcome. J. Reprod. Immunol. **83**:185-9.
11. **Gillin, F. D., D. S. Reiner, R. B. Levy, and P. A. Henkart.** 1984. Thiol groups on the surface of anaerobic parasitic protozoa. Mol. Biochem. Parasitol. **13**:1-12.
12. **Gupta, G., R. K. Jain, J. P. Maikhuri, P. K. Shukla, M. Kumar, A. K. Roy, A. Patra, V. Singh, and S. Batra**. 2005. Discovery of substituted isoxazolecarbaldehydes as potent spermicides, acrosin inhibitors and mild anti-fungal agents. Hum. Reprod. **20**:2301-8.
13. **Honey, K.** 2007. Microbicide trial screeches to a halt. J. Clin. Invest. **117**:1116.
14. **Jain, R. K., J. P. Maikhuri, S. T. Kiran Kumar, V. L. Sharma, A. K. Dwivedi, K. Mitra, V. K. Bajpai, and G Gupta**. 2007. Novel disulphide esters of carbothioic acid as potent, non-detergent spermicides with low toxicity to Lactobacillus and HeLa cells in vitro. Hum. Reprod. **22**:708-16.
15. **Jain, R. K., A. Jain, J. P. Maikhuri, V. L. Sharma, A. K. Dwivedi, S. T. Kiran Kumar, K. Mitra, V. K. Bajpai, and G. Gupta.** 2009. In vitro testing of rationally designed spermicides for selectively targeting human sperm in vagina to ensure safe contraception. Hum. Reprod. **24**:590-601.
16. **Jain, R. K., A. Jain, R. Kumar, V. Verma, J. P. Maikhuri, V. L. Sharma, K. Mitra, S. Batra, and G. Gupta. 2010.** Functional attenuation of human sperm by novel, non-surfactant spermicides: precise targeting of membrane physiology without affecting structure. Hum. Reprod. 2010. **25**:1165-76.
17. **Kiran, Kumar. S. T., V. L. Sharma, M. Kumar, P.K. Shukla, P. Tiwari, R. K. Jain, J. P. Maikhuri, D. Singh, G. Gupta, and M.M. Singh.** 2006. Synthesis of benzenepropanamine analogues as non-detergent spermicides with antitrichomonas and anticandida activities. Bioorg. Med. Chem. **14**:6593-600.
18. **Kumar, L., A. Sarswat, N. Lal, A. Jain, S. Kumar, S. T. Kiran Kumar, J. P. Maikhuri, A. K. Pandey, P. K Shukla, G. Gupta, and V. L. Sharma**. 2011. Design and synthesis of 3-(azol-1-yl)phenylpropanes as microbicidal spermicides for prophylactic contraception. Bioorg. Med. Chem. Lett. **21**:176-81.

19. **Kumar, V. S., V. L. Sharma, P. Tiwari, D. Singh, J. P. Maikhuri, G. Gupta, and M. M. Singh.** 2006. The spermicidal and antitrichomonas activities of SSRI antidepressants. Bioorg. Med. Chem. Lett. **16**:2509-12.

20. **Kumaria, N., A. K. Dwivedi, J. P. Maikhuri, G. Gupta, S. Habib, J. D.Dhar, and S. Singh**. Substituted acrylophenones and related mannich bases as possible spermicides and inhibitors of HIV envelope glycoprotein-CD4 interaction. Eur. J. Med. Chem. **37**:855-64.

21. **Leitsch, D., D. Kolarich, M. Duchêne.** 2010. The flavin inhibitor diphenyleneiodonium renders Trichomonas vaginalis resistant to metronidazole, inhibits thioredoxin reductase and flavin reductase, and shuts off hydrogenosomal enzymatic pathways. Mol. Biochem. Parasitol. **171**:17-24.

22. **Maikhuri, J. P., A. K. Dwivedi, J. D. Dhar, B. S. Setty, G. Gupta**. 2003. Mechanism of action of some acrylophenones, quinolines and dithiocarbamate as potent, non-detergent spermicidal agents. Contraception **67**:403-8.

23. **McClelland, R. S., L. Sangaré, W. M. Hassan, L. Lavreys, K. Mandaliya, J. Kiarie, J. Ndinia-Achola, W. Jaoko, and J. M. Baeten.** 2007. Infection with Trichomonas vaginalis Increases the Risk of HIV-1 Acquisition. J. Infect. Dis. 195:698-702.

24. **Mendoza-López, M. R., C. Becerril-Garcia, L. V. Fattel-Facenda , L. Avila-Gonzalez, M. E. Ruíz-Tachiquín, J. Ortega-Lopez, and R. Arroyo.** 2000. CP-30, a cysteine proteinase involved in Trichomonas vaginalis cytoadherence. Infect. Immun. **68**:4907-12.

25. **Miki, K., W. Qu, E. H. Goulding, W.D. Willis, D. O. Bunch, L. F. Strader, S. D. Perreault, E. M. Eddy, and D. A. O'Brien.** 2004. Glyceraldehyde-3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci U S A. **101**:16501-6.

26. **Müller. S., E. Liebau , R. D. Walter, R. L. Krauth-Siegel.** 2003. Thiol-based redox metabolism of protozoan parasites. Trends Parasitol. **19**:320-8.

27. **Owen, D. H., D. F. Katz.** 1999. A vaginal fluid simulant. Contraception **59**:91-5.

28. **Olsen, M. L., C. A. Cwiak, C. Koudelka, and J. T. Jensen.** 2007. Desired qualities and hypothetical contextual use of vaginal microbicides in a diverse sample of US women. Contraception **76**:314-8.

29. **Richardson, B. A., L. Lavreys, H. L. Martin, C. E. Stevens , E. Ngugi, K. Mandaliya, J. Bwayo, J. Ndinia-Achola, and J. K. Kreiss.** 2001**.** Evaluation of a low-dose nonoxynol-9 gel for the prevention of sexually transmitted diseases: a randomized clinical trial. Sex. Transm. Dis. **28**:394-400.

30. **Riddles, P.W., R. L. Blakeley, B. Zerner.** 1979. Ellman's reagent: 5,5'-dithiobis(2-nitrobenzoic acid)--a reexamination. Anal. Biochem. **94**:75-81.

31. **Rojas, L., I. Sariego, J. Fraga, C.A. Sarria, J. Sarracent, and L. Sanchez**. 2004. Use of in vitro cytoadherence assays in the comparative study of the virulence of isolates of Trichomonas vaginalis. Parasitol. Res. **93**:332–337.

32. **Shah, V., G. F. Doncel, T. Seyoum, K. M. Eaton, I. Zalenskaya, R. Hagver, A. Azim, and R. Gross.** 2005 Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrob. Agents Chemother. **49**:4093-100.

33. **Singh, S., G. Sedgh, R. Hussain.** 2010. Unintended pregnancy: worldwide levels, trends, and outcomes. Stud. Fam. Plann. **41**:241-50.

34. **Sobel, J. D., P. Nyirjesy, W. Brown.** 2001. Tinidazole therapy for metronidazole-resistant vaginal trichomoniasis. Clin. Infect. Dis. **33**:1341-6.

35. **Tarrant, M. E., S. Wedley, T. J. Woodage, and J. N. Green.** 1973. Antiparasitic nitroimidazoles. IV. Serum protein binding and mode of action of 2-(4'-carboxystyryl)-5-nitro-1-vinylimidazole. Biochem. Pharmacol. **22**:639-49.

36. **Tayal, S. C., S. A. Ochogwu, and H. Bunce.** Paromomycin treatment of recalcitrant Trichomonas vaginalis. 2010. Int. J. STD AIDS **21**:217-8.

37. **Tiwari, P., D. Singh, and M. M. Singh.** 2008. Anti-Trichomonas activity of Sapindus saponins, a candidate for development as microbicidal contraceptive. J. Antimicrob. Chemother. **62**:526–534.

568 **38. Upcroft, J. A, and P. Upcroft.** 2001. Drug susceptibility testing of anaerobic
569 protozoa. Antimicrob. Agents Chemother. **45**:1810-4.
570 **39. Upcroft, P., and J. A. Upcroft** . 2001. Drug targets and mechanisms of resistance in
571 the anaerobic protozoa. Clin. Microbiol. Rev. **14**:150-64.
572 **40. Vignini, A., E. Buldreghini, L. Nanetti, S. Amoroso, M. Boscaro, G. Ricciardo-**
573 **Lamonica, L. Mazzanti, and G. Balercia.** 2009. Free thiols in human spermatozoa:
574 are Na+/K+-ATPase, Ca2+-ATPase activities involved in sperm motility through
575 peroxynitrite formation. Reprod. Biomed. Online. **18**:132-40.
576 **41. Vogel, H.G. 2008.** Drug Discovery and Evaluation: Pharmacological Assays,
577 Springer, Germany.
578 **42. Westrop, G. D., I. Georg, and G. H. Coombs.** The mercaptopyruvate
579 sulfurtransferase of Trichomonas vaginalis links cysteine catabolism to the
580 production of thioredoxin persulfide. J. Biol. Chem. **284**:33485-94.
581 **43. WHO**. 1999. Global prevalence and incidence of selected curable sexually
582 transmitted diseases: overview and estimates. Geneva:World Health Organization.
583 **44. World Health Organization 2010** WHO laboratory manual for the examination
584 and processing of human semen. Fifth Edition. Available at:
585 http://whqlibdoc.who.int/publications/2010/9789241547789_eng.pdf
586 **45. Wright, J. M., L. A. Dunn, Z. Kazimierczuk, A. G. Burgess, K. G. Krauer, P.**
587 **Upcroft, and J. A.Upcroft.** 2010. Susceptibility in vitro of clinically metronidazole-
588 resistant Trichomonas vaginalis to nitazoxanide, toyocamycin, and 2-fluoro-2'-
589 deoxyadenosine. Parasitol. Res. **107**:847-53.
590 **46. Yu, K. and Y.W. Chien.** 1995. Spermicidal activity-structure relationship of
591 nonoxynol oligomers: Physicochemical basis. Int. J. Pharmaceut. **125**:81-90.

592
593

594 **Legend to figures**

595 **Figure 1**. Inhibition of [^{3}H]-*Trichomonas* cytoadherence to human cervical (*HeLa*) cells by
596 Compound-2 (pyrrolidinium pyrrolidine-1-carbodithioate) *in vitro*. Bars represent mean

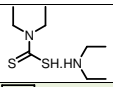
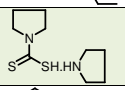
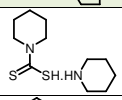
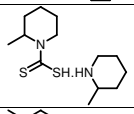
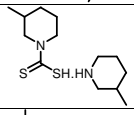
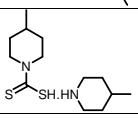
597 counts/minute (CPM) ± SE of three independent assays. Significant difference from control
598 (0.05% DMSO) is indicated as *P<0.05; **P<0.01; ***P<0.001.

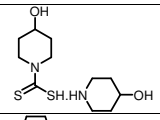
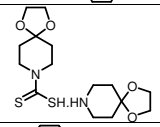
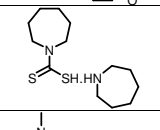
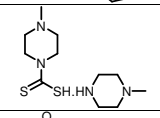
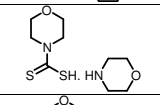
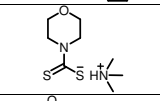
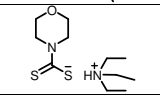
599

600 **Figure 2**. Inhibition of free thiols on human sperm by Compound-2 (pyrrolidinium pyrrolidine-
601 1-carbodithioate) at its spermicidal MEC (153 µM). Bars represent mean±SE of three
602 independent experiments using sperm from three different donors. Significant difference from
603 control (0.15% DMSO) is indicated as ***P<0.001.

604

605

Compound No.	Structure	IUPAC Name	MW	Spermicidal MEC (µM)	Anti-trichomonal MIC (µM) [MET-Susceptible]	Anti-trichomonal MIC (µM) [MET-Resistant]	Cytotoxic IC_{50} (µM) HeLa	Compatibility with *Lactobacillus* IC_{50} (µM)
1		diethylammonium diethylcarbamodithioate	222	188.0± 37.53 [3]	32.85± 12.41 [3]	93.84±18.76 [3]	1729.73 [3]	2617.12 [3]
2		pyrrolidinium pyrrolidine-1-carbodithioate	218	152.91±38.22 [3]	4.77 ±1.19 [3]	11.94±2.39 [3]	2229.36 [3]	1330.28 [3]
3		piperidinium piperidine-1-carbodithioate	246	169.38±33.87 [3]	8.46± 2.12 [3]	84.69±16.93 [3]	2577.24 [3]	3292.68 [3]
4		2-methylpiperidinium 2-methylpiperidine-1-carbodithioate	274	>10 mM [3]	30.41± 7.60 [3]	121.65±30.4 [3]	1645.99 [3]	1470.80 [3]
5		3-methylpiperidinium 3-methylpiperidine-1-carbodithioate	274	>10 mM [3]	243.31± 60.8 [3]	243.31±60.8 [3]	2587.59 [3]	2131.39 [3]
6		4-methylpiperidinium 4-methylpiperidine-1-carbodithioate	274	121.65± 30.41 [3]	>400 [3]	>400 [3]	1682.48 [3]	1711.68 [3]

7		4-hydroxypiperidinium 4-hydroxypiperidine-1-carbodithioate	278	8393.3± 4796.1 [3]	37.47± 7.49 [3]	>400 [3]	2100.72 [3]	>4000 [3]
8		1,4-dioxa-8-azonia-spiro[4.5]decane 1,4-dioxa-8- azaspiro[4.5]decane-8-carbodithioate	362	2302.03± 460.4 [3]	20.14± 7.61 [3]	23.02±5.75 [3]	2060.77 [3]	2204.42 [3]
9		azepanium azepane-1-carbodithioate	274	152.07± 30.41 [3]	76.03± 15.2 [3]	103.4±12.16 [3]	2649.64 [3]	591.24 [3]
10		4-methylpiperazin-1-ium 4-methylpiperazine-1-carbodithioate	276	1328.5±483.1 [3]	18.87± 3.77 [3]	332.13±30.1 [3]	>4000 [3]	>4000 [3]
11		morpholin-4-ium morpholine-4-carbodithioate	250	3333.3±666.6 [3]	16.67± 4.16 [3]	41.67±8.33 [3]	1704.00 [3]	1816.00 [3]
12		trimethylammonium morpholine-4-carbodithioate	222	1576.58± 675.67 [3]	18.77± 4.69 [3]	93.84±18.76 [3]	1738.74 [3]	1990.99 [3]
13		triethylammonium morpholine-4-carbodithioate	264	1010.1± 493.07 [3]	15.78± 3.94 [3]	39.46±7.89 [3]	1446.97 [3]	1863.64 [3]

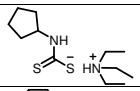
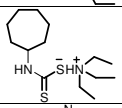
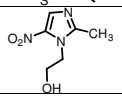
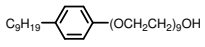
14		triethylammonium cyclopentylcarbamodithioate	262	>10 mM [3]	>400 [3]	>400 [3]	>4000 [3]	2160.31 [3]
15		triethylammonium cycloheptylcarbamodithioate	290	>10 mM [3]	>400 [3]	>400 [3]	>4000 [3]	2589.66 [3]
Metronidazole (MET)		2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol	171	>50 mM [3]	10.71± 0.97 [3]	340.83±48.6 [3]	>4000 [3]	>4000 [3]
Nonoxynol-9 (N-9)		26-(4-nonylphenoxy)-3,6,9,12,15,18,21, 24-octaoxa hexacosan -1-ol	616	243.18±81.05 [3]	70.25±5.40 [3]	75.65±5.40 [3]	53.82 [3]	52.68 [3]

Table-1. Structures of new compounds, their trichomonacidal and spermicidal activities, cyto-toxicity to cervical epithelial (*HeLa*) cells and compatibility with vaginal microflora (*Lactobacillus jensenii*). Mean±SE of three independent experiments. MW = Molecular Weight; MEC = minimum effective concentration; MIC = minimum inhibitory concentration.

Table 2. Susceptibility of *Trichomonas* vaginalis (susceptible and resistant strains) and human sperm to **compound-2** after its 60 min pre-incubation in simulated vaginal fluid (SVF, pH 4.2), and, under aerobic growth conditions of *Trichomonas*. [Mean±SE of 3 independent experiments; MET = metronidazole; MIC = minimum inhibitory concentration; MEC = minimum effective concentration]

	Trichomonacidal MIC (μM) [MET susceptible]		**Trichomonacidal MIC** (μM) [MET-resistant]		**Spermicidal MEC** [μM]	
	Comp-2	**Metronidazole**	**Comp-2**	**Metronidazole**	**Comp-2**	**Metronidazole**
Pre-incubation in SVF	2.98±0.59	7.59±1.51	9.54±2.39	311.61±19.47	145.2±42.56	>50 mM
Aerobic incubation	9.5±2.39	18.01±0.24	17.2±2.86	457±35.11	-	-