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# Effect of stereo and regiochemistry towards wild and multidrug resistant HIV-1 virus: viral potency of chiral PETT derivatives☆

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**Abstract**

Chiral derivatives of several substituted halopyridyl and thiazolyl PETT compounds were synthesized as non-nucleoside inhibitors of the reverse transcriptase (RT) enzyme of the human immunodeficiency virus (HIV-1). Molecular modeling studies indicated that because of the asymmetric geometry of the non-nucleoside inhibitors (NNRTI) binding pocket, the '*R*' stereoisomers would fit the NNRTI binding pocket of the HIV-1 RT much better than the corresponding '*S*' stereoisomers, as reflected by their $10^4$-fold lower $K_i$ values. The '*R*' stereoisomers of several PETT derivatives inhibited the recombinant RT in vitro with lower $IC_{50}$ values than their enantiomers. The active compounds were further evaluated for their ability to inhibit HIV-1 replication in human peripheral blood mononuclear cells (PBMCs). All the '*R*' isomers again showed potent anti-HIV activity and inhibited the replication of the HIV-1 strains $HTLV_{IIIB}$ in PBMCs at nanomolar concentrations whereas their enantiomers were less potent. The lead compounds for the respective groups were further tested against A17 (NNRTI-resistant, Y181C mutant RT), and A17Var (NNI-resistant Y181C ± K103N mutant RT) as well as multidrug resistant viral strains. The results indicated that the lead compounds were several logs more potent than the standard NNRTI drug nevirapine. Structure–activity relationship among the derivatives showed preference of pyridyl unit with halo substitutions primarily at 5-position demonstrating the importance of both the stereochemistry as well as regiochemistry. Our data provides experimental evidence that the stereochemistry and the regiochemistry of non-nucleoside inhibitors can profoundly affect their anti-HIV activity.


*Keywords:* HIV; Multidrug resistance; Non-nucleoside inhibitors; Regiochemistry; Stereochemistry; Thiourea

## 1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the causative agent for the transmission and development of the acquired immunodeficiency syndrome (AIDS) [1]. Current therapies target intervention in the replication cycle of the virus. The main targets in contemporary drug discovery efforts against HIV-1 is reverse transcriptase (RT), a vital enzyme that is responsible for the reverse transcription of retroviral RNA to proviral DNA [2–4]. Reverse transcriptase inhibitors constitute two types: nucleoside inhibitors such as AZT, 3TC and DDI, and non-nucleoside inhibitors (NNRTIs) such as nevirapine, delavirdine and efavirenz [5,6]. Combination therapies may be significant factor in the dramatic decrease of deaths from AIDS [7]. The most commonly used combinations include two nucleoside analogs, with or without a protease inhibitor [8]. In recent years, structure-based design has played an increasingly important role in the development of useful drugs, as demonstrated by the success of HIV-protease inhibitor design [9]. Nevirapine is currently the only non-nucleoside inhibitor compound that has been used in combination with AZT and/or protease inhibitors for the treatment of HIV-1. A new series of effective drug combinations most likely will involve other non-nucleoside inhibitors in combination with nucleoside and protease inhibitors as a triple-action regimen to combat the growing problem encountered during therapy due to the emergence of resistant mutant viral forms. NNRTIs inhibit HIV RT by altering either of the conformation or mobility of RT by binding to a specific allosteric site near the polymerase site, thereby resulting in non-competitive inhibition of the enzyme [10–12]. In a systematic search for potent anti-AIDS drugs, Bell et al. and Cantrell et al. [13,14] discovered the phenethylthiazolylthiourea (PETT)

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class of compounds as potent inhibitors of HIV-1 and disclosed the structure–activity relationship among various substituents in their structure. However, there was no report on the role of stereochemistry or regiochemistry towards anti-HIV activity of PETT derivatives. Following this lead and using rational drug design we have identified several structurally distinct thiourea compounds as potent NNRTIs for HIV-RT [15–21] and examined their structure–activity relationship. In order to examine the effect of stereochemistry of these PETT derivatives on HIV-activity we synthesized several chiral thiourea derivatives and examined their anti-HIV activity against the HIV-1 strain as well as multiple mutated strains. The following report gives the results obtained during the course of that study. Additionally, this study demonstrates for the first time that substitution on the ethyl linker unit of these PETT derivatives plays a significant role in their antiviral profile. It had been previously hypothesized that substitution on the aromatic and heterocyclic rings of the thiourea moiety imparted the biological activity, and no attention had been given to substitutions on the ethyl linker of the molecule.

## 2. Materials and methods

### 2.1. General synthesis of thiourea compounds

All chemicals were purchased from Aldrich (Milwaukee, WI) and were used without further purification. Unless otherwise noted, each reaction vessel was secured with rubber septa, and the reaction was performed under a nitrogen atmosphere. $^{1}$H, $^{13}$C NMR spectra were obtained on a Varian Mercury 300 instrument at ambient temperature in DMSO-$d_6$. Chemical shifts are reported as $\delta$ values in parts per million (ppm) downfield from tetramethylsilane ($\delta = 0.0$ ppm) as an internal standard or from the residual dimethylsulfoxide signal ($\delta = 2.49$ ppm for $^{1}$H NMR or $\delta = 39.7$ ppm for $^{13}$C NMR). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad peak. FT-IR spectra were recorded on a Nicolet Protégé 460 spectrometer. Mass spectra were performed on a Hewlett-Packard MALDI-TOF spectrometer (Model G2025A LD-TOF). Melting points were determined using a Melt John's apparatus and are uncorrected. HPLCs were done using a Hewlett-Packard 1100 series instrument consisting of an automatic sampler, an electronic degasser, a thermostatic control unit, and a diode array detector in conjunction with a Chemstation software assembly. The column used was an analytical RP-18 Lichrospher column, 5 μm (4.6 mm × 250 mm) and eluent was 35:65 water (0.1% acetic acid):acetonitrile. The flow rate was maintained at 1.0 ml/min and the detection wavelength was set at 275 nm. The column was maintained at room temperature throughout the analysis. Column chromatography was performed using silica gel obtained from the Baker Company. The solvents used for elution varied depending on the compound and included either one or a combination of the following: ethyl acetate, methanol, chloroform, hexane, methylene chloride, THF and ether. Condensing the respective chiral amines and thiocarbaimidazole derivatives of 5′- and 6′-substituted amino pyridines in anhydrous dimethylformamide made compounds. The chiral amines were purchased from Aldrich chemical company and were used without further purification. Thiazolyl and benzothiazolyl substituted thioureas were prepared in a similar fashion using thiocarbaimidazole derivatives of either substituted amino thiazoles or benzothiazoles, respectively.

A general synthesis for the compounds is as follows: thiocarbonyl di-imidazole and 2-amino-5-halo pyridine was added to 100 ml of dry acetonitrile under nitrogen atmosphere, and then stirred at room temperature for 12–15 h. The precipitate obtained was filtered, washed with cold acetonitrile, and dried thoroughly under vacuum to yield the thiocarbonyl intermediate. In the subsequent step, these intermediates were taken up in a dry flask under nitrogen, 50 ml of anhydrous dimethylformamide was added, and the contents were stirred for 30 min at room temperature. The corresponding chiral amine dissolved in 10 ml of dry dimethylformamide was added to this solution and the reaction mixture was heated to 110 °C for 15 h. The reaction mixture was cooled to room temperature, then poured into a crushed ice/water mixture and the contents were stirred for an additional hour. The precipitate was filtered, washed with cold water several times and dried under vacuum. The dried precipitate was then dissolved in chloroform and washed with brine, water and finally the separated chloroform layer was dried over anhydrous magnesium sulfate. Filtration and evaporation of the solvent yielded the target thiourea compound. Compounds were further purified using column chromatography on silica gel, and finally recrystallized using ethanol. A similar procedure was followed for other substituted compounds using either thiazolyl or benzothiazolyl precursors.

### 2.2. Stock $HTLV_{IIIB}$ virus

The HIV-1 strain $HTLV_{IIIB}$, which was propagated in CCRF-CEM, cells, was used in in-vitro assays of the anti-HIV-1 activity of the synthesized thiourea derivatives. Cell free supernatants of $HTLV_{IIIB}$-infected CCRF-CEM cells were harvested, dispensed into 1 ml aliquots, and frozen at −70 °C. Periodic titration of stock virus was performed by examining its cytopathic effects in MT-2 cells [22,23].

### 2.3. In vitro assays of anti-HIV-1 activity

Normal human peripheral blood mononuclear cells (PBMCs) from HIV-negative donors were cultured 72 h in RPMI 1640 supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS), 3% interleukin-2, 2 mM L-glutamine, 25 mM HEPES, 2 g/l $NaHCO_3$, 50 μg/ml

gentamicin, and 4 μg/ml phytohemagglutinin prior to exposure to HIV-1 at a multiplicity of infection (MOI) of 0.1 during a 1 h adsorption period at 37 °C in a humidified 5% $CO_2$ atmosphere. Subsequently, cells were cultured in 96-well microtiter plates (100 μm; $2 \times 10^6$ cells/ml) in the presence of various concentrations of thiourea analogues or nevirapine, trovirdine or AZT and aliquots of culture supernatants were removed from the wells on the 7th day after infection for p24 antigen assays, as previously described [22,23]. The applied p24 enzyme immunoassay (EIA) was the unmodified kinetic assay commercially available from Coulter Corporation/Immunotech, Inc. (Westbrooke, ME), which utilizes a murine mAb to HIV core protein coated onto microwell strips to which the antigen present in the test culture supernatant samples binds. Percent viral inhibition was calculated by comparing the p24 values from untreated infected cells (i.e. virus controls). In addition, the $IC_{50}$ values measuring the activity of compounds against recombinant HIV-1 RT (rRT) were determined using the Quan-RT assay system (Amersham, Arlington Heights, IL, USA), which utilizes the scintillation proximity assay principle [22,23].

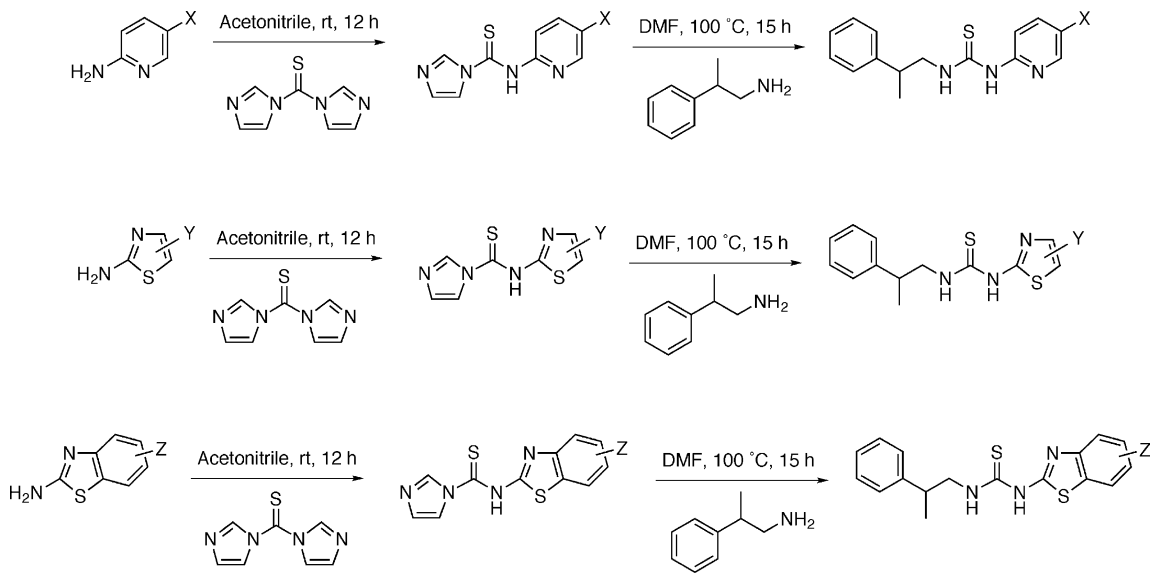
## 3. Results and discussion

For the synthesis of thiourea compounds we followed the general procedure shown in Scheme 1 as previously reported [14–16].

In our previous studies [15,16], we modified the structure of phenylethyl thiourea compounds by introducing various groups on the phenyl ring and evaluated their activity towards RT. In this study, we focused on the role of the chiral center of the phenyl ethyl thioureas for their NNRTI activity. Accordingly, we prepared the first group of chiral thiourea compounds to understand the role played by an extra methyl group, both '*R*' and '*S*' stereoisomers, in affecting their biological activities towards reverse transcriptase.

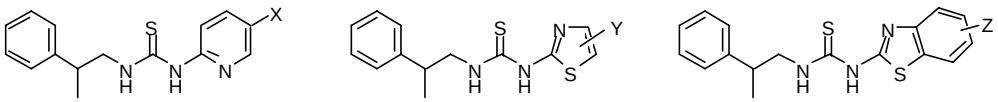
Among the ten β-methyl phenylethyl pyridyl thiourea compounds, the '*R*' stereoisomers of compounds with halogen (Br, Cl) or methyl substitutions respectively at 5-position, compounds **1**, **3** and **5**, were the most potent (Table 1). These compounds displayed nanomolar $IC_{50}$ values against recombinant RT in vitro. In contrast the '*S*' stereoisomers showed lower potency with $IC_{50}$ values in the range of 1–8 μM concentrations. Similarly, only the '*R*' stereoisomers of the β-methyl phenylethyl thiazolyl thioureas (**11** and **15**) exhibited anti-HIV activity (Table 1). Overall, the '*R*' stereoisomers of all compounds inhibited the recombinant RT in vitro with lower $IC_{50}$ values than their enantiomers.

Of the methyl phenyl thiourea (MPT) compounds (**1**–**19**) whose '*R*' stereoisomers exhibited nanomolar $IC_{50}$ values against recombinant RT, five compounds (**1**, **3**, **5**, **7**, and **9**) were further evaluated for their ability to inhibit HIV-1 replication in human peripheral blood mononuclear cells. These '*R*' stereoisomers were active anti-HIV agents and inhibited the replication of the HIV-1 strains $HTLV_{IIIB}$ (NNRTI-sensitive) at nanomolar concentrations. When examined for their ability to inhibit the replication of the HIV-1 strain, A17 (NNRTI-resistant, Y181C mutant RT) and A17Var (NNI-resistant, Y181C ± K103N mutant), majority of the '*R*' isomers showed activity from nanomolar to micromolar concentration range. The '*R*' stereoisomers of MPT compounds were relatively less potent against NNRTI-resistant HIV-strains than they were against $HTLV_{IIIB}$. Compound **3**, the 5-chloropyridyl isomer, was 380 times more active than nevirapine, 190 times more than delavirdine, and two times more active than trovirdine. We also examined the activity shown by these compounds against A17 variant HIV-1 strain and found the




Scheme 1.

Table 1
Effect of stereochemistry on anti-HIV activity of methyl phenylethyl substituted thiourea compounds



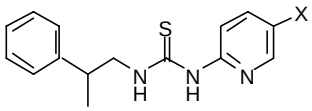
| Compound | Isomer | X | Y | rRT (μM) | RT (μM) HTLV $III_B$ | A17 (μM) | A17V (μM) |
|---|---|---|---|---|---|---|---|
| **1** | *R* | 5-Br | – | 0.42 | 0.003 | 0.67 | 1.08 |
| **2** | *S* | 5-Br | – | 1.90 | 0.030 | 1.18 | 1.23 |
| **3** | *R* | 5-Cl | – | 0.10 | <0.001 | 0.26 | 0.53 |
| **4** | *S* | 5-Cl | – | 1.39 | 0.038 | 0.54 | >100 |
| **5** | *R* | 5-Me | – | 0.52 | 0.003 | 1.08 | 1.84 |
| **6** | *S* | 5-Me | – | 7.70 | – | – | – |
| **7** | *R* | 6-Me | – | 16.71 | 0.04 | 1.40 | 1.19 |
| **8** | *S* | 6-Me | – | >100 | – | – | – |
| **9** | *R* | H | – | 8.40 | 0.028 | 2.34 | 0.54 |
| **10** | *S* | H | – | 3.50 | – | – | – |
| **11** | *R* | – | 4-Me | 5.05 | 0.055 | 6.85 | 1.10 |
| **12** | *S* | – | 4-Me | >100 | – | – | – |
| **13** | *R* | – | 4-Methylenecarbo | >100 | – | – | – |
| **14** | *S* | – | 4-Methylenecarbo | >100 | – | – | – |
| **15** | *R* | – | H | 10.02 | – | – | – |
| **16** | *S* | – | H | >100 | – | – | – |
| **17** | *R* | – | 4-Methylbenzo | >100 | – | – | – |
| **18** | *R* | – | Benzothiazolyl | >100 | – | – | – |
| **19** | *R* | – | 4,6-Me | 38.75 | – | – | – |
| Nevirapine | – | – | – | 23 | 0.034 | 100 | 100 |
| Trovirdine | – | – | – | 0.8 | 0.007 | 0.50 | 100 |
| Delavirdine | – | – | – | 1.5 | 0.009 | 50 | 100 |
| Efavirenz [27] | – | – | – | – | 0.0015– | – | 1.5 |
| Capravirin [28] | – | – | – | – | 0.005 | – | – |
| TMC-125 [29–31] | – | – | – | – | 0.002 | – | – |

The anti-HIV activity was measured by determining the inhibition of the HIV-1 strain $HTLV_{IIIB}$ in human PBMCs as previously described in detail [21,22]. The cell-free RT inhibition assays using recombinant RT(rRT) and the Quan-RT assay kit (Amersham, Arlington Heights, IL) were performed as reported [21,22].

entire series of these derivatives indicated activity in nanomolar concentrations whereas the standard drugs, namely nevirapine, trovirdine and delaviridine, were less potent. Based on the above experimental result we conclude that these β-methyl phenylethyl thiourea compounds may be useful candidates for further development especially since they showed remarkable in vitro activity against mutant strains of HIV-1. Table 2 shows the activity observed for β-methyl phenylethyl thioureas against RT MDR. It is evident from the table values that the halo pyridyl compounds exhibited potent activity at nanomolar concentrations. The β-methyl phenyl ethyl thiourea compounds were ten times more active compared to AZT, 65 times more potent than nevirapine and 19 times active than delavirdine indicating the role played by the structure of these compounds towards the mutant strains.

Our molecular modeling studies indicated that the '*R*' stereoisomers of chiral halopyridyl and thiazolyl thiourea compounds would fit the target NNRTI binding pocket on HIV-RT better than their enantiomers. Unfavorable interactions with the NNRTI binding pocket near the Y181 side chain would impair the binding of the '*S*' stereoisomer in its lower energy, ''staggered'' conformation. This steric hindrance could be relieved if the '*S*' stereoisomer adopted an energetically unfavorable ''eclipsed'' conformation. In either case, the estimated binding energies would be significantly higher for the '*S*' compounds compared to the '*R*' isomers. Fig. 1 shows a representative picture of

Table 2
Activity of β-methyl phenylethylthioureas against multidrug resistance strain of HIV-1



| Compound | Isomer | X | RTMDR (μM) |
|---|---|---|---|
| **1** | *R* | 5-Br | 0.02 |
| **2** | *S* | 5-Br | 0.17 |
| **3** | *R* | 5-Cl | 0.02 |
| **4** | *S* | 5-Cl | 0.16 |
| **5** | *R* | 5-Me | 0.05 |
| **7** | *R* | 6-Me | 0.10 |
| **9** | *R* | H | 0.19 |
| AZT | – | – | 0.15 |
| Nevirapine | – | – | 1.40 |
| Delavirdine | – | – | 0.40 |

The anti-HIV activity was measured by determining the inhibition of the replication of the strain in human PBMC [21,22].

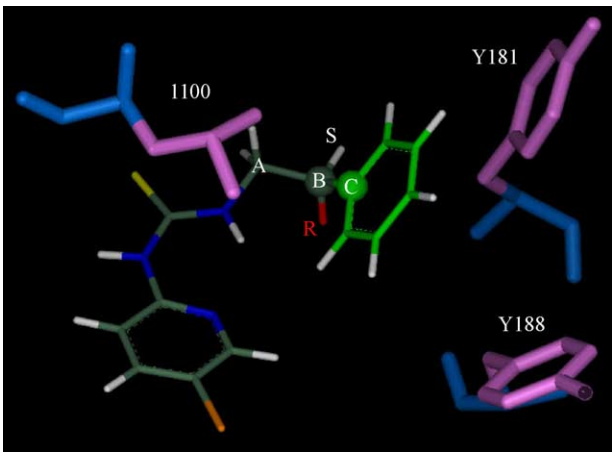



Fig. 1. Molecular model of a phenethyl pyridyl thiourea molecule that is docked into the non-nucleoside inhibitor reverse transcriptase binding pocket. The phenyl group is attached through a two-carbon linker marked as 'B' and 'A' in the figure. The chiral center in the molecule is labeled as 'B'. The pyridyl group is represented on the left hand side of the figure and is connected through thiourea unit.

phenylethyl pyridylthiourea molecule docked into the NNRTI binding site using previously described method [6,12].

Substitutions at the '*R*' and '*S*' positions would lead to different conformations of the phenethyl group in the Wing 2 region. The '*R*' stereoisomer is more energetically favored than the '*S*' stereoisomer, consistent with the predicted better-fit, and thus stronger binding, with the NNRTI binding pocket. However, the two-carbon linker between the phenyl group and the thiourea group is adjustable and more forgiving. The energetic difference is minimized by adopting an alternative conformation to maintain the tight fit with the binding pocket for both '*R*' and '*S*' stereoisomers, in comparison with those of the compounds with only a one-carbon spacing. The compounds with a one-carbon spacing are more rigid and their '*S*' stereoisomers have to overcome greater energetic barrier to accommodate the binding pocket. Based on this rationale we would expect that the '*R*' stereoisomers of β-methyl phenylethyl halopyridylthioureas may show greater potency compared to the '*S*' stereoisomers against reverse transcriptase, which is consistent with our experimental results. It is interesting to note that aza analogs of chiral ((2-phosphonomethoxy)propyl) guanines of a '*R*' stereoisomeric configuration were found to show potent anti-HIV activity as compared to the '*S*' isomers [24], another result that is consistent with our experimental data on chiral PETT derivatives.

### *3.1. Structure–activity relationship among chiral β-methyl phenethyl thioureas*

Among the chiral derivatives studied in the previous class of compounds it is evident that pyridyl compounds exhibit more potent activity compared to the thiazolyl as well as the benzothiazolyl compounds. Additionally, it was observed that halo-substitutions at the 5-position of the pyridyl ring enhanced the activity of these thioureas. Although a methyl substitution at the 6-position of the pyridyl ring was tolerated, once again the 5-position was preferred compared to the 6-position. This demonstrates the importance of substituent regiospecificity in addition to stereospecificity in these compounds.

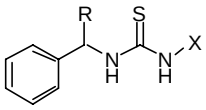
### *3.2. The activity of chiral α-methyl benzyl thioureas*

The synthesis followed the general procedure shown in Scheme 1 using the appropriate chiral amines and thiocarbonylimidazole derived from heterocyclic amines. Table 3 gives the anti-HIV activity observed for all the isomers of chiral α-methyl benzyl thiourea derivatives towards recombinant reverse transcriptase.

The α-methyl as well as α-ethyl groups were maintained constant and the pyridyl group was replaced by thiozolyl and benzothiazole rings. Additionally, we introduced several substituents in those rings in order to extend our study and have some understanding about structure–activity relationship among these derivatives. From Table 3 it is evident that all the '*R*' isomers were active whereas their enantiomers ('*S*') were inactive. This is in accordance with the results obtained from chiral PETT derivatives of β-methyl phenylethyl thiourea compounds as discussed previously (Table 1). Substitution on the pyridyl ring at 5-position was essential to retain the activity of the compounds. Among the substituents at 5-position, halo as well as alkyl substitutions yielded active derivatives. Among the methyl-substituted derivatives, the 5-methyl compound was more active than the 6-methyl substituted compound for these assays. Changing the pyridyl ring to thiazole ring and methyl substitution at 4-position of the thiazolyl ring did not measurably affect the activity of the compound. When we extended the study towards benzothiazolyl compounds, the activity of the compound decreased considerably indicating that this substitution is not beneficial for the PETT derivatives used in this study. Further introduction of groups on the benzothiazolyl functionality (compounds **38** and **43**) also did not improve biological activity.

The next aspect examined was the alteration of the chiral methyl group of these α-methyl benzyl thioureas using ethyl group instead. Unfortunately, we were able to only use a racemic mixture for this purpose due to non-availability of the individual isomeric amines. The thiazolyl compound (**20**) indicated an activity of 1.1 μM whereas the pyridyl compound (**28**) was 15 times less potent against recombinant reverse transcriptase. Interestingly, the bromo- and chloro-substituted pyridyl compounds (**25** and **26**) showed activity in the range of 3–5 μM, which was better than the unsubstituted pyridyl compound (**28**) (13.1 μM). Methyl substitution at 4-position of the thiazolyl compound indicated only moderate activity, however,

Table 3
Effect of stereochemistry on anti-HIV activity of α-methyl benzyl substituted thiourea compounds



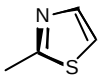
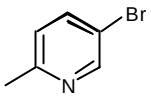
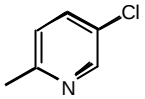
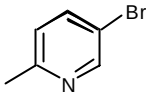
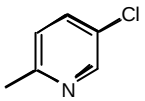
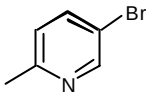
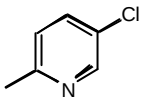
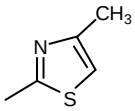
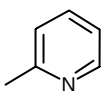
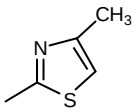
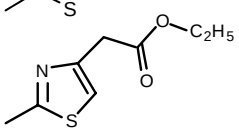
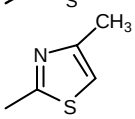
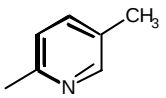
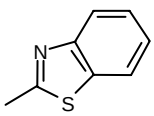
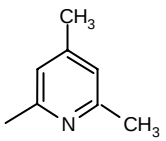
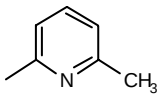
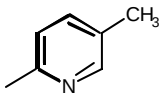
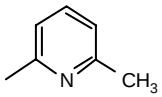
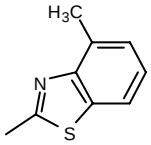
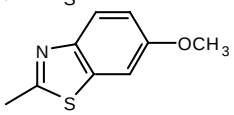
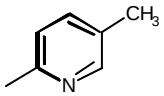
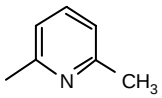
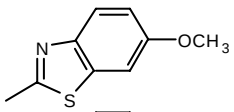
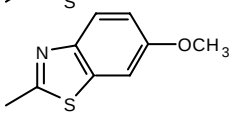
| Compound | R | Isomer | X | rRT (μM) | RT (μM) $HTLV_{IIIB}$ |
|---|---|---|---|---|---|
| **20** | Ethyl | *R*/*S* |  | 1.1 | <0.001 |
| **21** | Methyl | *R* |  | 1.6 | <0.01 |
| **22** | Methyl | *R* |  | 1.2 | <0.01 |
| **23** | Methyl | *S* |  | >100 | >1 |
| **24** | Methyl | *S* |  | >100 | – |
| **25** | Ethyl | *R*/*S* |  | 5.4 | 0.001 |
| **26** | Ethyl | *R*/*S* |  | 3.4 | 0.001 |
| **27** | Methyl | *R* |  | 0.8 | <0.001 |
| **28** | Ethyl | *R*/*S* |  | 13.1 | – |
| **29** | Methyl | *S* |  | >100 | – |
| **30** | Ethyl | *R*/*S* |  | 17.5 | 0.05 |
| **31** | Ethyl | *R*/*S* |  | 5.8 | 0.02 |
| **32** | Ethyl | *R*/*S* |  | 0.8 | 0.005 |
| **33** | Ethyl | *R*/*S* |  | >100 | – |
| **34** | Ethyl | *R*/*S* |  | >100 | – |

Table 3 (*Continued*)

| Compound | R | Isomer | X | rRT (μM) | RT (μM) $HTLV_{IIIB}$ |
|---|---|---|---|---|---|
| **35** | Methyl | *S* |  | >100 | – |
| **36** | Methyl | *R* |  | 1.5 | – |
| **37** | Ethyl | *R/S* |  | 5.7 | – |
| **38** | Methyl | *R* |  | 8.7 | – |
| **39** | Methyl | *S* |  | >100 | – |
| **40** | Methyl | *S* |  | >100 | – |
| **41** | Methyl | *R* |  | 1.7 | 0.025 |
| **42** | Ethyl | *R/S* |  | >100 | – |
| **43** | Methyl | *R* |  | >100 | – |
| Nevirapine | – | – | – | 23 | 0.034 |
| Trovirdine | – | – | – | 0.8 | 0.007 |
| Delvirdine | – | – | – | 1.5 | 0.009 |
| HI-240 | – | – | – | 0.6 | 0.001 |
| Efavirenz [27] | – | – | – | – | 0.0015 |
| Capravirin [28] | – | – | – | – | 0.005 |
| TMC-125 [29–31] | – | – | – | – | 0.002 |

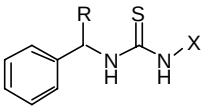
The anti-HIV activity was measured by determining the inhibition of the HIV-1 strain $HTLV_{IIIB}$ in human PBMCs as previously described in detail [21,22]. The cell-free RT inhibition assays using recombinant RT (rRT) and the Quan-RT assay kit (Amersham, Arlington Heights, IL) were performed as reported [21,22].

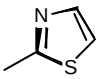
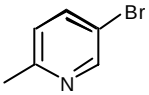
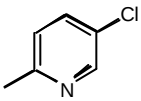
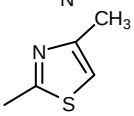
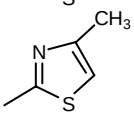
when carboethoxy group was introduced the activity decreased by 20-fold. Benzothiazolyl, dimethyl pyridyl, substitutions were also found to be detrimental for these sets of ethyl substituted compounds.

We next examined the ability of these novel thiourea compounds to inhibit the replication of the HIV-1 strain $HTLV_{IIIB}$ in human peripheral blood mononuclear cells (PBMCs). As shown in Table 3, few of the thiourea derivatives inhibited HIV-1 replication at nanomolar concentrations. Once again the '*R*' isomers were more active as compared to their enantiomers. The thiazolyl compound (**20**) was a potent compound with an activity of 0.001 μM in our assay. The structure activity relationship observed in the series was almost akin to that observed in the case of recombinant reverse transcriptase activity. It is also of note that the racemic ethyl substituted thioureas were very potent showing nanomolar level activity, implying that these compounds may be useful as lead compounds for future studies. In order to evaluate their clinical potential, we further examined their activity against a multidrug resistant virus (Table 4).

As a reference, we have also included the known antiviral compounds values for Table 4. Compound **27** was 1400 times more active than nevirapine, and 400 times more active than delavirdine. Similarly, compound **22** was 250 times more active than nevirapine and 80 times more active than delavirdine. In order to further evaluate the potential outcome of these chiral derivatives, a few of these

Table 4
Activity of α-methyl benzylthioureas against multidrug resistance and mutant virus strains of HIV-1



| Compound | R | Isomer | X | RTMDR (μM) | A17 (μM) | A17V (μM) |
|---|---|---|---|---|---|---|
| **20** | Ethyl | *R/S* |  | 1.130 | 2.0 | 1.50 |
| **21** | Methyl | *R* |  | 0.005 | 0.010 | 2.70 |
| **22** | Methyl | *R* |  | 0.010 | 0.20 | 10.20 |
| **27** | Methyl | *R* |  | 0.001 | 0.164 | 0.196 |
| **31** | Ethyl | *R/S* |  | 0.006 | 0.321 | 0.154 |
| Nevirapine | – | – | – | 1.400 | 100.0 | 100.0 |
| Trovirdine | – | – | – | – | 0.50 | 100.0 |
| Delavirdine | – | – | – | 0.400 | 50.0 | 100.0 |
| HI-240 | – | – | – | – | 0.20 | 41.0 |
| Efavirenz [27] | – | – | – | – | – | 1.5 |
| Capravirin [28] | – | – | – | – | – | – |
| TMC-125 [29–31] | – | – | – | – | – | – |

derivatives were tested against A17, A17 variant virus strains (Table 4). These compounds show promise indicating activity in nanomolar to micromolar concentration. As a whole, a trend emerges that a 5-substitution appears to be optimal in these thioureas. The stereochemistry also needs to have a '*R*' configuration for the best anti-HIV activity. This is in agreement with our previous hypothesis and strongly suggests that the stereochemistry of the thiourea derivatives plays a critical role in determining their activity towards HIV-1.

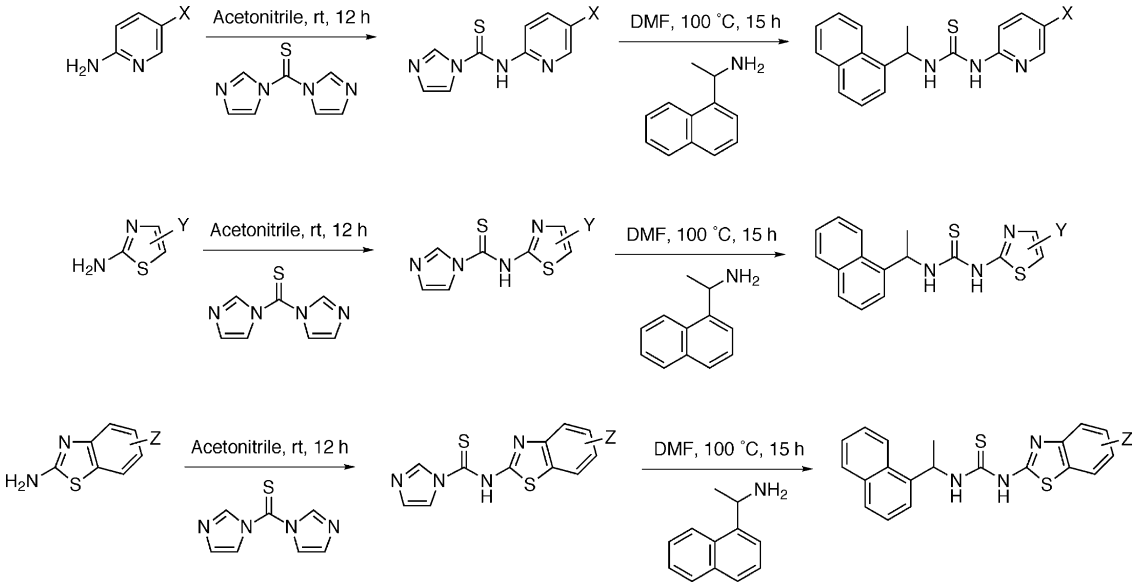
### 3.3. Molecular modeling studies

Molecular modeling studies indicated that the '*R*' stereoisomer would fit the target NNRTI binding pocket on HIV-RT much better than its enantiomer (Fig. 1) in the case of β-methyl phenyl ethyl thiourea derivatives. We further proposed that a similar trend is anticipated to occur in the case of α-methyl and α-ethyl benzyl thiourea derivatives. This was further confirmed by extending our examination towards cyclohexenyl, as well as naphthyl chiral.

This third class of naphthyl substituted chiral PETT compounds were synthesized using a similar method to the one described previously (Scheme 2). Among the six α-naphthyl ethyl pyridyl thiourea compounds, the '*R*' stereoisomers of compounds with halide (Cl (**44**), Br (**46**)) and methyl (**48**) substitutions at the 5-position were the most potent (Table 5). These compounds had nanomolar $IC_{50}$ values against recombinant RT in vitro. In contrast, none of the '*S*' stereoisomerisms were active against recombinant RT. Similarly, only the '*R*' stereoisomers of the five α-naphthyl ethyl thiazolyl thiourea compounds exhibited anti-HIV activity (Table 5). Overall, the '*R*' stereoisomers of all 11 compounds inhibited the recombinant RT in vitro with lower $IC_{50}$ values than their enantiomers.

Of the seven compounds whose '*R*' stereoisomers exhibited nanomolar $IC_{50}$ values against recombinant RT, five were further evaluated for their ability to inhibit HIV-1 replication in human PBMCs. All five '*R*' stereoisomers were active anti-HIV agents and inhibited the replication of the HIV-1 strains $HTLV_{IIIB}$ (NNRTI-sensitive), A17 (NNI-resistant, Y181C mutant RT), and A17Var (NNI-resistant, Y181C ± K103N mutant RT) in human PBMCs at nanomolar concentrations, whereas their enantiomers were inactive (Table 6).

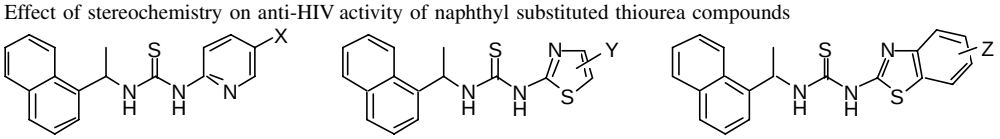
In addition we observed that the A17V (Y181C ± K103N) mutant was quite resistant to these compounds as seen in Table 6. Furthermore, in comparison with RTMDR, the Y181C ± K103N mutant displayed high resistance to all the compounds tested in the present study. For example, the Y181C ± K103N mutant displays 10 times greater resistance to compound **52**, and 50 times or greater resistance to **44**, **46** and **48**, respectively.




Scheme 2.

However, our lead compounds **44** and **52** were 3 orders of magnitude more potent than the standard NNRTI drug nevirapine against the NNRTI-resistant HIV-1 strains. Table 6 also shows the activity data observed for compounds against drug resistant laboratory strains of HIV-1 as well as a lead clinical HIV-1 isolates for AIDS patients. The structure–activity relationship once again established that a pyridyl ring with halo substitutions at the 5-position was essential to retain the anti-HIV activity of these naphthyl thiourea compounds. This is again consistent

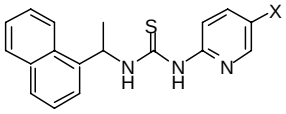
Table 5
Effect of stereochemistry on anti-HIV activity of naphthyl substituted thiourea compounds

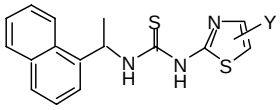


| Compound | X | Y | Z | Estimated $K_i$ (μM) | $IC_{50}$ rRT (μM) | $IC_{50}$ $HTLV_{IIIB}$ (nM) |
|---|---|---|---|---|---|---|
| **44** (*R*) | 5-Cl | – | – | 0.01 | 0.58 ± 0.18 | 1.0 ± 0 |
| **45** (*S*) | 5-Cl | – | – | >100 | >100 | – |
| **46** (*R*) | 5-Br | – | – | 0.01 | 0.67 ± 0.20 | 1.5 ± 0.6 |
| **47** (*S*) | 5-Br | – | – | >100 | >100 | – |
| **48** (*R*) | 5-Me | – | – | – | 0.45 ± 0.06 | 2.3 ± 0.3 |
| **49** (*S*) | 5-Me | – | – | >100 | >100 | – |
| **50** (*R*) | 6-Me | – | – | – | 1.03 ± 0.10 | 4.0 ± 3.7 |
| **51** (*S*) | 6-Me | – | – | >100 | >100 | – |
| **52** (*R*) | – | 4-Me | – | – | 1.17 ± 0.32 | 1.0 ± 0 |
| **53** (*S*) | – | 4-Me | – | >100 | >100 | – |
| **54** (*R*) | – | H | – | – | 12 | – |
| **55** (*S*) | – | 4-H | – | >100 | >100 | – |
| **56** (*R*) | 4,6-Dimethyl | – | – | – | 40 | – |
| **57** (*S*) | 4,6-Dimethyl | – | – | >100 | >100 | – |
| **58** (*R*) | – | 4-Methylene ethoxy | – | – | 0.89 ± 0.41 | 54.0 ± 16.0 |
| **59** (*S*) | – | 4-Methylene ethoxy | – | >100 | >100 | – |
| **60** (*R*) | – | – | H | – | 0.44 | 205.0 ± 95.3 |
| **61** (*S*) | – | – | H | >100 | >100 | – |
| **62** (*R*) | – | – | 4-Me | – | 10.2 | – |
| **63** (*S*) | – | – | 4-Me | >100 | >100 | – |
| **64** (*R*) | H | – | – | – | 1.21 | – |
| **65** (*S*) | H | – | – | >100 | >100 | – |
| Efavirenz [27] | – | – | – | – | – | 1.5 |
| Capravirin [28] | – | – | – | – | – | 5.0 |
| TMC-125 [29–31] | – | – | – | – | – | 2.0 |

The anti-HIV activity was measured by determining the inhibition of the HIV-1 strain $HTLV_{IIIB}$ in human PBMCs as previously described in detail. The cell-free RT inhibition assays using recombinant RT (rRT) and the Quan-RT assay kit (Amersham, Arlington Heights, IL) were performed as reported [21,22].

Table 6
Activity of lead CNT compounds against drug-resistant HIV-1 laboratory strains and primary HIV-1 isolates from AIDS patients




| Compound | X | Y | $IC_{50}$ (nM) | | | |
|---|---|---|---|---|---|---|
| | | | RTMDR[a] | A17[b] | A17V[c] | Patient HIV-1 isolates[d] ($n = 3$) |
| **44** (*R*) | 5-Cl | – | 1 ± 0 | 43 ± 14 | 367 ± 141 | 5 ± 4 |
| **46** (*R*) | 5-Br | – | 1 ± 0 | 105 ± 60 | 287 ± 177 | 1 ± 0 |
| **48** (*R*) | 5-Me | – | 3 ± 0 | 66 ± 22 | 543 ± 353 | 11 ± 9 |
| **52** (*R*) | – | 4-Me | 21 ± 10 | 145 ± 71 | 114 ± 50 | 11 ± 10 |
| Nevirapine | – | – | 155 | >1,000 | >10,000 | 9 ± 5 |
| AZT | – | – | 55 | – | – | 4 ± 1 |

The anti-HIV activity was measured by determining the inhibition of the replication of the indicated HIV-1 strains and clinical HIV-1 isolates in human PBMCs [20,21].
[a] V106A, T215Y, L74V.
[b] Y181C.
[c] Y181C, K103N.
[d] The patient HIV-1 isolates were BR/93/020 (L214F), BR/93/019(D67, T215D, K219 Q, L214F) and BR/93/029(L214F) obtained from NIH.

with the earlier results observed for the other chiral derivatives discussed previously.

Our molecular modeling studies again indicated that compound **52** (the '*R*' stereoisomer) would fit the target NNI binding pocket on HIV-RT much better than its enantiomer (compound **53**) (Fig. 2).

Based on our earlier hypothesis on chiral phenyl ethyl thiourea compounds (Fig. 1) we propose a similar scheme for naphthyl thiourea compounds. The predictions from the modeling studies are consistent with the experimental data obtained in cell-free RT inhibition assays and cellular HIV inhibition assays. Furthermore, these compounds are also very active against primary HIV-1 isolates of AIDS patients (Table 6), again demonstrating the importance of stereochemistry as well as regiochemistry in their structure.

The next class of chiral thioureas examined consisted of two cyclohexyl methyl halopyridyl thiourea compounds, two α-methyl benzylhalopyridyl thiourea compounds and one cyclohexyl ethyl thiazolyl thiourea compound. These were introduced to examine the effects of a non-planar group on the activity of the thiourea compounds. We followed the general synthetic procedure as discussed earlier. The isomeric purities of a few derivatives were further established by X-ray crystal structures. The structures of the chiral bromopyridyl thiourea compounds, *N*-[1-(1-(1*R*)-cyclohexylethyl)]-*N*-[2-(5-bromopyridyl)] thiourea (**66**) and *N*-[1-(1-(1*S*)-cyclohexylethyl)]-*N*-[2-(5-bromopyridyl)] thiourea (**67**), were resolved by X-ray crystallography (Fig. 3).

Both compounds adopt lower energy conformations in the crystalline state relative to the C14–C7–C8–C13 torsion angle (62.0(5)°) in **66** and the C14–C7–C8–C9 torsion angle (−62.4(5)°) in **67**, with staggering of the cyclohexyl and methyl groups on the chiral carbon. Our molecular modeling studies indicated that the '*R*' stereoisomer

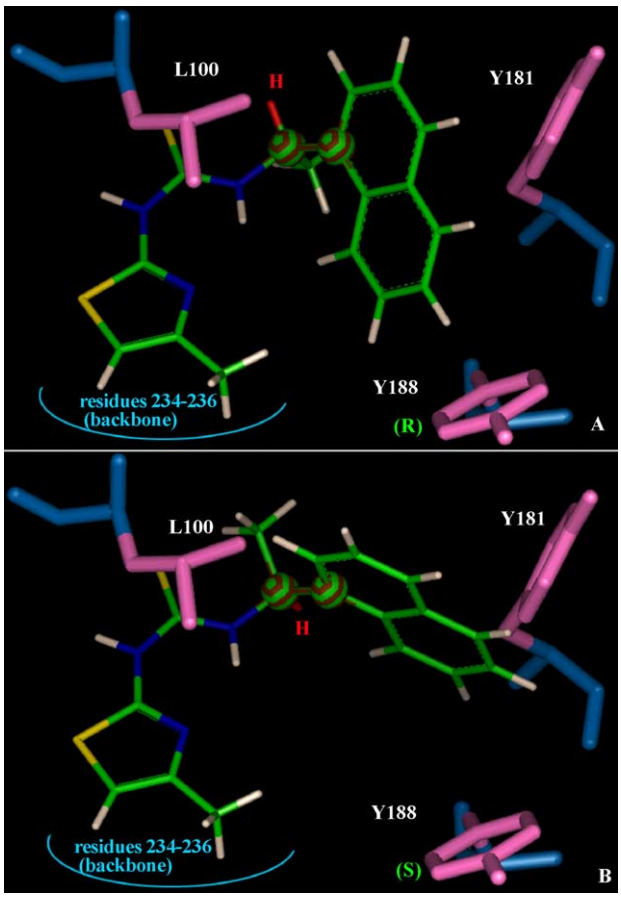



Fig. 2. Compounds **52** and **53** were docked into the NNRTI binding site of HIV reverse transcriptase. The stick models of **52** ('*R*' isomer, top) and **53** ('*S*' isomer, bottom) were docked into the NNRTI binding site as lower energy *staggered conformations* with respect to the naphthyl and methyl groups on the chiral carbon. Only the '*R*' isomer **40** provided a suitable fit into the NNRTI binding pocket. The '*S*' isomer **41** formed unfavorable steric interactions with binding pocket residues near the Y181 side chain.

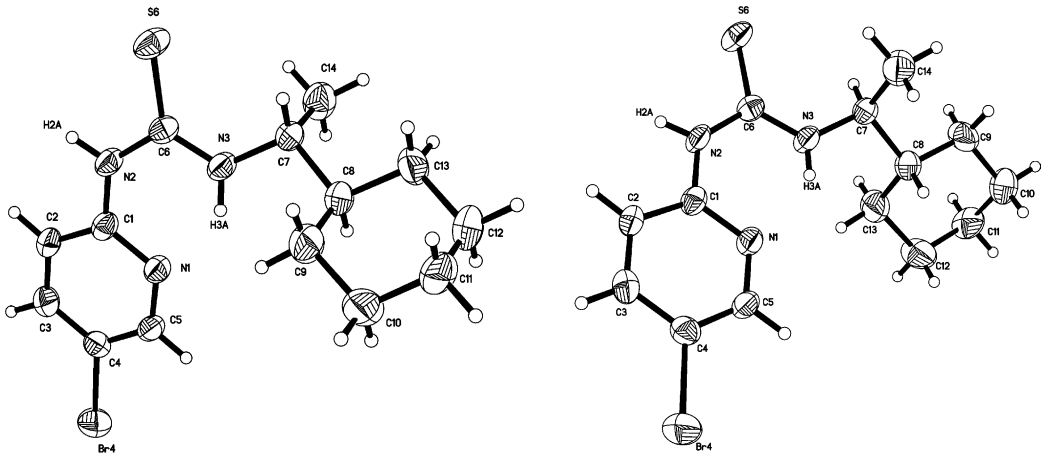



Fig. 3. X-ray crystal structures of compounds **66** and **67**. Thirty percent probability ellipsoids, $T = 22\ ^{\circ}C$. (A) Compound **66**: space group: $P3_22_1$, unit cell: $a = 9.2322(4)$ Å, $b = 9.2322(4)$ Å, $c = 32.9810(18)$ Å, $\alpha = 90^{\circ}$, $\beta = 90^{\circ}$, $\gamma = 120^{\circ}$, volume = 2434.5(2) Å$^3$, $Z = 6$, $\theta$ range for data collection = 1.85–28.17° ($\lambda = 0.71073$ Å), total reflections collected = 14,953, independent reflections = 3775 ($R_{int} = 0.030$), data/restraints/parameters = 3775/0/173, R1 ($I > 2\sigma(I)$) = 0.041, wR2 = 0.11, goodness-of-fit on $F^2 = 0.986$. Absolute structure parameter: 0.019(12). (B) Compound **67**: space group: $P3_12_1$, unit cell: $a = 9.2290(4)$ Å, $b = 9.2290(4)$ Å, $c = 32.979(2)$ Å, $\alpha = 90^{\circ}$, $\beta = 90^{\circ}$, $\gamma = 120^{\circ}$, volume = 2432.6(2) Å$^3$, $Z = 6$, $\theta$ range for data collection = 1.85–28.20° ($\lambda = 0.71073$ Å), total reflections collected = 14,952, independent reflections = 3792 ($R_{int} = 0.038$), data/restraints/parameters = 3792/0/181, R1 ($I > 2\sigma(I)$) = 0.041, wR2 = 0.099, goodness-of-fit on $F^2 = 0.913$. Absolute structure parameter: 0.014(12).

compound **66** would fit the target NNI binding pocket on HIV-RT much better than its enantiomer **67** (Fig. 4).

Unfavorable steric interactions with the NNI binding pocket residues near the Y181 side chain would impair the binding of '*S*' isomer in a lower energy, ''staggered'' conformation. This steric hindrance would be relieved if the '*S*' isomer adopts an energetically unfavorable ''eclipsed'' conformation. In either case, the estimated binding energy would be significantly higher for **67**, as reflected by the higher estimated $K_i$ value (Table 7). Similar assumptions could be made in favor of the '*R*' stereoisomer in modeling studies of the chiral chloropyridyl thiourea compounds, *N*-[1-(1-(1*R*)-cyclohexylethyl)]-*N*-[2-(5-chloropyridyl)] thiourea (**68**) and *N*-[1-(1-(1*S*)-cyclohexylethyl)]-*N*-[2-(5-chloropyridyl)] thiourea (**69**). Modeling studies indicated that the methyl group on the chiral carbon of **66** and **68** promotes the strong binding to the NNI binding pocket via van der Waals contacts with residue V179. Since this methyl group is 7 Å away from Y181 (as measured from the carbon atom of the methyl group to the Cγ position of the protein residue), 9 Å from Y188, 8.5 Å from V106, and 6.5 Å from K103, its favorable influence on the binding of compounds **66** and **68** to RT should not be affected by frequently encountered mutations involving these residues. The control compounds with unsubstituted pyridyl rings were predicted to fit poorly into the NNI binding pocket (Table 7). The accuracy of the predictions from the modeling studies was evaluated in cell free RT inhibition assays. As presented in Table 7, compounds **66** and **68**, with estimated $K_i$ values 100-fold lower than those of the '*S*'

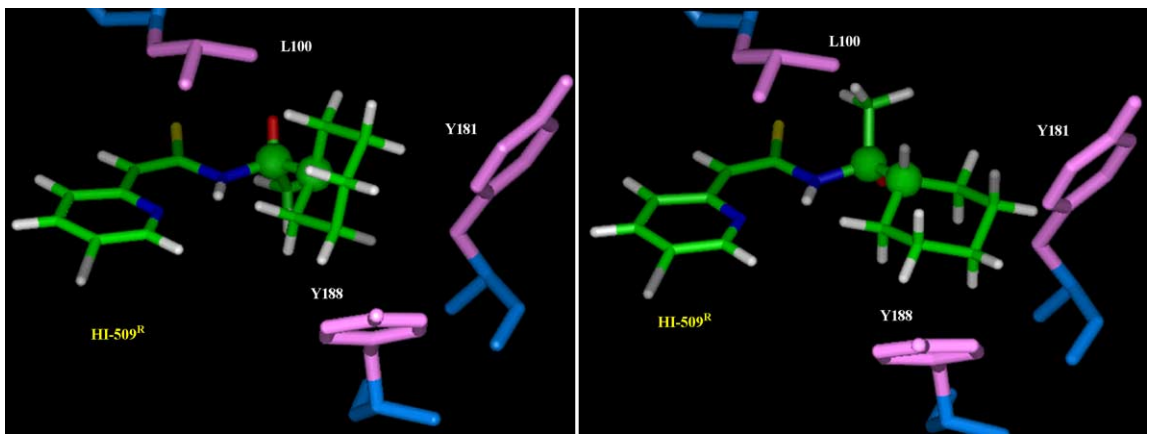
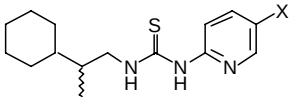
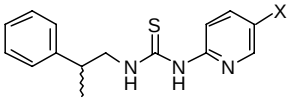



Fig. 4. Binding of thiourea compounds to NNRTI binding pocket of HIV-RT. Compounds **66** and **67** were docked into the NNRTI binding site of HIV reverse transcriptase. The stick models of **66** ('*R*' isomer, left) and **67** ('*S*' isomer, right) were docked into the NNRTI binding site as lower energy *staggered conformations* with respect to the cyclohexane and methyl groups on the chiral carbon. Only the '*R*' isomer (**66**) provided a suitable fit into the NNRTI binding pocket. The '*S*' isomer (**67**) formed unfavorable steric interactions with binding pocket residues near the Y181 sidechain.

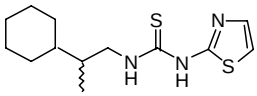
Table 7
Effect of stereochemistry on anti-HIV activity of thiourea compounds



X = Cl [**66**, **67**]
X = Br [**68**, **69**]



X = Cl [**21**, **22**]
X = Br [**23**, **24**]



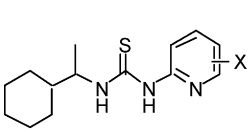
[**72**, **73**]

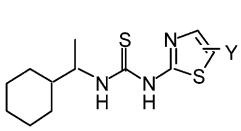
| Compound | Isomer | X | Estimated $K_i$ (μM)[a] | $IC_{50}$ rRT (μM) | $IC_{50}$ $HTLV_{IIIB}$ (μM) |
|---|---|---|---|---|---|
| **66** | *R* | Br | 1.1 | 1.2 | 0.001 |
| **67** | *S* | Br | >100 | >100 | >1 |
| **68** | *R* | Cl | 1.2 | 1.4 | 0.025 |
| **69** | *S* | Cl | >100 | >100 | >1 |
| **70** | *S* | H | >100 | >100 | >1 |
| **71** | *R* | H | >100 | >100 | >1 |
| **21** | *R* | Br | – | 1.6 | 0.01 |
| **23** | *S* | Br | – | >100 | >1 |
| **22** | *R* | Cl | – | 1.2 | 0.01 |
| **24** | *S* | Cl | – | >100 | ND |
| **72** | *R* | – | 12.0 | 13.0 | 0.001 |
| **73** | *S* | – | >100 | >100 | >1 |
| Efavirenz [27] | – | – | – | – | 0.0015 |
| Capravirin [28] | – | – | – | – | 0.005 |
| TMC-125 [29–31] | – | – | – | – | 0.002 |

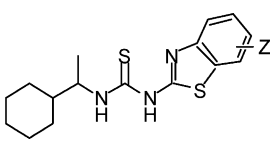
The anti-HIV activity was measured by determining the inhibition of the HIV-1 strain $HTLV_{IIIB}$ in human PBMCs as previously described in detail [21,22]. The cell-free RT inhibition assays using recombinant RT (rRT) and the Quan-RT assay kit (Amersham, Arlington Heights, IL) were performed as reported [21,22].
[a] $K_i$ values were estimated based on our previously published procedures [10–12].

stereoisomers, inhibited recombinant RT in vitro with 100-fold lower $IC_{50}$ values. We next examined the ability of '*R*' stereoisomers **66** and **68** to inhibit the replication of the HIV-1 strain $HTLV_{IIIB}$ in human peripheral blood mononuclear cells (PBMCs). Both compounds inhibited HIV-1 replication with $IC_{50}$ values of 0.001 and 0.025 μM, respectively. In contrast, the $IC_{50}$ values of the '*S*' stereoisomers **67** and **69** and the control compounds with unsubstituted pyridyl rings (**70** and **71**) were >1 μM (Table 7). Similarly the '*R*' stereoisomers (but not '*S*' stereoisomers) of the α-methyl benzyl halopyridyl compounds **21** and **22** exhibited potent activity both in cell-free RT inhibition assays and cellular HIV-1 replication assays (Table 7). The substitution of the pyridyl ring of **66** and **68** with a thiazolyl ring (compound **72**) resulted in 10-fold higher $K_i$ values and 10-fold higher $IC_{50}$ values in cell free RT inhibition assays. The '*S*' stereoisomer (**73**) with an estimated $K_i$ value of >100 μM did not exhibit any RT inhibitory activity even at 100 μM.

In order to further validate the structure activity relationship we prepared some of the substituted cyclohexenyl thiourea compounds and examined their antiviral activity towards recombinant reverse transcriptase. Introduction of a methyl group as well as a benzothiazolyl unit in the structure shown below did not yield active compounds.







In fact, compounds **74**–**81** were inactive (see supplementary material) demonstrating the critical structural requirements for the anti-HIV activity of these chiral thioureas.

Taken together, these results provide evidence that the stereochemistry of a thiourea compound can profoundly affect its ability to fit into the NNRTI binding pocket of RT and therefore its anti-HIV activity. However, the structure–activity relationship studies should be interpreted with due caution since no activity testing was performed on purified mutant RT proteins.

### *3.4. Comparing the anti-HIV activity of the chiral thiourea derivatives with known anti-HIV agents*

Having established the potent anti-HIV activity of the chiral thiourea compounds examined in this study, we next compared them with known anti-HIV agents. For this study we selected several new NNRTI that are currently undergoing clinical trials. Comparing the activities towards wild RT by Evafirvenz ($IC_{50}$ = 1.5 nM), Capravarin ($IC_{50}$ = 5 nM), TMC-125 ($IC_{50}$ = 2 nM) and our lead compounds, the methylphenyl substituted compounds **1** ($IC_{50}$ = 3 nM) and **3** ($IC_{50}$ = 1 nM), we find that they have similar activities (Table 1). However, our lead compounds are more potent than Evafirvenz against mutant strains.

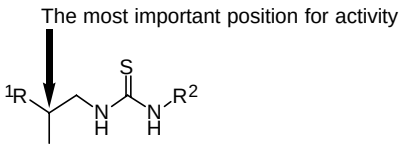
Additionally, some of the α-ethylbenzyl substituted thioureas, namely **20**, **25** and **26**, and a number of the '*R*' isomer naphthly substituted thioureas (**44**, **46**, **48**, **50**, and **52**) also indicted anti-HIV activity with an $IC_{50} = 1$ nM against wild RT. However, unlike the methylphenylthioureas, the naphthyl derivatives when compared to Evafirvenz had lower activity against mutant strains. Overall, these results indicate that further structural modification of these thiourea compounds may result in potent anti-HIV agents.

### 3.5. Stability of chiral thiourea compounds

Related to our study on the effectiveness of these chiral thiourea compounds as anti-HIV agents, we have also evaluated their stability under varying temperature and humidity conditions. In brief, the results indicated that these compounds are quite stable for a period of at least 1 year. Analysis of samples by HPLC also indicated negligible degradation of these compounds. In addition, we have also evaluated the transport of these types of compounds by examining their pharmacodynamics as well as pharmacokinetic profiles as reported in our earlier publications [25,26].

## 4. Conclusion

In summary, our data establishes the stereochemistry of the thiourea compounds as a major determinant of their potency as NNRTI. Molecular modeling studies indicated that the two carbon-linker between the phenyl group and the thiourea groups is adjustable and more forgiving than a methyl linkage. In addition, the energetic difference is minimized by adopting an alternative conformation to maintain the tight fit with the binding pocket. Out of the more than eighty thiourea compounds studied so far in our laboratory, it appears that the most important feature for activity is the '*R*' isomer of the ethyl linker, as illustrated as follows:




Irrespective of the substitution on the ethyl linker ($R^1$), the compound proved to be active against HIV-1 demonstrating the fact that these substitutions are not as important as the presence of the ethyl linker. Among the chiral methyl benzylthioureas studied compounds **20**, and **25–27** were the most potent compounds in the series. Additionally, substitution at the 5-position of the pyridyl ring at $R^2$ enhanced the activity of the compounds. We conclude that the stereochemistry is the key determinant factor of the NNRTI activity of these thiourea compounds. This conclusion parallels our modeling predictions for a tight fit in the NNRTI binding pocket due to an '*R*' isomer methyl substitution of the ethyl linker unit of these thiourea derivatives.

The toxicity and phamacodynamic features of the chiral thiourea derivatives will be the subject of future studies in order to assess their bioavailability before clinical evaluation. It will be important to determine if the in vitro activity observed for these compounds can be achieved in vivo without unacceptable toxicity. Based on the results presented in our earlier publications we believe this goal can be accomplished [25,26] and further work is in progress to formulate active thiourea derivatives for preclinical evaluations.

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