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REVIEW

Novel tight binding PETT, HEPT and DABO-based non-nucleoside inhibitors of HIV-1 reverse transcriptase

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Abstract

Non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are a key component of effective combination antiretroviral therapies for HIV/AIDS. NNRTIs despite their chemical diversity, bind to a common allosteric site of HIV-1 RT, the primary target for anti-AIDS chemotherapy, and noncompetitively inhibit DNA polymerization. NNRTIs currently in clinical use have a low genetic barrier to resistance and therefore, the need for novel NNRTIs active against drug-resistant mutants selected by current therapies is of paramount importance. We describe the chemistry and biological evaluation of highly potent novel phenethylthiazolylthiourea (PETT), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) and dihydroalkoxybenzyloxopyrimidine (DABO) derivatives targeting the hydrophobic binding pocket of HIV-1 RT. These NNRTIs were rationally designed by molecular modeling and docking studies using a novel composite binding pocket that predicted how drug-resistant mutations would change the RT binding pocket shape, volume, and chemical make-up and how these changes could affect NNRTI binding. Several ligand derivatization sites were identified for docked NNRTIs that fit the composite binding pocket. The best fit was determined by calculating an inhibition constant (Ludi K_i) of the docked compound for the composite binding pocket. Compounds with a Ludi K_i of < 1 μM were identified as the most promising tight binding NNRTIs. These NNRTIs displayed high selective indices with robust anti-HIV-1 activity against the wild-type and drug-resistant isolates carrying multiple RT gene mutations. The high rate of treatment failure due to the emergence of drug resistance mutations makes the discovery of broad-spectrum PETT, HEPT and DABO-based NNRTIs useful as a component of effective combination regimens.

Keywords: *HIV/AIDS, combination regimen, computer-aided drug design, non-nucleoside inhibitors, reverse transcriptase, thiourea compounds, inhibition*

Introduction

Human immunodeficiency virus type 1 (HIV-1) which causes AIDS has become the leading infectious cause of death worldwide [1]. Data from the World Health Organization AIDS Epidemic Update at the end of 2005 list 3.1 million deaths, 42 million people currently living with AIDS, and 4.9 million people becoming infected with HIV-1 [2]. Current treatment of HIV-1 infected patients involves the use of highly active antiretroviral therapy (HAART) regimens, which generally comprise at least 3 classes of drugs belonging to at least 2 of the currently available antiretroviral classes. The use of multiple-drug combination ART has been highly efficient in reducing viral replication and increasing $CD4^+$T-cell numbers, resulting in significant decreases in AIDS-associated morbidity and mortality in high-income countries [3–5]. However, with the HIV-positive population still expanding globally, especially in low-income countries, the annual number of AIDS deaths can be expected to increase for many years unless more effective provision of HAART begins to slow

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the death rate [6,7]. Even in high-income countries, the infection rates continue to rise largely due to widespread access to combination ART, which prolongs the lives of HIV-positive people who are able to transmit the drug-resistant virus to treatment-naive individuals [8]. Also, virological failure may appear after exposure to suboptimal therapy or as a consequence of poor compliance with treatment, allowing drug-resistant viruses to emerge [9]. Consequently, drug-resistant variants are frequently present in both recently and chronically infected treatment-naive patients. It is estimated that 1.6 million people are living with HIV-1 in North America and Western Europe - a figure that includes 65000 who were newly infected in 2004 [10]. At least 1 of 10 antiretroviral-naive patients in Europe carries HIV-1 with ≥ 1 drug-resistance mutation [11]. In the US, among viremic patients, an estimated 76% have resistance to one or more antiretroviral drugs [12].

Clinical advantages of NNRTIs

The 21 anti-retroviral drugs currently in use either in clinical trials or licensed for AIDS therapy generally fall into four major categories, i.e., eight nucleoside/-nucleotide reverse transcriptase (RT) inhibitors (NRTIs), three non-nucleoside RT inhibitors (NNRTIs), nine protease inhibitors (PI), and one fusion inhibitor [13,14]. The virologic goal of HIV management with combination ART is to achieve durable suppression of HIV-1 RNA to undetectable levels with minimal toxicity. Current treatment guidelines recommend HAART that comprises three classes of antiretroviral drugs such as NRTI, NNRTI, and PI [15–17]. Initial HAART recommended a combination of a single PI and 2 NRTIs [18]. Both older studies [19] and recent studies [20] involving treatment-naive patients have confirmed that NNRTIs and PIs have similar potency. However, complicated dosing regimens, coupled with long-term side effects of PIs, including dyslipidemia and abnormal body fat distribution, have in turn favored an initial antiretroviral regimen that substitutes PIs with NNRTIs [21–23]. Several randomized clinical trials in antiretroviral therapy-naïve patients have demonstrated that the replacement of a PI with an NNRTI can alleviate toxicities, facilitate compliance with treatment regimens, and improve quality of life [24–28].

NNRTIs in clinical use

NNRTIs are chemically diverse, largely hydrophobic compounds, which comprise over 30 different classes [29,30]. Unlike NRTIs, NNRTIs do not require sequential intracellular phosphorylation to exert their antiretroviral effect, are noncompetitive inhibitors of RT activity with respect to deoxynucleoside triphosphate (dNTP) substrate and template/primer, and are relatively noncytotoxic. NNRTIs bind to a hydrophobic pocket close to but distinct from the polymerase active site in the p66 RT subunit and inhibit enzyme activity by mediating allosteric changes in the RT [31–34]. The NNRTIs currently approved for use in HAART include nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV) [35–37]. A regimen consisting of two NRTIs (lamivudine, stavudine) and NNRTI (NVP) has been shown to be efficacious and safe in both treatment-experienced and treatment-naïve patients, regardless of baseline viral loads [25–28]. HAART regimens that combine the NNRTI EFV with two NRTIs have been shown to be more effective in durably suppressing HIV-1 in treatment-naïve patients and NRTI-experienced patients as well as or better than unboosted PI-based regimens [19,20,38–40]. The US guidelines currently recommend initiating ART with 2 NRTIs plus a single NNRTI or a ritonovir-boosted PI (http://www.aidsinfo.nih.gov/guidelines) [41]. These recommendations are based on the clinical trials in which 60% to 90% of patients had HIV-1 RNA levels below the assay detection limit in 48 weeks [38,42,43].

NNRTI-associated mutations

Initial clinical use of NNRTIs as monotherapy and selection of drug-resistant variants in cell culture resulted in the rapid emergence of highly drug-resistant variants due to single amino acid changes in the NNRTI binding pocket that directly affect drug binding [31,32,44–47]. Subsequently, dozens of mutant strains have been characterized as resistant to first generation NNRTIs, including L100I, K103N, V106A, E138K, Y188I/C and Y188H [48–54]. In patients treated with NNRTIs, high incidence of NNRTI-associated mutations are found mainly at codons 103, 181, and 190: (i) high incidence of Y181C, G190A, and K103N mutations in patients failing NVP therapy [55–57]; (ii) K103N in patients failing EFV therapy [55,58]; and (iii) Y181C and K103N in patients failing DLV therapy [59,60]. Additional mutations, particularly T215Y/F, M41L, L210W, H208Y, and V118I, are associated with hypersusceptibility to NNRTIs [61,62]. In particular, the K103N and Y181C mutants are the most difficult to treat, because they are resistant to most of the NNRTIs that have been examined. [49–54]. Also, the prevalence of a large degree of cross-resistance between these three NNRTIs precludes their sequential use in cases of virological failure [63,64].

Nevertheless, the success of NNRTIs for the clinical management of AIDS has led to the computer-aided design and chemical synthesis of a second- and third-generation of potent NNRTIs [65–69]. In a systematic search for potent anti-AIDS drugs, Bell et al. [67] and Cantrell et al. [68] discovered the

phenethylthiazolylthiourea (PETT) class of compounds as potent inhibitors of HIV-1 and disclosed a structure–activity relationship (SAR) among various substituents in their structure. Following this lead and using rational drug design, we have identified several structurally distinct thiourea compounds as potent NNRTIs against drug-sensitive, drug-resistant, and multidrug-resistant strains of HIV-1 [70–87].

Strategies for design and synthesis of novel PETT derivatives

The retrovirus reverse transcribes its RNA genome catalyzed by RT enzyme into double-stranded DNA upon infection of permissive host cells. The HIV-1 RT is encoded as part of the Gag-Pol precursor protein Pr160Gag-Pol. During and after assembly of the virus particle, Pr160Gag-Pol is cleaved by the viral protease to liberate a 66-kDa RT subunit. Subsequent cleavage of the C-terminal domain of p66 produces the 51-kDa RT subunit. The two different subunits dimerize in the virion and form the functional RT p51/p66 heterodimer [88]. It has been proposed that NNRTIs interfere with reverse transcription by altering either the conformation or mobility of RT rather than directly preventing the template-primer binding [89]. Specifically, binding of a NNRTI to the NNRTI binding site (approximately 10 Å away from the polymerase catalytic site) inhibits RT by interfering with the mobility of the "thumb" and/or position of the "primer grip" (residues 229–231), which interact with the DNA primer strand (Figure 1A).

Computer programs can be used to identify unoccupied (aqueous) space between the van der Waals surface of a compound and the surface defined by residues in the binding site. These gaps in atom-atom contact represent volume that could be occupied by new functional groups on a modified version of the lead compound. More efficient use of the unoccupied space in the binding site could lead to a stronger binding compound if the overall energy of such a change is favorable. A region of the binding pocket which has unoccupied volume large enough to accommodate the volume of a group equal to or larger than a covalently bonded carbon atom can be identified as a promising position for functional group substitution. Functional group substitution at this region can constitute substituting something other than a carbon atom, such as oxygen. If the volume is large enough to accommodate a group larger than a carbon atom, a different functional group, which would have a high likelihood of interacting with protein residues in this region, may be chosen. Features, which contribute to interaction with protein residues and identification of promising substitutions, include hydrophobicity, size, rigidity, and polarity. The combination of docking, binding affinity (K_i) estimation, and visual representation of sterically

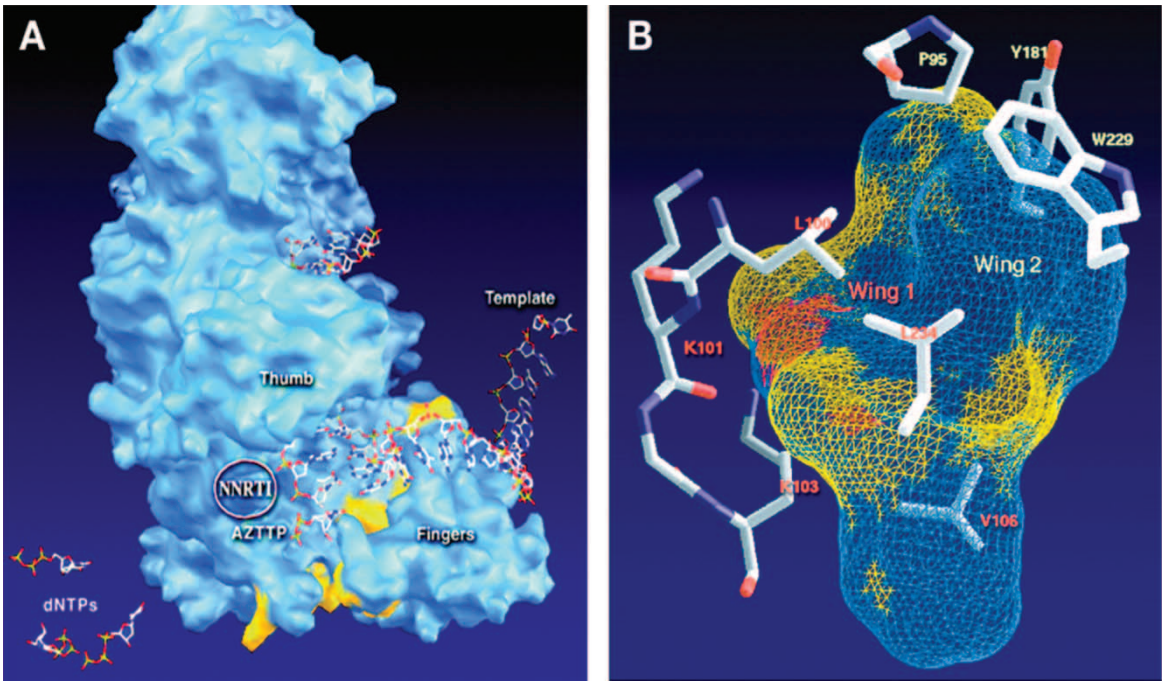


Figure 1. (A) Three-dimensional model of HIV-1-RT showing the RT binding site for NNRTI (circled). The site for nucleoside inhibitors is labeled dNTP which includes the 3′ terminus of DNA. Features describing the geometry of the binding region include the "thumb", "palm", "fingers", and "hinge" region of RT. (B) The composite binding pocket of the NNRTI active site of HIV-1 RT. Composite binding pocket of NNRTI active site of HIV-1 RT is illustrated as grid lines representing the collective van der Waals is overlaid with the residues, which constitute Wing 1, and Wing 2 of the "butterfly" shaped binding pocket. Surface is color-coded: red for hydrogen bonding, blue for hydrophobic, and yellow for polar groups.

allowed room for improvement permits prediction of potent derivatives.

The strategy employed for systematic synthesis of novel PETT derivatives was based on the crystal structure of RT-NNRTI complexes and extensive analysis of SAR of promising RT inhibitors. Because the NNRTI binding pocket model based on an individual RT-NNRTI crystal structure [31–33, 90–92] would have limited potential for predicting the binding of new, chemically distinct inhibitors, a composite binding pocket or a composite molecular surface was constructed using the NNRTI binding site coordinates of multiple and varied RT-NNRTI structures [70–73]. The binding pocket generated from nine different structures of RT complexed with 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT), 6-benzyl-2-(ethoxymethyl)-5-isopropyluracil (MKC), 6-benzyl-1-[(benzyloxy)methyl]-5-isopropyluracil (TNK), α-anilinophenylacetamide (APA), NVP (dipyridodiazepinone derivative), N-ethyl NVP derivative, 9-Cl-tetrahydrobenzodiazepine derivative (9-Cl TIBO), 9-Cl TIBO, and 8-Cl TIBO structures) revealed a larger than presumed NNRTI binding pocket not shown or predicted by any of the individual structures alone (Figure 1B). This novel composite binding pocket, together with a computer docking procedure and a structure-based semi-empirical score function was used to predict the energetically favourable position of novel phenethyl-thiazolyl-thiourea (PETT), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) and dihydro-alkoxy-benzyl-oxopyrimidine (DABO), as well as other novel compounds, in the NNRTI binding site of RT. The practical utility of this novel composite model was illustrated and validated by the observed superior potency of new PETT, HEPT, and S-DABO derivatives as anti-HIV agents [70–87].

NNRTI binding pocket

The structurally diverse NNRTIs bind to an allosteric site of HIV-1 RT [31,93,94], which is ~10 Å away from the polymerase active site in the "palm" domain of the p66 subunit (Figure 1A). The crystal structure and *in vitro* biochemical analyses indicate that the p66 subunit of RT is primarily responsible for the enzyme's polymerase and ribonuclease H activities. The polymerase domain is further divided into the "fingers", "palm", "thumb", and connection subdomains (Figure 1A). NNRTI binding induces rotamer conformation changes in amino acid residues (Y181 and Y188) and makes the "thumb" region of the enzyme more rigid [90,91]. Both events alter the substrate-binding mode and/or affect the translocation of the double strand, which are critical for the polymerase function, thereby leading to a noncompetitive inhibition of the enzyme [33,34].

Drug-resistant RT mutants have been proposed to obstruct the binding of NNRTIs. Most mutations conferring resistance to NNRTIs are directly in contact with the NNRTI molecule, and thus are associated with changes in the binding of NNRTI to HIV-1 RT [95–98]. For example, primary mutations associated with resistance to NVP involve residues K103, V106, V108, Y181, Y188, and G190, which have van der Waals contact with the NNRTI. The mutations of these residues lead to the weakening of the NNI binding to RT. Currently used NNRTIs, DLV, EFV, and NVP are less active against RT with primary mutations K103N or Y181C [50–64].

Rational design of tight binding thiourea NNRTIs

The novel computer model of the NNRTI binding pocket of HIV-1 RT, which was constructed by superimposing nine individual RT-NNRTI crystal structures and generating a Van der Waals surface that encompassed all the overlaid ligands, revealed a different and unexpectedly larger NNRTI binding site than shown in or predictable from any of the individual structures [70–73]. This served as a probe to more accurately define the potentially usable space in the binding site (Figure 1B). This composite NNRTI binding pocket model was used to design potent NNRTIs against wild type RT and drug-resistant RT mutants [70–87]. Fixed docking in the Affinity program within InsightII [99] was used for docking of NNRTIs into the NNRTI binding site. Molecular modeling and score functions such as molecular surface area, the buried surface, and inhibitory constants (Ludi K_i values) were used to analyze how drug-resistant mutations would change the RT binding pocket shape, volume, and chemical make-up of PETT NNRTIs, and how these changes could affect NNRTI binding [70–73]. The calculated K_i values of the positioned thiourea NNRTIs were evaluated by using the Ludi score function [100]. The Insight II program [99] used for docking and Ludi scoring employed a calibration procedure during its establishment, which involved calculation of the K_i values of 45 protein-ligand complexes having known K_i values and known crystal structures, and comparing the calculated K_i values to the experimentally determined K_i values [101,102].

Docking studies with the PETT derivative, trovirdine (TRV), revealed multiple sites, which can be used for the incorporation of larger functional groups. In the composite binding pocket, the docked TRV molecule showed a lot of usable space surrounding the pyridyl ring, (R_2–R_6), the ethyl linker (R_7) and near the 5-bromo position (R_8) (Figure 2). It was hypothesized that efficient use of this space by strategically designed functional groups would lead to high affinity binding and ultimately result in better

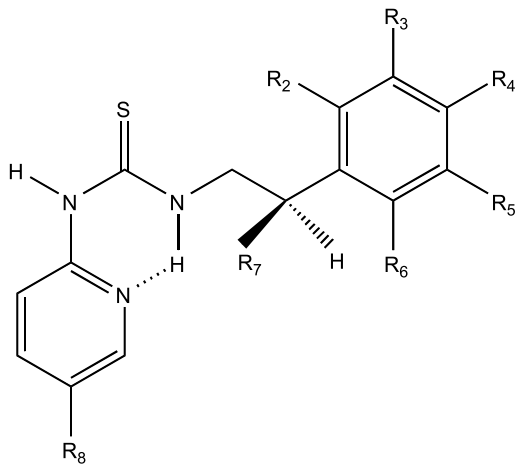


Figure 2. Potential modification sites for PETT derivatives.

inhibitors. Modeling studies suggested that designs using the space available in these regions, including (i) substitutions at R_2–R_6; (ii) substituting heterocyclic rings for the pyridyl ring of TRV; (iii) substitutions at R_7; (iv) substitutions at R_8; and (v) maintaining the intramolecular hydrogen bond would lead to potent RT inhibitors.

PETT NNRTIs can be viewed as two chemical moieties linked together by a thiourea group (Figure 3). The left side of the molecule is either a 2-aminothiazole group (PETT) or a 5-bromopyridyl group (TRV). Both these groups are capable of forming an intramolecular hydrogen-bonded heterocyclic ring [70–73]. The right side of the molecule is a pyridyl or phenyl ring separated from the thiocarbonyl group by an ethyl linker. Trovirdine docked into the NNRTI binding site of RT had a higher binding score than the parent compound (PETT) and fits into the "butterfly"-shaped binding region with one part residing in Wing 1 and the other in Wing 2. Docking studies with PETT and TRV revealed that the Wing 2 region defining space of the "butterfly"-shaped binding pocket has a substantial molecular volume (~160 Å) surrounding the phenyl ring of PETT compounds that can be more efficiently occupied by a larger functional group to achieve a high binding affinity even against the problematic Y181C and Y188C RT mutants. The binding of NNRTI forces RT residue W229 to change its position slightly and causes residues Y181 and Y188 to rotate into another rotamer conformation. Consequently, the binding pocket would be substantially larger than it was before NNRTI binding, forcing the primer-template into an inactive binding conformation and rendering the protein inactive. This volume change is a direct consequence of the different positions of the Y181, Y188, and W229 sidechains before and after the NNRTI binding. When Y181 and Y188 are mutated to cysteine residues, the volume change due to NNRTI binding is smaller and the impact of NNRTI inhibiting the RT mutants would be attenuated. This idea fits well with adding a larger functional group at the Wing 2 region. Therefore, NNRTI, which has a maximum occupancy at the Wing 2 region, was predicted to have an advantage against Wing 2 mutants, such as the Y181C and Y188C mutants.

Accordingly, an ideal NNRTI should be: (i) highly potent against wild-type RT and therefore, can afford a considerable activity loss against mutants (i.e. a picomolar-level inhibitor against wildtype RT may still be effective against RT mutants at nanomolar levels); (ii) maximize the occupancy at Wing 2 region, which will have an advantage against the Wing 2 mutants, and; (iii) the substitutions should match with the chemical nature of the residue that is potentially mutated in the RT mutant.

Tight binding thiourea NNRTIs

Using the composite binding pocket described above, a series of thiourea NNRTIs were synthesized that

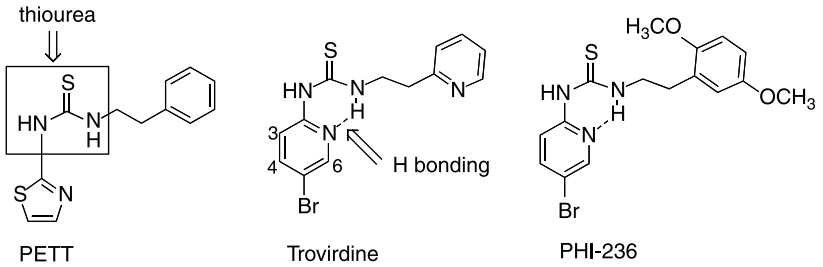


Figure 3. Rational design of thiourea NNRTIs. Phenethylthiazolylthiourea (PETT) NNRTIs can be viewed as two chemical moieties linked together by a thiourea group. The left side of the molecule shown above is either a 2-aminothiazole group (PETT) or a 5-bromopyridyl group (TRV). Both these groups are capable of forming an intramolecular H-bonded heterocyclic ring. A more efficient use of such sterically allowed unoccupied spatial gaps in the binding site was achieved by replacing the 2-pyridyl ring of trovirdine with 2,5-dimethoxy-substituted phenyl ring (PHI-236) to yield potentially more active NNRTI with larger molecular surface areas, higher binding scores, and lower K_i value.

Table I. Anti-HIV-1 activity of phenyl-ring substituted thiourea NNRTIs.

Compd*	R	IC_{50} (μM)						SI‡
		K_i†	rRT	$HTLV_{IIIB}$ (p24)	A17 (p24)	A17V (p24)	MDR (p24)	
PHI-236	2-5-dimethoxy	0.07	0.1	<0.001	0.14	8	0.005	10^5
PHI-240	2-fluoro	0.8	0.6	<0.001	0.33	35	0.006	10^5
PHI-241	3-fluoro	0.6	0.7	<0.001	ND	ND	0.02	10^5
PHI-244	4-methyl	0.25	0.1	0.007	0.07	ND	>0.001	10^5
PHI-253	2-chloro	0.5	0.7	<0.001	ND	ND	0.004	10^5
TRV	NA	0.7	0.8	0.007	0.5	>100	0.02	10^4
NVP	NA	ND	23	0.034	>100	>100	5	10^3
DLV	NA	ND	1.5	0.009	50	>100	0.4	10^2

TRV, trovirdine; NVP, nevirapine; DLV, delavirdine. MDR, multidrug resistant; rRT, recombinant HIV-1 reverse transcriptase.
* Specific PETT derivatives discovered included: PHI-236: N-[2-(2,5-dimethoxyphenethyl)]-N′-[2-(5-bromopyridyl)] thiourea; PHI-240: N-[2-(2-fluorophenethyl)]-N′-[2-(5-bromopyridyl)] thiourea; PHI-241: N-[2-(3-fluorophenethyl)]-N′-[2-(5-bromopyridyl)] thiourea; PHI-244: N-[2-(4-methylphenethyl)]-N′-[2-(5-bromopyridyl)] thiourea; and PHI-253: N-[2-(2-chlorophenethyl)]-N′-[2-(5-bromopyridyl)] thiourea. † Ludi K_i values were calculated based on modified empirical score function in Ludi program. ‡ SI (selectivity index) = IC_{50} [MTA]/IC_{50}[p24]. NA, not applicable. ND, not determined.

yielded high binding affinity for HIV-1 RT and robust anti-HIV-1 activity [70,73–87]. The thiourea NNRTIs were more potent against drug-sensitive and multidrug-resistant strains of HIV-1 than the three classes of NNRTIs currently in clinical use to treat HIV infections.

Heterocyclic ring substituted thioureas

The effect of systematic substitutions of the phenyl ring of TRV with various heterocyclic rings was studied. This provided an alternative strategy to fit the compound into the relatively flexible and spacious Wing 2 region. In the subsequent modeling studies, these heterocyclic rings, which have a larger volume than the pyridyl ring of TRV, were shown to better fill the Wing 2 region of the composite binding pocket. The energetically favored docked position of the thioureas in the NNRTI binding pocket was determined and a Ludi score was assigned and an estimation of the inhibition constant (K_i value) was determined [77,81,100]. The accuracy of the predictions of the modeling studies was evaluated in anti-HIV-1 assays.

The SAR studies affecting the potency of PETT derivatives with substitutions on various positions of the phenyl ring revealed the following:

i) methoxy substitution is more favourable at the meta position than at the ortho or para positions;
ii) fluorine substitution is favourable at ortho and meta positions but not at the para position;
iii) chlorine substitution is favourable only at the ortho position; and
iv) a hydrophobic group is more desirable than a polar group or hydrophilic group at the para position. These results were generally consistent with predictions made during modeling.

Substitution of the phenyl ring with various functional groups had a major impact on the anti-HIV-1 activity of pyridyl thioureas. Among the 14 phenyl ring-substituted pyridyl thiourea compounds, functionalization of the phenyl ring with 2,5-dimethoxy, 2-fluoro, 3-fluoro, and 2-chloro was associated with enhanced anti-HIV activity ($IC_{50[rRT]} = 0.1\,\mu M$ to $0.7\,\mu M$; $IC_{50[p24]} = < 0.001\,\mu M$; Table I). In addition, substitutions with 3-methoxy, 4-methyl, and 4-chloro functional groups also resulted in potent inhibitors of HIV-1 ($IC_{50[rRT]} = 0.1\,\mu M$ to $2.5\,\mu M$; $IC_{50[p24]} = 0.001\,\mu M$ to $0.007\,\mu M$) [70,75,77,79,80,83]. By comparison, the 4-hydroxyl and 2-nitro substituted phenyl thioureas were inactive with $IC_{50[rRT]}$ values of $> 80\,\mu M$ and $IC_{50[p24]}$ values of $> 100\,\mu M$, respectively.

PHI-236 (N-[2-(2,5-dimethoxy phenylethyl)]-N′-[2-(5-bromopyridyl)]- thiourea)

PHI-236 displayed high affinity (Ludi $K_i = 0.07\,\mu M$) for the NNRTI binding pocket of HIV-1 RT and abrogated HIV-1 replication at nanomolar concentrations ($IC_{50[p24]} = < 1\,nM$) without evidence of cytotoxicity, and with an unprecedented selectivity index of >100000 [80]. Molecular modeling studies revealed that replacement of a pyridyl ethyl group of TRV with an 2,5-dimethoxy-substituted phenyl ring would provide favourable contacts with binding site residues and lead to stronger binding to RT [75]. In fact, the addition of 2,5-dimethoxy groups in PHI-236 increased the molecular volume in the Wing 2 region of the binding site by 18 Å. Thus, PHI-236, which has a maximum occupancy at the Wing 2 region and more closely in contact with residues L100 and L234, was predicted to have an advantage against Wing 2 mutants, such as the Y181C and Y188C mutants. An energy-minimized model of PHI-236 in the RT

binding site revealed the largest molecular surface area in contact with the protein and thus achieved the highest lipophilicity score. The larger surface area and favourable chemical properties of PHI-236 contributed to a better lipophilic score and better Ludi K_i value than TRV.

The trend of the calculated Ludi K_i values based on the modeling studies predicted the trend of the experimentally determined IC_{50} values with surprising accuracy. Compounds that better fit the composite binding pocket when compared to TRV had lower calculated Ludi K_i values and rRT IC_{50} as well as p24 IC_{50} values. Indeed, a comparison of 14 pyridyl thiourea NNRTIs revealed that the observed rRT IC_{50} values significantly correlated with Ludi K_i values ($r^2 = 0.942$; $P = < 0.0001$; Figure 4A). Similarly, the p24 antigen values correlated significantly with Ludi K_i values ($r^2 = 0.981$; $P = < 0.0001$; Figure 4B). These results confirmed the hypothesis that the binding interactions predicted based on our modeling studies largely account for the superior anti-HIV-1 activity of thiourea NNRTIs.

Activity of PHI-236 against drug-resistant HIV-1 mutants

PHI-236 was active against laboratory and primary clinical isolates with variable resistance to thymidine analogues (ZDV/STV) [87]. The activity of PHI-236 against genotypic NNRTI-resistant HIV-1 strains (A17, A17 variant) carrying clinically relevant mutations (Y181C and K103N + Y181C, respectively) were superior when compared to the activity of the three NNRTIs in clinical use. In p24 antigen assays, PHI-236 was not only more potent than TRV and ZDV against the drug-sensitive HIV-1 strain, human T-cell lymphotropic virus type III ($HTLV_{IIIB}$), it was also 500–1000 times more effective than DLV or NVP against the NNRTI-resistant Y181C mutant HIV-1 strain A17. Most importantly, PHI-236 was highly effective (5 nM) against the multidrug-resistant HIV-1 strain (RT-MDR) with multiple mutations involving the RT residues M41L, L74V, V106A, and T215Y. The activity of PHI-236 against RT-A17 variant with K103N or Y181C mutations was superior to that of other NNRTIs tested.

Notably, PHI-236 was highly active against 17 NRTI- and NNRTI-resistant primary clinical HIV-1 isolates tested with 2 to 7 thymidine analogue mutations (TAM) in amino acid sequence 20–219 (TAMs: M41L, E44D, D67N, T69D, K70R, L74V, K103N, F116S, M184V, Y181C, L210W, T215Y, or K219Q) with a mean $IC_{50[p24]}$ value of $0.043 \pm 0.02\,\mu M$ (range $0.00004\,\mu M$ to $0.4\,\mu M$). PHI-236 was 30- to 70000-fold more potent than ZDV against these isolates originating from South America, Asia, and the sub-Saharan Africa. Notably, PHI-236 was active at low micromolar concentrations against four isolates tested (L567-1, S159-2, U612 and X165-09) that harboured problematic NNRTI-resistant mutations K103N or Y181C (mean $IC_{50[p24]}$ value = $0.18\,\mu M$; range 0.014 to $0.4\,\mu M$) [87]. This is particularly relevant because a high percentage of newly infected individuals harbour NRTI/NNRTI-resistant mutants with increased incidence of HIV subtypes [103–106]. Subtype B predominates in

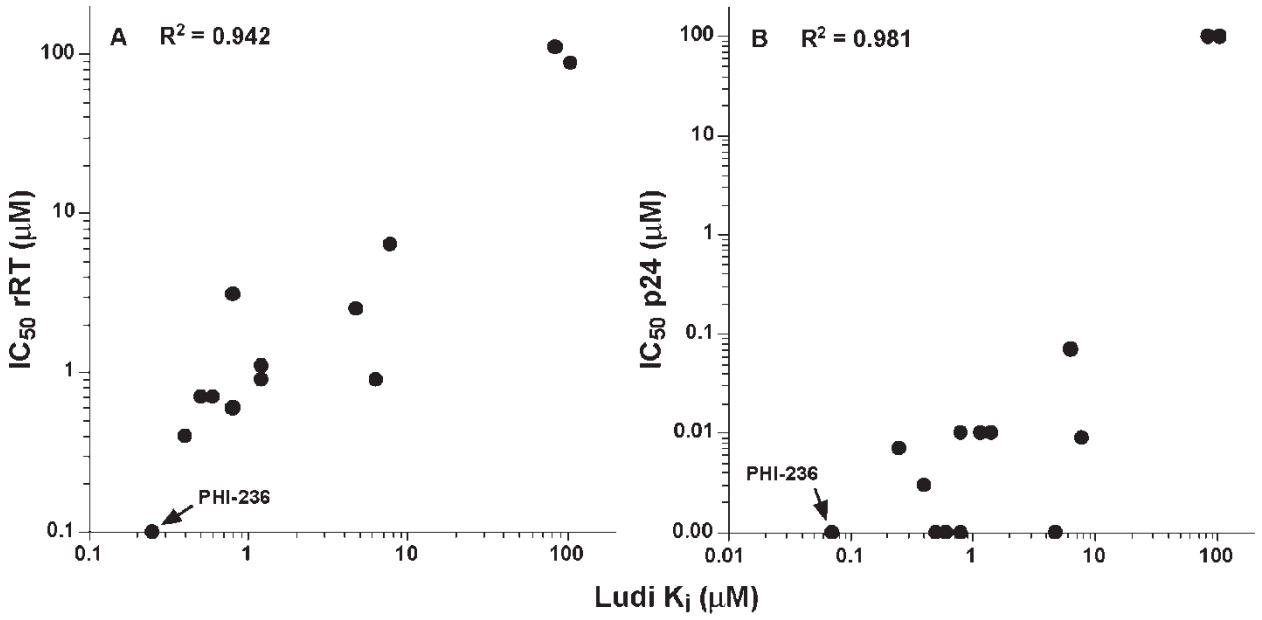


Figure 4. Correlation between lower Ludi K_i values and HIV-1 rRT IC_{50} or p24 IC_{50} values. The data point represents the calculated Ludi K_i values and experimental rRT [A] and p24 antigen [B] inhibitory values for 14 rationally designed phenyl ring-substituted pyridyl thiourea compounds. Ludi K_i values were calculated based on the empirical score function in the Ludi program [99–102]. Ideal H-bond distances and angles between compounds and protein are assumed in all cases for Ludi K_i calculations.

North America and Europe [103]. However, HIV-1 subtype B currently accounts for only 12% of the estimated 42 million HIV infected individuals worldwide and the vast majority of new infections are caused by non-subtype B HIV-strains.

In a syncytial focus (plaque) formation assay using CD4-expressing HeLa cells (HT4-6C), PHI-236 inhibited the infectivity of 16 NRTI and NNRTI-resistant primary clinical HIV-1 isolates tested carrying 2-5 TAMs at nanomolar concentrations. PHI-236 was 270-fold more potent than ZDV (mean $IC_{50[p24]}$ values 0.009 vs. 2.44 μM, $P < 0.0001$) against ZDV-resistant 16 primary clinical HIV-1 isolates originating from South America, Asia, and sub-Saharan Africa. Notably, the phenotypically highly ZDV-resistant isolates (G910-6, G704-2, G780-1, and J179-1) carrying 4-5 TAMs, respectively, were inhibited by PHI-236 with an average IC_{50} value of 0.005 μM (range 0.004 to 0.008 μM). PHI-236 was active at low micromolar concentrations ($IC_{50} = 0.014$ μM) against L567-1 and U612-2 harbouring NNRTI-resistant mutations K103N and Y181C, respectively. These findings established that PHI-236 is a potent antiviral agent against drug-sensitive, NRTI/NNRTI-resistant, and multidrug-resistant strains of HIV-I.

The improved anti-HIV activity of PHI-236 against RT mutants is consistent with the structural analysis of PHI-236 binding to RT based on X-ray crystallographic data [75]. The hydrogen (H) bonds involved in the intramolecular interaction between a thiourea NH and the pyridyl N [N–H...N = 2.671] locks the molecule into a relatively planar conformation. A second H bond between a thiourea N atom and the thiocarbonyl-S atom [N–H2...S = 3.403] allows the formation of inversion-related H bonded dimers. This compact conformation would allow PHI-236 to more easily fit into the NNRTI binding site (Figure 5A).

PHI-244 (N-[2-(4-methylphenethyl)]-N′-[2-(5-bromopyridyl)] thiourea)

PHI-244 effectively inhibited replication of the HIV-1 strain $HTLV_{IIIB}$ in PBMC with an IC_{50} value of 0.007 μM, which is equal to the IC_{50} value of TRV (Table I). However, PHI-244 was 20-times more effective than TRV against RT-MDR and 7-times more potent than TRV against A17 with an Y181C mutation [83]. PHI-244 was not cytotoxic to leukocytes even at a 100 μM concentration. Thus, the selectivity index for PHI-244 was (i) >10000 against the wild-type HIV-1 strain $HTLV_{IIIB}$; (ii) >71000 against the multidrug-resistant V106A mutant strain RT-MDR, and (iii) >1000 against the NNRTI-resistant Y181C mutant strain A17. These findings established that the para-methyl substituted phenyl thiourea is an effective NNRTI against drug-sensitive, NNRTI-resistant, and multidrug-resistant strains of HIV-1.

The para-substituted group lies within a hydrophobic region indicated by the composite NNRTI binding pocket. This region contains the aromatic

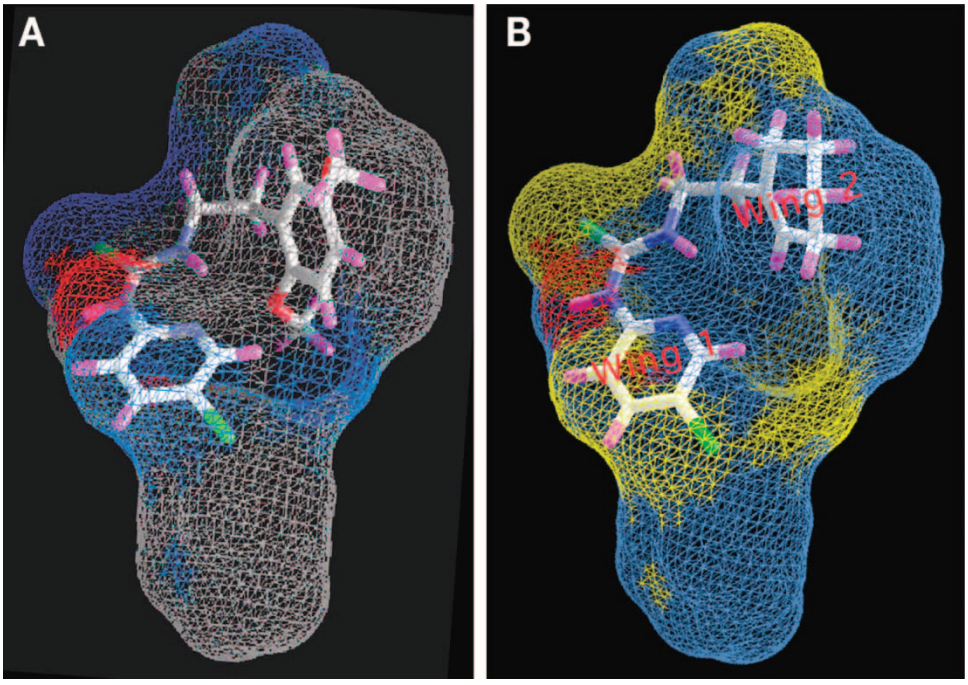


Figure 5. Composite binding pocket of NNRTI active site of HIV-1 RT with PHI-236 (A) or PHI-346 (B) docked into the NNRTI binding site. The binding pocket is illustrated as grid lines representing the collective van der Waals surface. A. Red represents hydrogen-bonding region, gray represents the hydrophobic region, and blue represents the hydrophilic region. B. Blue represents the hydrophobic region, red represents the hydrogen-bond region, and yellow represents the polar region.

rings of residues Y188 and W229, which would interact favourably with a hydrophobic group (Figure 6A). Therefore, the observed inhibition level of the para-substituted compounds against the wild-type HIV-1 is generally proportional to the hydrophobicity of the para-substituted group. The potency of the para-substitued compounds is consistent with the following trend: hydrophobic group > polar group > hydrophilic group.

The para-methyl group in PHI-244 was predicted to reside closer to the Y181 residue (C-to-CI distance + 5.5 Å) than to the V106 residue (C-to-CI distance + 8.1 Å) (Figure 6B). As a result, the Y181 mutation reduced the potency of PHI-244 more than the V106 mutation. This reasoning is consistent with the observation that the Y181C mutant strain (A17 strain) has 10-fold resistance to PHI-244 whereas the V106A mutant strain (RT-MDR) showed no resistance, and in fact shows improved activity (Table I). The apparent van der Waals contact between the compound and tyrosine residue 181 after its mutation to a smaller cysteine residue may help explain the observed resistance. The superior activity of PHI-244 against the V106A RT mutant relative to the wild-type RT is likely due to its repositioning in the binding site, leading to improved contacts between the para-methyl group and Y188 resulting in a better binding affinity.

The addition of a paramethyl group in PHI-244 increased the molecular volume in the Wing 2 region of the binding site by 18 Å. In the NNRTI-resistant A17 strain (Y181C mutation), the Wing 2 region of the mutant becomes larger when Y181 is mutated to a smaller cysteine residue, as observed in the crystal structure of the Y181C mutant. These results showing a 7-fold higher potency of PHI-244 relative to TRV are consistent with our previously reported hypothesis that an NNRTI compound containing larger (and compatible) functional groups at the Wing 2 region of the binding site can provide better inhibitor activity against these HIV-1 RT mutants.

To further understand the structure-function relationships of RT-NNRTIs and to discover novel, effective NNRTIs, the pyridyl ring of TRV was replaced with one of eight different heterocyclic substituents, including: (i) the heterocyclic amines pyrrolidine, 1-methyl-pyrrolidine, morpholine, imidazole, indole; (ii) heterocyclic aromatic groups furan and thiophene; and (iii) the aromatic aldehyde piperonyl. Additionally, the role of the chiral center of the phenyl ethyl thioureas was evaluated for their NNRTI activity (Figure 7).

The piperidine, piperazine and morpholine rings of thiourea NNRTIs are puckered and therefore occupy a larger overall volume than the planar pyridyl ring of TRV and are in close contact with residues Leu234 and Leu100, the latter of which can mutate to isoleucine, frequently found in a drug-resistant RT mutant strain. The encouraging results from efforts to make modifications perpendicular to the ring plane introduce new possibilities to develop more potent inhibitors of RT. The heterocyclic rings which are conformationally more flexible than an aromatic ring may have the advantage of fitting an uncompromising

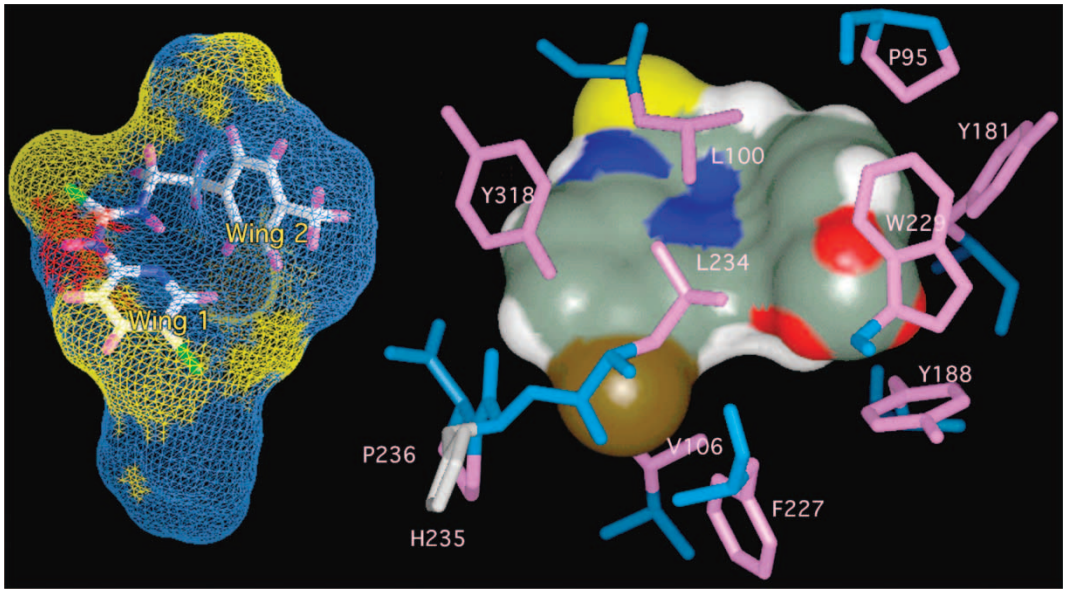
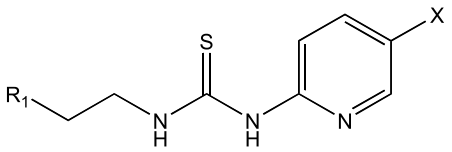


Figure 6. (A). Composite binding pocket of the NNRTI binding site of HIV-1 RT with stick model of PHI-244 docked in the composite binding pocket. A blue surface represents the hydrophobic region of the binding site, red is the hydrogen-bonding region, and yellow is the polar region. The para-methyl group of PHI-244 is compatible with the hydrophobic area of the Wing 2 region. (B). Connolly surface representation of PHI-244 in the NNRTI binding site. The molecular surface area associated with hydrogen atoms on the methyl group is colored in red. Nitrogen atoms are colored in blue, bromine in brown, sulfur in yellow, carbon in gray, and other hydrogens in white. The residues in contact with PHI-244 are labeled and shown in stick models (pink for side-chains and steel-blue for main chains).



R_1 = Phenyl, 4-methoxy, 4-fluoro, 4-chloro, 4-bromo, 4-nitro, 4-methyl, or 4-hydroxyphenyl

Figure 7. Potential modification sites of phenethyl-thiourea NNRTIs.

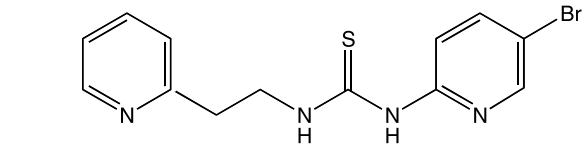
binding pocket more effectively, despite the expense paid for loss of entropy upon binding. Various combinations of double substitutions at axial or equatorial position of these heterocyclic rings would generate derivatives with a broader range of curvatures than TRV derivatives and would serve to better fit Wing 2 which itself contains some curvature. As predicted, these compounds were more potent than TRV for inhibition of HIV-1. Our lead heterocyclic PETT derivatives, N-[2-(1-piperidinylethyl)]-N′-[2-(5-bromopyridyl)]-thiourea and N-[2-(1-morpholinylethyl)]-N′-[2-(5-bromopyridyl)]-thiourea elicited potent anti-HIV activity with IC_{50} values of <1 nM and showed no detectable cytotoxicity [73].

PHI-346 (N-[2-(1-cyclohexenyl)ethyl]-N′-[2-(5-bromopyridyl)]-thiourea)

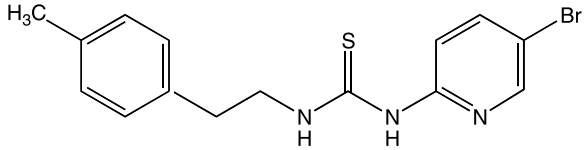
Docking studies using the computer-generated model of the NNRTI binding pocket suggested that the replacement of the planar pyridyl ring of TRV with a nonplanar cyclohexenyl ring, which occupies larger volume, would better fit the spacious Wing 2 region of the NNRTI binding pocket (Figure 5B). Modeling studies indicated that the cyclohexenyl group is slightly better than the pyridyl group of TRV relative to its hydrophobic interactions with the RT residues. The preferred compounds were 5-bromo (PHI-346) and 5-chloro (PHI-445) functionalized cyclohexenyl ring-substituted thioureas [78] (Figure 8). PHI-346 makes 94 hydrophobic contacts with the surrounding RT residues, including P95, Y181, L100, V179, and Y188, which translates into a 3.0 log unit gain in the final interaction score [78].

The composite binding pocket also indicated a region in Wing 1, which would be compatible with polar atoms; this region corresponds to the predicted location of the bound halogen atoms of PHI-346 (Br) and PHI-445 (Cl). The bromine atom of PHI-346 makes 21 contacts with 7 RT residues including H235, L234, and V106, because of its large van der Waal radius. The estimated values for the hydrophobic score function in log units were 10.2 for PHI-346, and 9.7 for PHI-445, whereas the estimated values for the polar score function in log units were 1.7 for PHI-346 as well as PHI-445. The estimated K_i values were 0.16 μM for PHI-346 and 0.50 μM for PHI-445 (Table II).

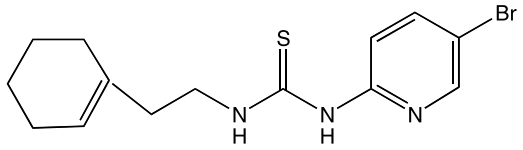
The cyclohexenyl-substituted thiourea NNRTI, PHI-346 had a better K_i value than the 0.64 μM for TRV. As predicted based on the estimated Ludi K_i value of 0.16 μM, PHI-346 was a potent inhibitor of drug-sensitive and drug-resistant HIV-1 strains [78]. Functionalization at the 5′-position of the pyridyl ring of cyclohexenyl ring-containing thioureas with a Br atom led to a significant increase in anti-HIV-1 activity as well as gain of spermicidal function [107]. PHI-346 was 3-times more effective against RT-MDR than it was against $HTLV_{IIIB}$. PHI-346 was 20-times more potent than TRV, 200-times more potent than ZDV,



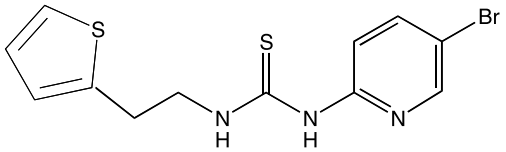
Trovirdine



PHI-244



PHI-346



PHI-443

Figure 8. Structures of heterocyclic and alicyclic ring-substituted thioureas.

Table II. Anti-HIV-1 activity of cyclohexenyl and thiophene-ethyl-thiourea NNRTIs.

		IC_{50} (μM)						
Compd	X	K_i	rRT	$HTLV_{IIIB}$ (p24)	A17 (p24)	A17V (p24)	MDR (p24)	SI
PHI-346	Br	0.16	0.4	0.003	ND	18.7	0.001	10^5
PHI-347	CF_3	0.63	4.0	0.079	0.3	>100	0.038	10^5
PHI-445	Cl	0.50	0.5	0.003	0.068	30	0.001	10^5
PHI-443	Br		5.3	0.03	0.048	3.26	0.004	10^5

300-times more potent than MKC-442, 400-times more potent than DLV, and 5000-times more potent than NVP against RT-MDR [78]. These findings established PHI-346 as a potent inhibitor of drug-sensitive as well as multidrug-resistant stains of HIV-1.

When PHI-346 was docked into the NNRTI binding site of RT, it fitted into the butterfly-shaped binding region with one part of the molecule residing in Wing 1 and the other in Wing 2 (Figure 5B). The docking results indicated that the cyclohexenyl group of PHI-346 is situated in the Wing 2 region of the NNRTI binding pocket, providing contact with RT residues including Y181 (Figure 9A). In addition, the cyclohexenyl group contains more ring hydrogens than the heterocyclic pyridyl ring and therefore, has more hydrogen atom-mediated contacts and fewer carbon atom-mediated contacts with RT residues than TRV. Because the cyclohexenyl rings are conformationally more flexible than aromatic ring-containing thiourea derivatives, they are likely to have an added advantage by being able to fit an uncompromising binding pocket more effectively than conventional NNRTIs.

PHI-443 (N′-[2-(2-thiophene)ethyl]-N′-[2-(5-bromopyridyl)] thiourea)

PHI-443 is a rationally designed thiophene thiourea NNRTI with potent activity against NRTI-resistant, NNRTI-resistant, and multidrug-resistant HIV-1 [76,86]. Docking studies with PHI-443 indicated that the thiophene ring situated in the Wing 2 region of the NNRTI binding pocket provides better contact with RT residues including Y181. The thiophene group of PHI-443 was found to be located in close proximity to the Y181 residue (Figure 9B). In this docked position, the sulfur (S) atom of the thiophene ring is only 4.4 Å away from the C atom of the Y181 residue, which is mutated to an S atom in the RTY181C mutant strains (A17 and A17 variant). The S atom of the thiophene group is more compatible with the S-containing cysteine 181 residue than the pyridyl group of TRV.

PHI-443 effectively inhibited the replication of the HIV-1 strain $HTLV_{IIIB}$ in human PBMC with an IC_{50} value of 0.03 μM. It was almost as potent against the NNRTI-resistant HIV-1 strain A17 (IC_{50}: 0.04 μM

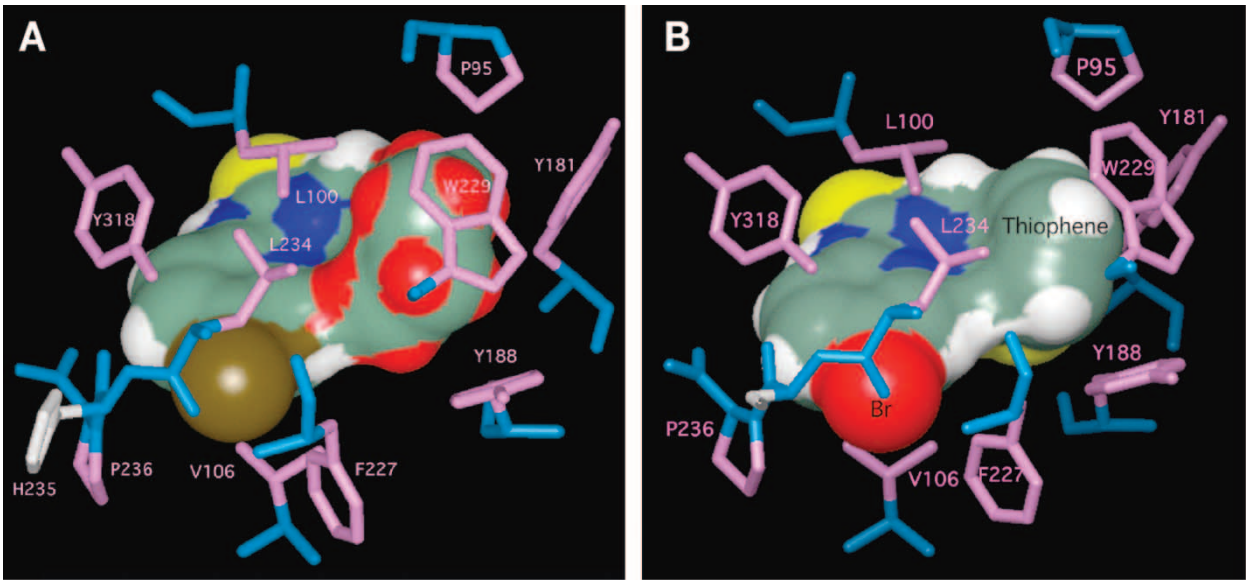


Figure 9. Connolly surface representation of PHI-346 (A) or PHI-443 (B) in the NNRTI binding site. 7A. The molecular surface area associated with hydrogen atoms on cyclohexenyl ring are colored in red. Other surface colors: nitrogen is blue, bromine is brown, sulfur is yellow, carbon is gray, and other hydrogens are white, respectively. The residues in contact with Br atom and cyclohexenyl group are labeled and are shown in stick model (pink for side chains and steel-blue for mainchains). 7B. The molecular surface area associated with bromo atom is colored red. Other surface colors: nitrogen in blue, bromine in red, sulfur in yellow, carbon in gray and other hydrogens in white. The residues in contact with PHI-443 compound are labeled and are shown in stick model (pink for side chains and steel-blue for main chains).

vs. 0.03 μM) and inhibited the replication of the NNRTI-resistant HIV-1 strain, A17 variant with an IC_{50} value of 3.2 μM whereas the IC_{50} values of TRV, NVP, and DLV were all >100 μM. PHI-443 was 10-times more potent against RT-MDR relative to its activity against the $HTLV_{IIIB}$ strain.

Thus, PHI-443 was 5-times more potent than TRV, 50-times more potent than ZDV, 75-times more potent than EFV, 100-times more potent than DLV, and 1250-times more potent than NVP against RT-MDR [86]. PHI-443 was 10-times more potent than TRV, 1042-times more potent than DLV and 2083-times more potent than NVP against strain A17. Similarly, PHI-443 was more effective than TRV, DLV, and NVP against the problematic NNRTI-resistant HIV-1 strain A17-variant. Notably, PHI-443 was active against genotypically and/or phenotypically NRTI/NNRTI-resistant 23 primary clinical HIV-1 isolates (subtypes A, B, F, and G carrying 2–7 TAMs) tested with a mean IC_{50} value of 0.02 μM (range 0.00004 μM to 0.24 μM). In addition, PHI-443 inhibited the infectivity of nine NRTI-resistant primary clinical HIV-1 isolates (carrying 2–7 TAMs) tested in a syncytial focus (plaque) formation assay using the CD4-expressing HeLa cell line (HT4-6C) with a mean IC_{50} value of 0.03 μM (range 0.005 μM to 0.18 μM).

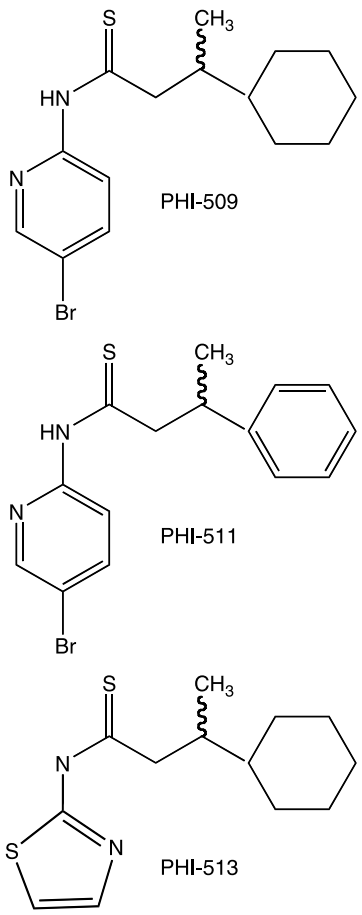


Figure 10. Chemical structures of (*R*)-isomers of halopyridyl (PHI 509/511) and thiazolyl (PHI-513) thiourea NNRTs.

Anti-HIV activity of (*R*)-isomers of halopyridyl and thiazolyl thioureas

The stereochemistry of halopyridyl and thiazolyl thiourea NNRTIs is a major determinant of their anti-HIV-1 potency. The lead compounds discovered were a cyclohexylethyl halopyridyl thiourea (PHI-509R, N-[1-(1-(1R)-cyclohexylethyl)]-N′-[2-(5-bromopyridyl)] thiourea), an α-methylbenzyl halopyridyl thiourea, (PHI-511R, N-[1-(1R)-(1-α-methylbenzyl]-N′-[2-(5-bromopyridyl)]thiourea) and a cyclohexyl ethyl thiazolyl thiourea, and (PHI-513R, N-[1(1-(1R)-cyclohexylethyl)]-N′-[2-(thiazolyl)]thiourea) (Figure 10).

Computer generated stick models of the test compounds docked in the NNRTI binding pocket indicated that the (*R*)-isomers would fit the target NNRTI binding pocket on HIV-1 RT much better than then enantiomers, as reflected by their 10^4-fold lower Ludi K_i values [82]. Modeling studies indicated that the methyl group on the chiral carbon of PHI-509R likely promotes its strong binding to the NNRTI binding pocket *via* van der Waals contacts with residue V179. Since this methyl group is 7 Å away from Y181, 9 Å from Y188, 8.5 Å from V106, and 6.5 Å from K103, as measured from the C atom of the methyl group to the C quadrate position of the protein residue, its favourable impact on the binding of PHI-509R to RT should not be affected by frequently encountered mutations involving these residues [82].

Accordingly, PHI-509R with an estimated K_i value of 100-fold lower than the S-isomer, inhibited recombinant RT *in vitro* with 100-fold lower IC_{50} values (Table III). The control compounds with unsubstituted pyridyl rings did not exhibit detectable RT inhibitory activity. Both PHI-509R and PHI-513R

Table III. Anti-HIV-1 activity of (*R*)-isomers of halopyridyl thiourea NNRTIs.

		IC_{50} (μM)				
Compd	K_i	rRT	$HTLV_{IIIB}$	A17	A17V	RT-MDR
PHI-509R	1.1	1.2	0.001	0.4	10.0	0.2
PHI-511R	ND	1.6	0.01	0.01	2.7	0.005
PHI-513R	12.0	13.0	0.001	0.9	5.8	5.6

inhibited HIV-1 replication with IC_{50} value of 0.001 μM. Similarly the (*R*)-isomers (but not (*S*)-isomers) of the α-methyl benzyl halopyridyl compound PHI-511 exhibited potent activity both in cell-free RT inhibition assays and cellular HIV-1 replication assays (Table III). The substitution of the pyridyl ring of PHI-509R with a thiazolyl ring (PHI-513R) resulted in a 10-fold higher K_i value and a 10-fold higher IC_{50} value in cell free RT inhibition assays. The (*S*)-isomers with estimated K_i values of >100 μM, did not exhibit any RT inhibitory activity, even at 100 μM. The most active agent, PHI-511R was found to be 10000-times more active than NVP, 5000-times more active than DLV, and 50-times more active than TRV against HIV-1 strain A17. PHI-511R inhibited the A17 variant with an IC_{50} value of 2.7 μM. These results provided unprecedented evidence that the stereochemistry as well as regiochemistry of a thiourea compound can profoundly affect its ability to fit into the NNRTI binding pocket of RT, and therefore, affect its anti-HIV-1 activity.

Additionally, the structural features of the "bridge" between the pyridyl and phenyl moieties of PETT-related thiourea compounds were shown to significantly affect their anti-HIV-1 potency. Among the 10 β-methyl phenylethyl pyridyl thioureas evaluated, the (*R*)-stereoisomers of compounds with halogen (Br, Cl) or methyl substitutions respectively, at 5-position were the most potent with nanomolar IC_{50} values against recombinant RT *in vitro* [85]. The 5-chloropyridyl isomer was 380-times more active than NVP, 190-times more than DLV, and two times more active than TRV. The β-methyl phenylethyl pyridyl thioureas were 65 times more potent than NVP and 19-times more active than DLV against RT-MDR.

Molecular modeling studies indicated that the (*R*)-stereoisomers of chiral halopyridyl and thiazolyl thiourea compounds would fit the target NNRTI binding pocket on HIV-1 RT better than their enantiomers [85,108–114]. The pyridyl thioureas exhibited more potent activity than the thiazolyl or benzothiazolyl thioureas. The 5-substitution of the pyridyl ring substantially enhanced the anti-HIV-1 activity indicating the importance of substitutent regiospecificity in addition to the stereospecificity of thiourea compounds [108–113]. Among the various substituted thiazolyl thioureas designed, synthesized and tested for anti-HIV-1 activity,

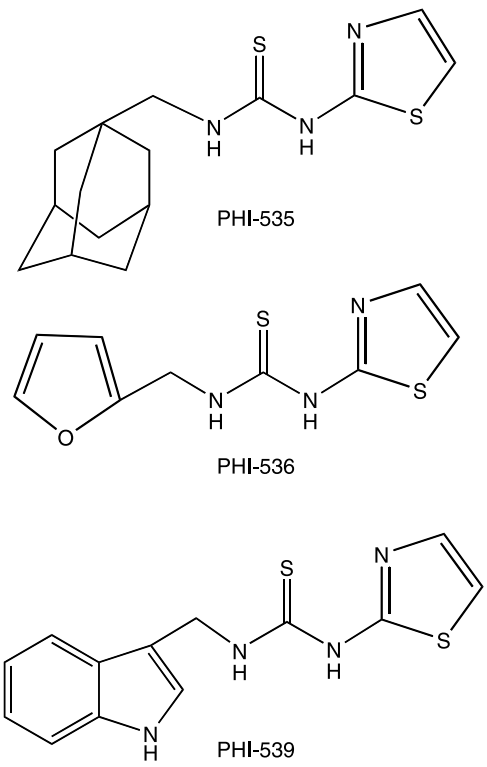


Figure 11. Chemical structures of chiral thiazolyl thiourea NNRTIs.

PHI-535, N-[1-(1adamantyl)methyl]-N′-[2-(thiazolyl)thiourea; PHI-536, N-[1-(1-furoylmethyl)]-N′-[2-(thiazolyl)thiourea and PHI-539 N-[2-(2-indole)ethy]-N′-[2-(thiazolyl)thiourea were potent against wild type as well as A17 and A17 variant mutant strains, consistent with the prediction that a larger Wing 2 group is advantageous for inhibition of these mutant strains (Figure 11). The most promising compound, PHI-536, showed potency against two NNRTI-resistant isolates at nanomolar to low micromolar concentrations [85] (Table IV).

Anti-HIV activity of aromatic and heterocyclic thiazolyl thioureas

Chiral derivatives of several substituted heterocyclic and alicyclic thiazolyl thiourea compounds were designed, synthesized, and tested for anti-HIV-1 activity. Six lead compounds were identified that showed subnanomolar IC_{50} values for the inhibition of

Table IV. Anti-HIV-1 activity of thiazolyl thiourea NNRTIs.

		$IC_{50[p24]}$ (μM)			
Compd	Substituent	$HTLV_{IIIB}$	A17	A17V	SI
PHI-535R	Adamantyl methyl	<0.001	0.6	1.3	4×10^4
PHI-536R	Furoyl methyl	<0.001	2.0	0.6	10^5
PHI-539R	Indole ethyl	<0.001	2.2	3.7	3×10^4

HIV-1 replication with CC_{50} values ranging from 28 to $>100\,\mu M$ [85]. The lead compounds, especially PHI-535, PHI-536, and PHI-539 were potent against wild-type HIV-1 as well as A17 and A17 variant mutant strains, consistent with our prediction that a larger Wing 2 group is advantageous for the inhibition of these RT mutant strains (Figure 11). PHI-536 was the most potent NNRTI with nanomolar IC_{50} values. It was 9–34 times more potent than the standard NNRTIs, NVP and DLV (Table IV).

Anti-HIV activity of piperidinylethyl and fluorophenethyl thioureas

Among the piperidinylethyl, phenoxyethyl, and fluorophenethyl bromopyridyl thiourea compounds synthesized and evaluated for anti-HIV-1 activity, PHI-516, N-[2-(2-methylpiperidinylethyl)]-N′-[2-(5-bromopyridyl)] thiourea, PHI-565, β-fluoro [2-phenethyl]-N′-[2-(5-bromopyridyl) thiourea, and PHI-566, β-fluoro[2-phenethyl]-N′-[2-(5-chloropyridyl) thiourea (Figure 12), were the most potent with IC_{50} values of $<0.001\,\mu M$ with a SI of >30000 (Table V).

These compounds were at least 4–5-fold more active in inhibiting HIV-1 replication compared to the phenoxyethyl thiourea compounds. Further, TRV, which lacks the fluoro substitution on the ethyl linker group, was at least 7-fold less active than PHI-565 and PHI-566. Both compounds inhibited RT-MDR with submicromolar IC_{50} values. PHI-566 was 14-fold more potent than NVP and 4-fold more potent than DLV. PHI-565 was 7-fold more potent than NVP and 2-fold more potent than DLV. Both compounds inhibited the Y181C mutant HIV-1 strain A17 with an IC_{50} value of $0.1\,\mu M$. PHI-565 and PHI-566 were 200-fold more potent than NVP ($IC_{50} = 21\,\mu M$) and 500-fold more potent than DLV ($IC_{50} = 50\,\mu M$). PHI-516 was not active against A17 and was less active than NVP or DLV against RT-MDR [110].

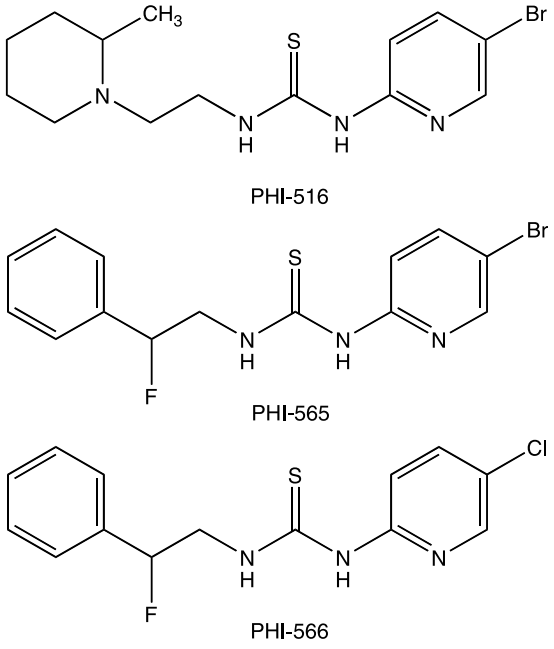


Figure 12. Chemical structures of piperidinylethyl and fluorophenethyl thioureas.

Table V. Anti-HIV-1 activity of piperidinylethyl and fluorophenethyl thioureas.

	$IC_{50[p24]}$ (μM)				
Compound	$HTLV_{IIIB}$	A17	RT-MDR	CC_{50}	SI
PHI-516	<0.001	>100	1.9	50	>50000
PHI-565	<0.001	0.1	0.2	50	>50000
PHI-566	<0.001	0.1	0.1	30	>30000

Anti-HIV activity of α-methyl benzyl thiourea and β-methyl phenylethyl pyridyl thioureas

Among the several (*R*)- and (*S*)-stereoisomers of β-methylphenylethyl pyridyl) thiourea compounds, the (*R*)-stereoisomers with halogen or methyl substitutions at the 5-position of their pyridyl ring were the most potent with nanomolar IC_{50} values against rRT (Figure 13). The lead compound, N-[(2R)-β-methylphenylethyl]-N′-[2-(5-chloropyridyl)thiourea was 380-fold more active than NVP and 190-fold more potent than DLV against A17. It was >200-fold more potent than NVP and DLV against A17V and exhibited remarkable activity against RT-MDR strain.

Among the several chiral α-methyl benzyl thiourea compounds designed and tested, substitution on the pyridyl ring at 5-position especially halo and alkyl was essential for the anti-HIV-1 activity [109] (Figure 13). The 5-methyl compound was the most active. Changing the pyridyl ring to thiazole ring did not affect the activity of the compound. The R isomers, PHI-567, N-[1-(1R)-β-methylbenzyl]-N′-[2-(5-bromopyridyl)thiourea; PHI-568, N-[1-(1-(1R)-β-methylbenzyl]-N′-[2-(5-chloropyridyl)thiourea; and PHI-611, N-[1-(1R)-β-methylbenzyl]-N′-[2-(4-methylthiazolyl)thiourea exhibited potent activity against NNRTI-resistant strains A17, A17V as well as RT-MDR (Table VI).

Molecular modeling studies indicated that the R stereoisomers of chiral halopyridyl as well as chiral thiazolyl thiourea compounds would fit the target NNRTI binding pocket on HIV-1 RT much better than their enantiomers [108–115]. 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 energetically more favored than the S stereoisomer, consistent with the better-fit and thus stronger binding with the NNRTI

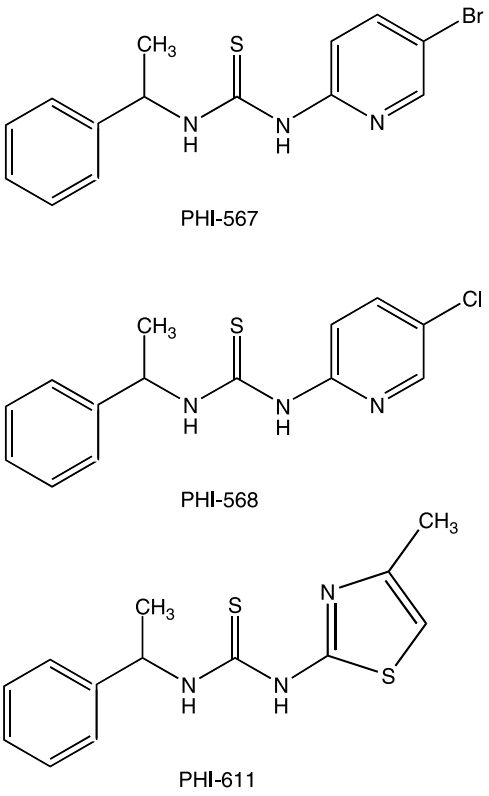


Figure 13. Chemical structures of three chiral α-methyl benzyl thiourea NNRTIs.

binding pocket. A similar trend was anticipated for α-methyl benzyl thiourea compounds since they differ from β-methyl phenyl thiourea compounds only by a carbon atom.

Tight binding HEPT NNRTIs

New HEPT derivatives synthesized included compounds with added groups at the N-1 (Y-R_3) and C-5 (R_1) positions and those having oxygen (X or Y) atoms replaced by sulfur (Figure 14). Substitution of oxygen by S can aid binding by decreasing the desolvation energy involved in binding. These modifications enabled the HEPT derivative to fit favourably into the "butterfly"-shaped RT-NNRTI binding site, with the benzyl ring residing in one wing and thymine ring in the other. In all HEPT derivatives, the benzyl ring is near Trp229 and the N-1 group is near Pro236, a typical position observed in crystal structures. The modeling calculations, along with the application of the constructed binding pocket, provided a guideline for the synthesis of lead compounds designed to have potent anti-HIV-1 activity. The choice of compounds was also based on synthetic feasibility. The lead HEPT compound was 6-benzyl-5-isopropyl-1[(methylthio)-methyl]-2-thiouracil.

Table VI. Anti-HIV-1 activity of (*R*)-isomers of α-methyl benzyl thiourea NNRTIs.

	IC_{50} (μM)				
Compound	rRT	$HTLV_{IIIB}$	A17	A17V	RT-MDR
PHI-567R	1.6	<0.01	0.01	2.7	0.005
PHI-568R	1.2	<0.01	0.2	10.2	0.01
PHI-611R	0.8	<0.001	0.16	0.19	0.001

The region of the NNRTI site of HIV-1 RT located near the thymine ring nitrogen N-1 of the HEPT analogues contains a Pro236 loop region, which is large enough to accommodate N-1 substituents. When an inhibitor binds to the NNRTI site of HIV-1 RT, the presence of a hydrophobic N-1 substituent could influence the Pro loop of this flexible region and provide additional hydrophobic contact leading to stronger binding. Docking results indicated that substitution at N-1 also helps the molecule position itself to achieve the best fit within the pocket.

The Ludi analysis showed a substantial increase in contact (Lipo score) between the compound and the pocket and the calculation suggested an increase in hydrophobic contact and stronger binding when the substituent on the N-1 tail (R_3) is larger than a methyl moiety. The Tyr183 residue of the HIV-1 RT is located in the catalytic region, which has a conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif characteristic of RT. Therefore, the displacement of this tyrosine residue can interfere with catalysis and render the HIV-1 RT protein inactive.

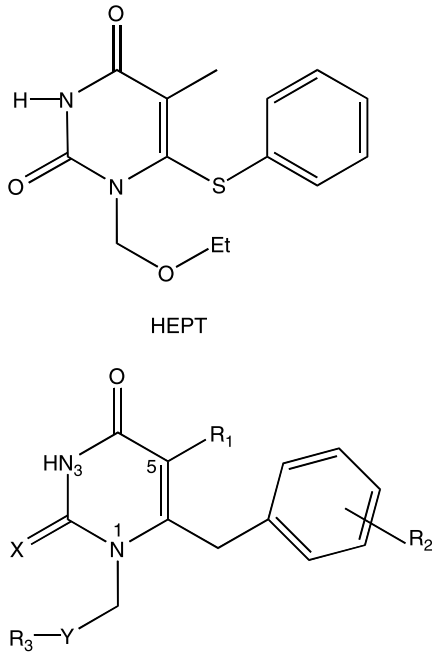


Figure 14. Potential modification sites of HEPT derivatives.

It has been suggested that bulky substituents at the 5-position of the thymine ring (R_1) could indirectly accomplish this goal by displacing Tyr181, which is near Tyr183. The composite binding pocket shows sufficient room for at least a 3-carbon group in this region. The addition of a methyl, ethyl or isopropyl group on the 5- position of the thymine ring would lead to a higher affinity for the relatively hydrophobic environment. Ludi analysis showed that the hydrophobic contact increases as hydrophobic groups at the 5- position (R_1) get bulkier. As it binds to the site, the ethyl or isopropyl group causes the nearby Tyr181 residue to rotate away from the inhibitor. This change in conformation in turn affects the positions of the neighbouring Tyr183 and Tyr188, which can lead to the inactivation of HIV-1 RT.

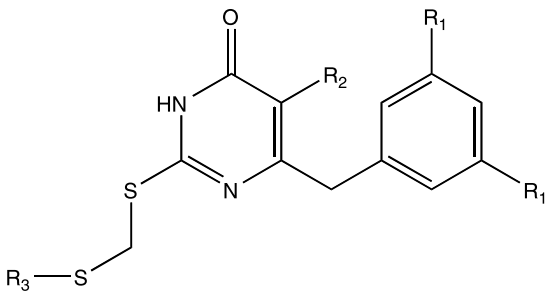


Figure 15. Potential modification sites of DABO derivatives.

Tight binding DABO NNRTIs

Detailed analysis of HEPT binding revealed that the N1 substituents of HEPT derivatives occupy the same region of the binding site as the thio (S2) substituents of DABO compounds. Therefore, new DABO derivatives were designed and their binding into the NNRTI site of RT modeled using the crystal structure coordinates of the RT-MKC complex and a molecular docking procedure [71,72]. The final coordinates of the docked molecules were then superimposed into the composite binding pocket to evaluate the fit within the RT-NNRTI pocket. The docked DABO molecule showed significant space surrounding the 6-benzyl ring and the 5- position of the thymine ring, which led to our design and synthesis of new DABO derivatives [72]. Structure–activity relationship profile of DABOs together with molecular modeling studies on their putative binding mode have shown that the presence of a C_2-alkoxy (DABOs) or C_2-alkylthio (S-DABOs) side chain is a structural determinant for the anti-HIV-1 activity [72].

Modeling studies predicted that the addition of a methyl, ethyl, or isopropyl group at the 5- position of the thymine ring would lead to higher affinity for the NNRTI binding pocket (K_i values 0.05–0.56 μM) as well as conformational rotation of the 6-benzyl ring, which affects the positions of nearby Tyr181, Tyr183 and Tyr188 residues (Figure 15). Biological evaluation indicated that the lead DABO derivative, PHI-280 (5-isopropyl-2-[(methylthiomethyl)thio]-6-(benzyl)-pyrimidin-4-(1H)-one) elicited potent anti-HIV-1 activity with an IC_{50} value of <1 nM for inhibition of HIV-1 replication and showed no detectable cytotoxicity ($CC_{50} > 100$ μM) (Table VII). PHI-280 was >4-fold more active than MKC-442. PHI-281 (5-isopropyl-2-[(methylthiomethyl)thio]-6-(3,5-dimethylbenzyl)-pyrimidin-4-1 H-one), which differs from PHI-280 by the addition of two methyl groups to the benzyl ring resulted in a larger molecular surface area, more hydrophobic contact with the NNI binding pocket, lower K_i values and was predicted to be more potent than PHI-280 based on the modeling studies.

The Tyr183 residue of the HIV-1 RT is located in the catalytic region, which has a conserved YMDD motif characteristic of reverse transcriptases. Therefore, the displacement of this tyrosine residue can interfere with catalysis and render the HIV-1 RT protein inactive. Bulky substituents at the 5- position of the thymine ring could indirectly accomplish such inactivation by displacing Tyr181, which is near Tyr183. The composite binding pocket showed sufficient space for at least a 3-carbon group at the 5- position. The addition of a methyl, ethyl or isopropyl group at the 5- position of the thymine ring was expected to lead to higher affinity for the relatively hydrophobic environment at this location of the binding pocket. The favourable hydrophobic contact increases, as the hydrophobic group at the 5- position gets bulkier. As the DABO derivative binds to the site, the isopropyl group can also cause the nearby Tyr181 residue to rotate away from the inhibitor.

Modeling studies showed that this change in conformation in turn affects the positions of neighboring

Table VII. Anti-HIV-1 activity of DABO derivatives.

Compd	R_1	R_2	K_i	IC50 (μM) RRT	p24	A17	A17V	MDR	CC_{50}	SI
PHI-280	H	I-Pr	0.56	5.6	<0.001	>100	>100	28	>100	$>10^5$
PHI-281	3,5-Me	I-Pr	0.05	7.0	0.016	3	55	7	>100	$>10^5$

Tyr183 and Tyr188, which may contribute to the inactivation of HIV-1 RT. The benzyl ring of PHI-280/281 is located near a region surrounded by the hydrophobic ring planes of residues Trp229, Pro95, Y188, and Y181. The analysis of PHI-280/281 in the composite binding pocket suggested that the benzyl ring would be located on the boundary of the pocket, near residue Y188. A para substituent of the ring is situated perpendicular to the ring plane of nearby Trp229, within van der Waals contact, and leaves a lot of space unfilled between the compound and Pro95. With a slight conformational rotation of the benzyl ring, PHI-281, with the addition of two methyl groups, was found to better fill the composite binding pocket [69,70]. Such observations indicate that further modifications to the benzyl ring could lead to even more potent inhibitors.

Pharmacology and toxicity

The thiourea class of NNRTIs is novel and there have been no reports to suggest that they may be toxic to humans. The thiourea NNRTIs are structurally and biologically very different from other NNRTIs such as TRV, which cause hepatotoxicity. The *in vivo* toxicity, pharmacokinetic features, tissue distribution, and metabolism of four representative thiourea NNRTIs (PHI-236, PHI-240, PHI-346, and PHI-443) has been examined in mice using a sensitive HPLC-based quantitative detection method [74,81,86,87,116–118]. At the dose range tested (10 to 100 mg/kg) thioureas NNRTIs were nontoxic to mice when administered intravenously or intraperitoneally. Thiourea NNRTIs showed favorable pharmacokinetics and did not cause acute or subacute toxicity in mice. Pharmacokinetic studies following oral or mucosal administration of thiourea NNRTIs (250–400 mg/kg bolus dose) indicated lack of toxicity.

Thiourea NNRTIs as anti-HIV microbicides

Rationally designed NNRTIs that display high binding affinity for HIV-1 RT and robust anti-HIV-1 activity against the wild type and drug-resistant strains without cytotoxicity are sought as molecular virucides or microbicides intended to prevent the sexual transmission of HIV-1 *via* semen [86,87,119–125]. The rationale for the development of tight binding NNRTIs as microbicides is that unlike NRTIs they do not require metabolic activation to achieve antiviral activity. Therefore, tight binding NNRTIs might exert their activity against cell free and cell-associated HIV-1 within the vaginal or rectal cavity. Additional criteria for an NNRTI to be an ideal microbicide are: (i) ability to rapidly cross membrane barriers; (ii) prolonged or irreversible inhibition of HIV-1 RT activity; (iii) rapid virucidal activity without metabolic activation; (iv) retain antiviral activity under acidic conditions; (v) stable under various climatological temperatures; (vi) minimal binding to genital tract components; (vii) retain virucidal activity following drug removal; (viii) lack systemic absorption to prevent drug resistance; and (ix) should not perturb the vaginal mucosa and normal vaginal flora.

Membrane permeable tight binding NNRTIs have the unique ability to inactivate cell-free as well as cell-associated HIV-1 in semen without metabolic activation [86,87,90–96]. NNRTIs currently under development as experimental microbicides include thiourea-PETT derivatives (PHI-236, PHI-346, and PHI-443), urea-PETT derivatives (MIV-150), oxypyrimidines (S-DABOs), thiocarboxanilides (UC-781) and diarylpyrimidines (TMC-120). Unlike the currently used detergent-type spermicidal antimicrobials, thiourea NNRTIs show high SI against normal human female genital tract epithelial cells [82,86,87]. In short-term subchronic studies, thiourea NNRTIs administered intravaginally at doses of 0.5%, 1%, and 2% *via* a gel-microemulsion to test animal species failed to induce any signs of toxicity [118]. Repeated intravaginal exposure of mice and rabbits to increasing concentrations of thiourea NNRTI for up to 13 weeks had no adverse effect on their subsequent reproductive capability, perinatal outcome, growth, and development of offspring [86,87,118]. Collectively, these findings demonstrate that repetitive intravaginal administration of thiourea NNRTI to yield effective antiviral concentrations is not associated with local, systemic, or reproductive toxicity. The antiviral efficacies of mucoadhesive formulations of tight binding NNRTIs have been studied in animal models and three (MIV-150, TMC120 and UC-781) have entered Phase I clinical trials in humans [126,127].

Conclusion

The success of NNRTIs for the clinical treatment of AIDS has led to extensive structural and molecular modeling studies of HIV-1 RT-NNRTI complexes and chemical synthesis of second- and third-generation NNRTIs. Rationally designed NNRTIs deduced from changes in binding pocket size, shape, and residue character that result from clinically observed NNRTI resistance associated mutations exhibit high binding affinity for HIV-1 RT and robust anti-HIV activity against the wild-type and drug-resistant strains without cytotoxicity. The thiourea NNRTIs were more potent against drug-sensitive and multidrug-resistant strains of HIV-1 than the three classes of NNRTIs currently in clinical use to treat HIV infections. Membrane permeable tight binding NNRTIs have utility as topical anti-HIV-1 microbicides since they can inactivate cell-free as well as cell-associated HIV-1 in semen without metabolic activation. These NNRTIs are expected to inactivate RT enzymatic activity in a highly efficient manner, thus eliminating

the ability of the virus to initiate a new round of infection. The high rate of treatment failure due to the emergence of drug resistance mutations makes the discovery of these broad-spectrum NNRTIs useful as a component of combination therapy for the prevention as well as treatment of both antiretroviral-naive and antiretroviral-experienced HIV-1 infected patients.

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