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Dithiocarbamate-Thiourea Hybrids Useful as Vaginal Microbicides Also Show Reverse Transcriptase Inhibition: Design, Synthesis, Docking and Pharmacokinetic studies[#]

Veenu Bala,[a,f] Santosh Jangir,[a] Dhanaraju Mandalapu,[a] Sonal Gupta,[a,f] Yashpal S. Chhonker,[c,f] Nand Lal,[a] Bhavana Kushwaha,[b] Hardik Chandasana,[c,f] Shagun Krishna,[d] Kavita Rawat,[e] Jagdamba P. Maikhuri,[b] Rabi S. Bhatta,[c,f] Mohammad I. Siddiqi,[d] Rajkamal Tripathi,[e] Gopal Gupta,[b] and Vishnu L. Sharma[a,f,*]

[a]*Medicinal & Process Chemistry Division, CSIR-Central Drug Research Institute, Lucknow-226031 (India)*
[b]*Endocrinology Division, CSIR-Central Drug Research Institute, Lucknow-226031 (India)*
[c]*Pharmacokinetics and Metabolism Division, CSIR-Central Drug Research Institute, Lucknow-226031 (India)*
[d]*Molecular & Structural Biology Division, CSIR-Central Drug Research Institute, Lucknow-226031 (India)*
[e]*Toxicology Division, CSIR-Central Drug Research Institute, Lucknow-226031 (India)*
[f]*Academy of Scientific and Innovative Research (AcSIR), New Delhi-110001 (India)*

ABSTRACT Prophylactic prevention is considered as the most promising strategy to tackle STI/HIV. Twenty-five dithiocarbamate-thiourea hybrids (**14-38**) were synthesized as woman controlled topical vaginal microbicides to counter *Trichomonas vaginalis* and sperm along with RT inhibition potential. The four promising compounds (**18**, **26**, **28** and **33**) were tested for safety through cytotoxic assay against human cervical cell line (*HeLa*) and compatibility with vaginal flora, *Lactobacillus*. Docking study of most promising vaginal microbicide (**33)** revealed that it docked in a position and orientation similar to known reverse transcriptase inhibitor Nevirapine. The preliminary *in vivo* pharmacokinetics of compound **33** was performed in *NZ*-rabbits to evaluate systemic toxicity in comparison to Nonoxynol-9.

Keywords: Dithiocarbamate, Thiourea, Non-nucleoside reverse transcriptase inhibitor (NNRTI), Spermicidal, Microbicidal, Nonoxynol-9.

**Corresponding author. Medicinal and Process Chemistry Division, Central Drug Research Institute, Sector 10, Jankipuram ext., Lucknow, Uttar Pradesh 226031, India.*
Tel.: 91-522-2772450; Ext. 4671; Fax: 91-522-2771941 E-mail address: vlscdri1@rediffmail.com; vlscdri@gmail.com

The acquired immune deficiency syndrome (AIDS) epidemic continues to spread throughout the world and especially imposes a particular burden on women and girls. According to UNAIDS 2013 estimate nearly 52% of all individuals living with HIV are now women of reproductive age (15–44 years).[1] They acquire the virus largely by heterosexual contacts, documented as the dominant mode of this pandemic spread.[2] However, male condoms may reduce the risk of HIV infection by 80-90% but they are beyond the control of women.[3] Thus, women urgently need self controlled methods to protect themselves against HIV infection. In the absence of an effective prophylactic HIV vaccine, prevention of new infections has become a priority. It was thought worthwhile to integrate HIV prevention and reproductive health services including unintended pregnancy protection for women as both are related with unprotected sex. Topical vaginal microbicide is one of the most promising female-controlled approaches effective against STIs (Sexually transmitted infections) including HIV in conjunction with pregnancy protection via sperm immobilization.[4, 5]

A safe, efficacious and women friendly vaginal microbicide has yet not been available because microbicides that are undergoing preclinical and human clinical trials possess detergent action. Nonoxynol-9 (N-9), the only recommended microbicide for protection against sexual transmission of HIV-1 resulted in compromised vagina owing to detergent action.[6, 7] Moreover, studies found that with frequent use, N-9 causes mucosal erosion and local inflammation of the female reproductive tract, which increases susceptibility to HIV-1 and other viral infections. Thus, clinical safety becomes a serious liability for vaginal products.[8-10]

Anti-HIV-1 potential of reverse transcriptase (RT) inhibitors in genital tract secretions has already been established. [11, 12] So, it was attempted to synthesize novel vaginal microbicides with RT inhibition as best suited prophylactic approach against vaginal HIV-1 transmission. [13, 14] The success of thiourea derivatives as NNRTIs [15, 16] and ongoing research interest in the development of dithiocarbamates (Figure 1) as potent vaginal anti-trichomonal spermicides [17-20] led us to hybridize these scaffolds in one chemical entity (alkyl 4-(alkyl/arylcarbamothioyl)piperazine-1-carbodithioate, Figure 1) to achieve dual action. Anti-HIV spermicidal potential of thiourea moiety has been recognized. [21-23] Considering the well-known "butterfly" conformation of NNRTIs, [24] like piperazine derivative, Delavirdine (Figure 1) these diverse scaffolds were synthesized and evaluated for their *in vitro* RT inhibition assay to ascertain their anti HIV-1 potential. Anti-*Trichomonas* and spermicidal activity of synthesized compounds was also evaluated against *Trichomonas vaginalis* and human sperm, respectively. The structure activity relationship (SAR) has also been discussed. The promising compounds were also assessed for their *in vitro* cytotoxicity profile on vaginal flora (*Lactobacillus*) and human cervical epithelium (*HeLa*) cells because they are intended to be used vaginally. Pharmacokinetics of most potent compound versus N-9 was evaluated in female Newzealand (*NZ)* rabbits to examine its absorption into systemic circulation and subsequent exposure in blood plasma through vaginal wall. Moreover, a docking study was also carried out to find a suitable correlation of most active compound with NNRTIs activity and inhibition of the prospective receptor.

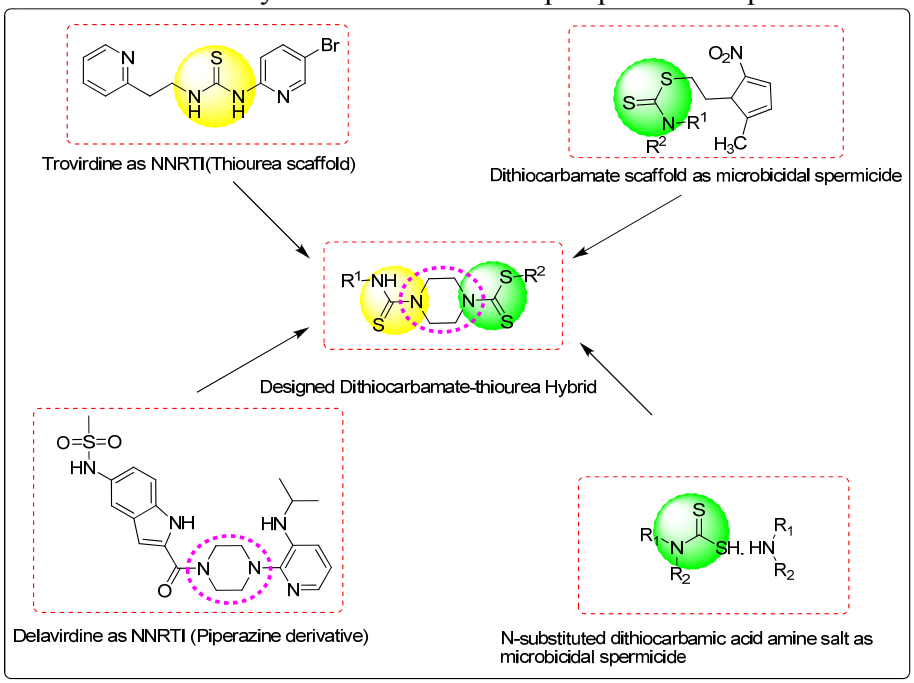
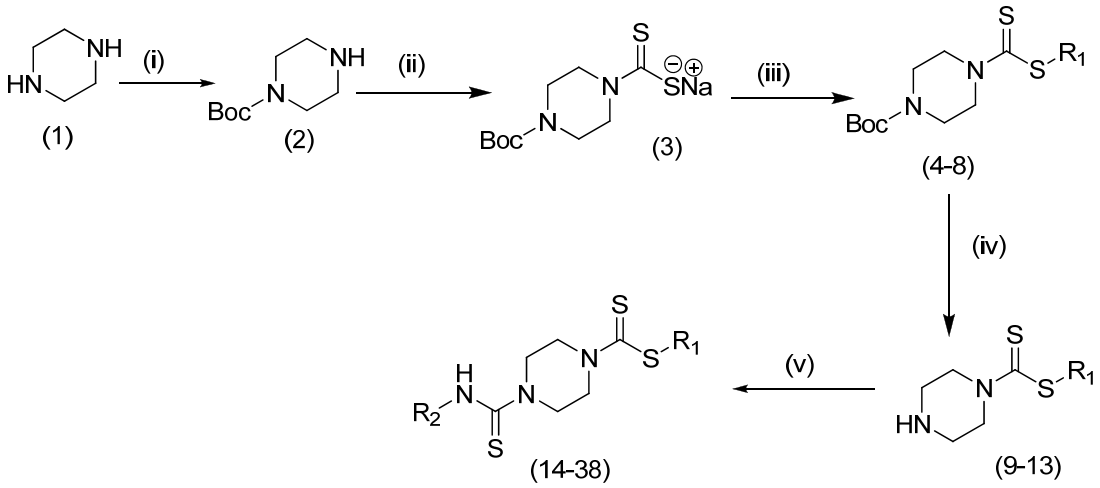


Figure 1. *Designing of Dithiocarbamate-Thiourea hybrids*

The alkyl 4-(alkyl/arylcarbamothioyl)piperazine-1-carbodithioate (**14–38**) were synthesized according to the strategy depicted in Scheme 1. Sodium 4-(*tert*-butoxycarbonyl)piperazine-1-carbodithioate (**3**) was synthesized by reaction of N-Boc piperazine with carbon disulfide and sodium hydroxide. Reaction of 3 with different alkyl halide in the presence of triethylamine in methanol at rt yielded *tert*-butyl 4-(alkylthiocarbonothioyl)piperazine-1-carboxylate (**4–8**). Treatment with trifluoroacetic acid (TFA) in dichloromethane (DCM, 0-5 °C) and aqueous sodium bicarbonate ($NaHCO_3$) resulted in Boc deprotection and gave alkyl piperazine-1-carbodithioate (**9–13**). Compounds **9–13** afforded alkyl 4-(alkyl/(aryl)alkyl carbamothioyl)piperazine-1-carbodithioate **(14–38)** on treatment with substituted isothiocyanates in ethanol at room temperature.

Scheme 1. *Synthesis of substituted Dithiocarbamate-Thiourea hybrids*[a]



[a]Reagents and conditions: (i) di-tert-butyl dicarbonate, methanol, rt, 4 h; (ii) CS_2, NaOH, ethyl acetate, 0-5°C, 3 h; (iii) alkyl halide, triethyl amine, methanol, rt, 4-5 h; (iv) TFA, DCM, aq. $NaHCO_3$; (v) Isothiocyanate, ethanol, rt, 1-2 h.

The structures of all newly synthesized compounds were confirmed by ^{1}H-NMR, ^{13}C-NMR, IR, mass spectrometry (ESMS and HRMS) and elemental analysis (see supporting information).

The compounds presented in this study namely alkyl 4-(alkyl/aryl carbamothioyl) piperazine-1-carbodithioate (**14–38**) were evaluated for anti-*Trichomonas* and spermicidal potential along with RT inhibitory activity by using enzymatic RT assay (Table 1).

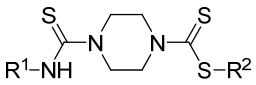
Trichomonas vaginalis causes punctuate hemorrhages giving HIV open access to its target T-lymphocytes. These hemorrhages leak the virus and cause them to concentrate in the infected area, which increase HIV transmission by 2 to 3 fold.[25] All the synthesized compounds (**14–38**) except **25** and **36** exhibited anti-*trichomonas* activity ranging from 7.78-500 µg/mL. Remarkable activity (better than N-9, MIC 20 µg/mL) was observed in compounds **28**, **33** and **14** which were active at 7.78 µg/mL, 15.56 µg/mL and 15.56 µg/mL respectively.

A potent contraceptive (spermicidal) activity in microbicides would attract users, especially those at a high risk of acquiring HIV/STD, and may improve compliance in usage. Spermicides capable of killing 100% human sperm almost instantaneously at physiological concentrations *in vitro* are likely to provide adequate pregnancy protection *in vivo*. Among synthesized twenty five

compounds, thirteen compounds (**14**, **16**, **18**, **23, 26-31, 33, 36** and **38**) exhibited spermicidal activity (Table 1) at 0.025%-2% (w/v) concentration and irreversibly immobilized 100% normal human spermatozoa. Out of these thirteen compounds, four compounds (**18**, **26, 28** and **33**) were active at conc. 0.025-0.05% comparable or even more active than marketed spermicide N-9 (MEC, 0.05%). Two compounds (**18** and **33**) demonstrated extremely potent spermicidal activity at MEC 0.025%.

All the compounds inhibited the RT ranging 1.90-44.67% at 100 µg/mL concentration. The compounds that showed moderate inhibitory activity (>30%) were **16**, **18, 20**, **29**, **31**, **33** whereas the control NNRTIs marketed drug Nevirapine (NVP) showed 99.6% inhibition. The enzyme assay results were visualized in combination with anti-*trichomonas* and spermicidal activity. The results are summarized in Table 1 along with standard drug NVP.

Table 1. *Anti-Trichomonas, spermicidal activity and RT inhibition assay of compounds* ***14–38***

Compound			Anti-*Trichomonas* activity MIC[a] (µg/ml)	Spermicidal activity MEC[b] (%)	% RT inhibition (100 µg/ml)[c]
	R^1	R^2			
14	Benzyl	Propyl	15.56	1%	20.56
15	Phenyl	Propyl	500	>2%	21.47
16	3-pyridyl	Propyl	125	0.5%	35.39
17	Phenethyl	Propyl	250	>2%	28.44
18	Benzoyl	Propyl	62.5	0.025%	31.42
19	Benzyl	Butyl	500	>2%	14.24
20	Phenyl	Butyl	125	>2%	30.87
21	3-pyridyl	Butyl	250	>2%	14.92
22	Phenethyl	Butyl	250	>2%	13.47
23	Benzoyl	Butyl	125	1%	27.8
24	Benzyl	Hexyl	250	>2%	27.33
25	Phenyl	Hexyl	>500	>2%	1.90
26	3-pyridyl	Hexyl	125	0.05%	26.14
27	Phenethyl	Hexyl	250	1%	14.84
28	Benzoyl	Hexyl	7.78	0.05%	23.19
29	Benzyl	Octyl	250	1%	36.46
30	Phenyl	Octyl	125	1%	23.96
31	3-pyridyl	Octyl	62.5	2%	44.67
32	Phenethyl	Octyl	250	>2%	15.67
33	Benzoyl	Octyl	15.56	0.025%	41.29
34	Benzyl	Benzyl	125	>2%	9.11

35	Phenyl	Benzyl	250	>2%	21.9
36	3-pyridyl	Benzyl	>500	2%	25.77
37	Phenethyl	Benzyl	62.5	>2%	11.85
38	Benzoyl	Benzyl	62.5	1%	12.35
Nevirapine					99.6
Nonoxynol-9			20	0.05	

[a]MIC-Minimum inhibitory concentration, [b]MEC-minimum effective concentration, [c]Inhibition (%) = observed inhibition(%) − negative control (%) of each tested compound. (All data presented over here are averages of three different observations)

This study included 1,4-disubstituted piperazine compounds (**14-38**) having a substituted thiourea at the N^1-position while substituted dithiocarbamates at N^4-position. The thiourea substituents R^1 were benzyl, phenyl, 3-pyridyl, phenethyl and benzoyl while the dithiocarbamate substituents R^2 have been propyl, butyl, hexyl, octyl, and benzyl. The anti-*Trichomonas* activity results suggested that the alkyl substituents at N^4-carbodithioate group were preferred over benzyl substituent. The hexyl substituent at N^4-position (R^2) of piperazine resulted in most active compound **28** (MIC, 7.78 μg/mL) followed by propyl/octyl substitution (compound **14** and **33**, 15.56 μg/mL). On the other hand the benzoyl group at N^1-thiourea substituent was most effective (**18**, **23**, **28**, **33** and **38**). The results of spermicidal activity showed that the preference of alkyl chain at N^4-carbodithioate was octyl > hexyl > propyl > benzyl > butyl whereas benzoyl was most suitable group at N^1-thiourea substituent. Two compounds (**26** and **28**) were as active as N-9 (MEC, 0.05%) while two others (**18** and **33**) exhibited two fold activity.

Among twenty five newly synthesized dithiocarbamate-thiourea hybrids (**14-38**), six compounds (**16**, **18, 20**, **29**, **31 and 33**) showed 30.87-44.67 % RT inhibition at 100 μg/mL. The thiourea moiety was thought responsible for RT inhibition. The activity with respect to the substituent at N^1-position (R^1) of piperazine was of the following order: 3-pyridyl (**31**, 44.67%) > benzoyl (**33**, 41.29%) > Benzyl (**29**, 36.46%) > phenyl (**20**, 30.87%) > phenethyl (**17**, 28.44%). It seems that structural combination of 3-pyridyl/benzoyl/benzyl at N^1 and octyl carbodithioate at N^4-position was most desirable for RT inhibition.

The structure of most active compounds (**18** and **33**) suggested that benzoyl thiourea at N^1 and propyl/octyl carbodithioate at N^4-position of piperazine were most required for sperm immobilization. The vaginal microbicidal activity comparisons of these compounds were made in view of all three activities together to achieve the aim to arrive at a pharmacophore possessing multiple activities. The study resulted in twelve dually active compounds (**14**, **16**, **18**, **23**, **26**-**31**, **33** and **38**) of which the most promising compound was octyl 4-(benzoylcarbamothioyl) piperazine-1-carbodithioate (**33**) as it showed anti-*Trichomonas* (15.56 μg/ml), spermicidal (0.025%) activity and RT inhibition (41.29%).

The compounds synthesized as vaginal microbicide with RT often also target other cells of vagina, such as cervico-vaginal epithelia and *lactobacilli* that are crucial in maintaining natural barrier to invasions by pathogens, especially the HIV. The most active compounds (**18**, **26, 28** and **33**) were evaluated for their safety towards human cervical cell line (*HeLa*) and compatibility with vaginal flora (*Lactobacillus,* Figure 2) at 200 μg/mL concentration by cell-viability assay. These compounds did not affect the viability of *HeLa* cells or growth of *Lactobacilli* during 24 hours of incubation and therefore appeared apparently much safer for vaginal use. These safety results support the utility of synthesized compounds as anti HIV-1

microbicide. Comparative *HeLa* cell survival after treatment with N-9 and most promising compound **33** is shown in Figure 3.

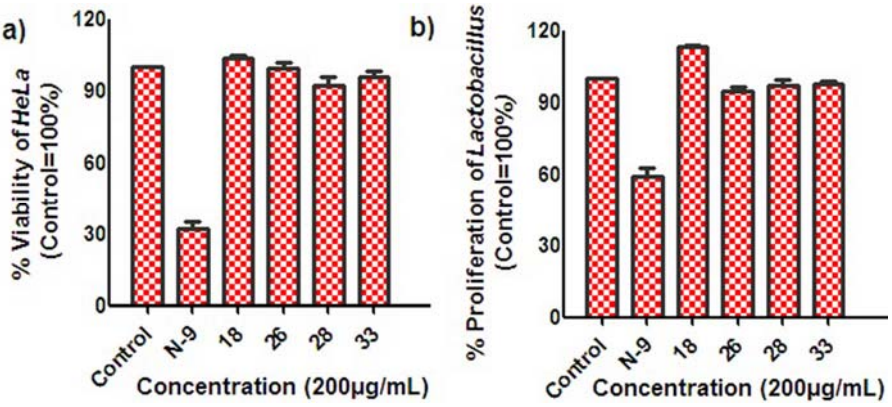


Figure 2. *(a) Cytotoxicity of compounds towards Human cervical cell lines (HeLa) (b) Compatibility with normal vaginal flora (Lactobacillus)*

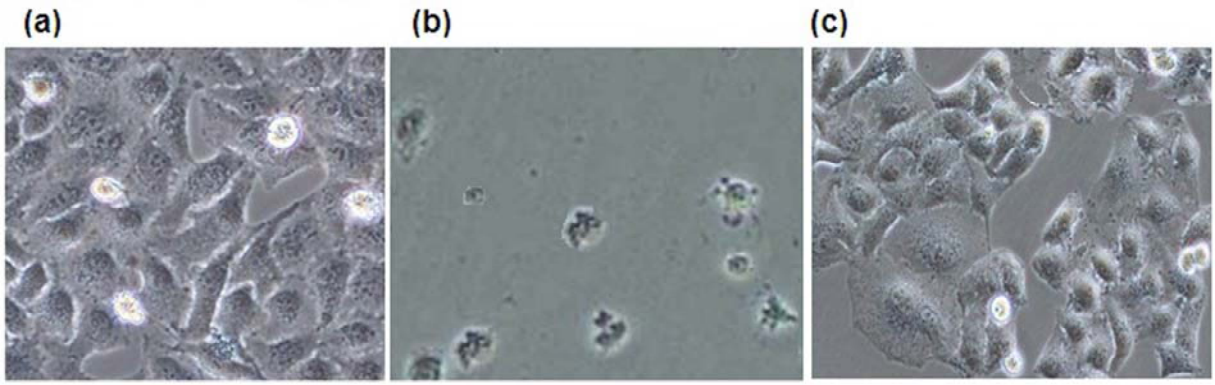


Figure 3*. Morphological changes of HeLa cells exposed to compound **33** at 200µg/mL for 24 h. (a) Control- HeLa cells incubated without any compound treatment (b) Cells treated with N-9 (c) Cells treated with most promising compound **33***

Docking study was performed to gain an insight into the binding mode and interactions of the most promising compound **33** with HIV-1 Reverse Transcriptase. The HIV-1 RT protein is a heterodimeric protein that consists of two units p66 and p51 with a catalytic and allosteric site.[26] The inhibitors of HIV-1 RT can occupy either the catalytic site or the allosteric site. The allosteric site is the binding site of various NNRTIs including Nevirapine (NVP). Previous studies done on the NNRTIs showed that these inhibitors occupy a hydrophobic pocket.[27] For our docking studies, initially NVP was extracted from crystal structure and re-docked into the active site of the crystal to obtain the conformation similar that in crystal structure to validate our docking program. Autodock4.2 successfully reproduced the conformation of crystal structure. For the docking simulations, the active site was selected by taking a 6.5 Å area around the co-crystallized ligand NVP.[28-30]

The docked complex of compound **33** is stabilized mainly by considerable hydrophobic interactions. To study the binding mode of compound **33**, the docked conformation was

compared with the crystal structure of HIV-1 RT. The residues involved in hydrophobic interaction include Leu100, Lys101, Val106, Val179, Tyr181, Tyr188, and Leu234 from p66 subunit and Glu138 from p51 unit. Figure 4 shows the binding mode of compound **33** comparable to NVP. It is clear from the figure that compound **33** occupies almost the same position as that of NVP in crystal structure. The benzoyl moiety present in this compound formed a cavity with Tyr188, Phe227, Trp229 and Leu234 residues, while the octyl group is oriented towards Leu100, Lys103 and Val106 residues, which is necessary for the stabilization of the complex.

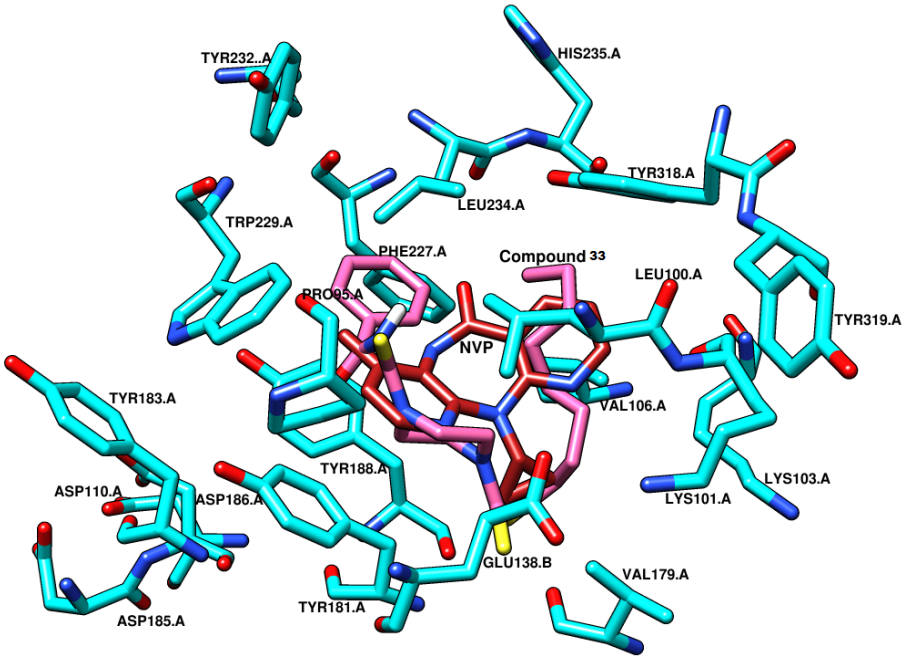


Figure 4. *Docked conformations of compound **33** (pink) superimposed on NVP (Brown). NVP is displayed for comparison.*

Stability and quantification of the extent of drug substance absorption after vaginal administration is essential in determining the strategy of nonclinical pharmacology/toxicology development. Simulated vaginal fluid (SVF, pH 4.2) was considered as a perfect media to determine the stability of molecules that are intended for intra-vaginal use.[31] The compound **33** was incubated in SVF at a final concentration 100 ng/mL for stability study. Samples were removed at 0, 5, 15, 30, 45, and 60 min. The concentration of compound **33** was determined by LC-MS/MS. The stability data have shown that compound **33** was stable in SVF upto 1.0 h. This behavior of compound **33** is favorable for its vaginal administration and supports its efficacy as vaginal microbicide with RT inhibition potential.

A preliminary vaginal pharmacokinetics study was carried out to monitor drug substance in blood plasma. LC-MS/MS assay using positive ion electro-spray ionization (ESI) in multiple reaction monitoring (MRM) mode was found appropriate to develop selective, sensitive and reproducible analysis of compound **33**, N-9 and internal standard (IS) in rabbit plasma. The pharmacokinetic analysis was processed non-compartmental model using Phoenix WinNonlin (version 6.3, Pharsight, MountainView, CA). The linear trapezoidal method with linear interpolation was used to calculate pharmacokinetic parameters. The mean peak plasma concentration (C_{max}) 4.87 ± 0.37 ng/mL of N-9 was achieved after vaginal dosing at 1.0 h. The

terminal half-life of plasma concentration of N-9 was 1.45± 0.07 h. The systemic absorption of compound **33** was below the limit of quantitation (Figure 5). After vaginal administration, the presence of compound **33** was very less in systemic circulation might be suggestive of minimal systemic adverse effects or minimal toxic effect. In summary, together with pharmacological findings, this study could provide useful clues and guidance, such as dosage regimen and application strategy of compound **33** as a lead candidate to be used as vaginal microbicide.

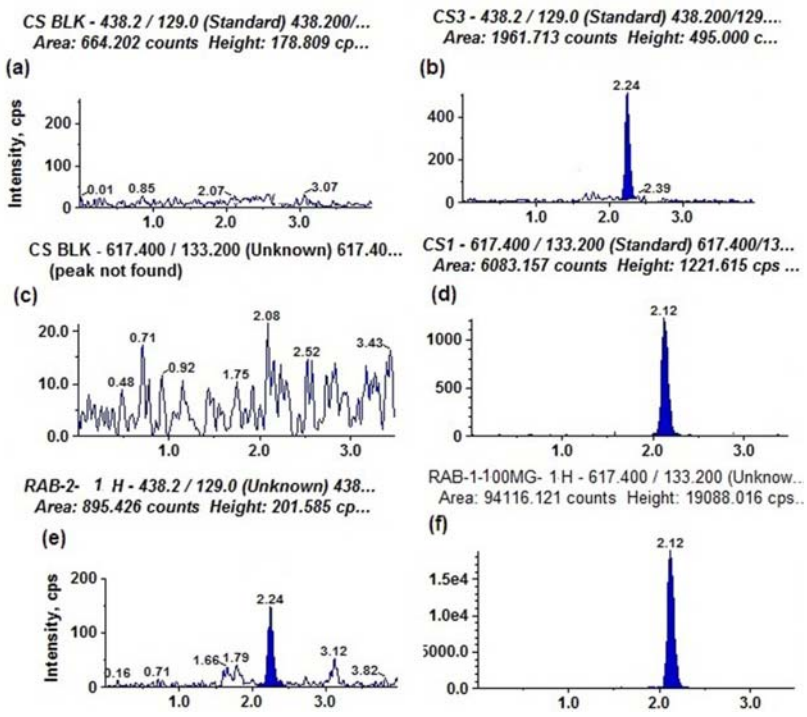


Figure 5. *Representative MRM ion-chromatograms of (a) blank plasma (compound **33**), (b) compound **33** spiked in plasma, (2.12 min) (c) blank plasma (N-9), (d) N-9 spiked in plasma, (e) PK sample at 1h of compound **33** and (f) PK sample at 1 h of N-9.*

In a systemic effort to identify a non-detergent prophylactic vaginal microbicide potentially capable of preventing sexual transmission of HIV/STDs as well as providing fertility control, a series of dithiocarbamate-thiourea hybrids as alkyl 4-(alkyl/aryl carbamothioyl)piperazine-1-carbodithioate (**14–38**) were synthesized and evaluated for multiple activities (anti-*Trichomonas,* spermicidal and RT inhibition). The intended dual action was achieved and several compounds exhibited all the three activities with remarkable safety against cervico-vaginal epithelial cells and vaginal flora. Among the safest structures, most promising compound **33** exhibited considerable RT inhibition, anti-*Trichomonas* and spermicidal activities, which could be attributed to its utility as vaginal microbicide. Proposed docking analysis of **33** is notable and can be utilized as a guideline for designing of promising vaginal microbicide with RT inhibition potential. Compound **33** was also found pharmacokinetically safe in comparison to N-9 due to least vaginal absorption to systemic circulation. Further lead optimization is underway to develop novel vaginal microbicide possessing RT inhibitory activity in order to empower women to deal independently with their reproductive health and fertility.

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Supplementary data

Supplementary data like scan copies of ^{1}H NMR spectra of **2–13** and ^{1}HNMR, ^{13}C NMR and HRMS spectral data of compounds **14–38** associated with this article can be found in the on-line version.

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