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RATIONAL DESIGN OF N-[2-(2,5-DIMETHOXYPHENYLETHYL)]-N'-[2-(5-BROMOPYRIDYL)]-THIOUREA (HI-236) AS A POTENT NON-NUCLEOSIDE INHIBITOR OF DRUG-RESISTANT HUMAN IMMUNODEFICIENCY VIRUS

Chen Mao,[a,b] Elise A. Sudbeck,[a,b] T. K. Venkatachalam,[a,c] and Fatih M. Uckun,[a,d]*
[a]Drug Discovery Program, Departments of [b]Structural Biology, [c]Chemistry and [d]Virology, Hughes Institute, St. Paul, MN 55113, U.S.A.

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Abstract: The novel thiourea compound N-[2-(2,5-dimethoxyphenylethyl)]-N'-[2-(5-bromopyridyl)]-thiourea (**HI-236**) targeting the non-nucleoside inhibitor (NNI) binding pocket of HIV-1 reverse transcriptase (RT) was rationally designed using a computer model of the NNI binding pocket. The NNI binding pocket model takes into consideration changes in binding pocket size, shape, and changes in residue character that result from clinically-observed NNI resistance-associated mutations of HIV RT. RT assays revealed that **HI-236** was not only more potent than trovirdine, MKC-442, and AZT against the drug-sensitive HIV-1 strain $HTLV_{IIIB}$, it was also 50–100 times more effective than delavirdine or nevirapine and twice as effective as our recently reported lead compound N-[2-(2-fluorophenethyl)]-N'-[2-(5-bromopyridyl)]-thiourea (**HI-240**) against the NNI-resistant Y181C mutant HIV-1 strain A17. Most importantly, **HI-236** was highly effective against the multidrug-resistant HIV-1 strain RT-MDR with multiple mutations involving the RT residues 74V, 41L, 106A, and 215Y. The activity of **HI-236** against RT-MDR was superior to that of other anti-HIV agents tested, which are listed in the following order: **HI-236** (IC_{50}: 5 nM) > **HI-240** (IC_{50}: 6 nM) > trovirdine (IC_{50}: 20 nM) >AZT (IC_{50}: 150 nM) > MKC-442 (IC_{50}: 300 nM) > delavirdine (IC_{50}: 400 nM) > nevirapine (IC_{50}: 5 μM).

The three categories of agents currently in use which are effective against the human immunodeficiency virus (HIV) are nucleoside analogs (such as 3'-azido-3'-deoxythymidine, AZT), protease inhibitors (such as nelfinavir), and the recently introduced non-nucleoside reverse transcriptase inhibitors (NNI or NNRTI) such as nevirapine. The high replication rate of the virus unfortunately leads to drug-resistant genetic variants (mutants), especially when selective pressure is introduced therapeutically.[1] Recently, we reported a novel computer model for the NNI binding pocket of HIV reverse transcriptase (RT), which provides an effective tool for rational design of NNI.[2–4] We utilized this novel composite binding pocket, together with a computer docking procedure and a structure-based semi-empirical score function, as a guide to predict energetically favorable positions of new NNI compounds in the NNI binding site of RT and reported the synthesis of a series of potent compounds that abrogated HIV replication in peripheral blood mononuclear cells at nanomolar concentrations without evidence of cytotoxicity.[2–4] In the present study, we applied this modeling procedure to design effective compounds against clinically observed drug-resistant HIV-1 isolates.

Our modeling studies revealed some important details regarding RT mutations leading to NNI resistance. One observation is that the Wing 2 region of the composite binding pocket consists of multiple aromatic residues including Y181, Y188 and W229. Residues in Wing 2 that are located in the β12 and β13 hairpin turn, (W229, for example), are conserved in RT because mutations would affect its grip on the primer strand and severely decrease polymerase activity.[5] Residues Y181 and Y188 occupy a substantial volume within the binding pocket. Any mutations at Y181 and Y188 would involve a smaller residue in the Wing 2 region, which is indeed the case for the Y181C, Y188C, and Y188H mutations in drug-resistant HIV strains. These mutations occupy a smaller volume of Wing 2, leading to a larger unoccupied volume in the binding pocket. An inhibitor that lacks a compatible functional group to interact with the mutated residues of Y181C, Y188C, and Y188H (a nonpolar

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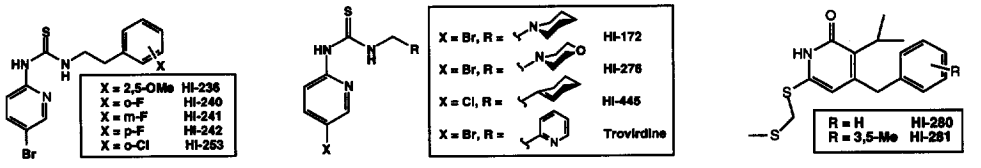
group in the case of Y181C and Y188C, and an aromatic group in the case of Y188H) and that lacks a large enough group to provide surface contact with the mutated region could result in drug resistance. This may explain the poor activity of nevirapine and delavirdine against the Y181C RT mutant (Table 1). As for other RT mutants, the V106A mutation introduces a smaller aliphatic residue, which leads to a slightly larger binding pocket volume in this region. As is the case for the Y181 and Y188 mutants, an inhibitor that does not sufficiently fill the additional volume in the V106A mutant binding pocket with a compatible (hydrophobic) functional group and that does not provide adequate van der Waal's (VDW) contact with A106 may result in lower activity against the V106A RT mutant. For example, V106A can exhibit reduced VDW contact between the linker region located between Wing 1 and Wing 2 and the NNI compounds nevirapine or delavirdine. This loss of hydrophobic contact considerably reduces (but does not abolish) the RT inhibitory activity of the NNI (Table 1). Residue K103 of the wild-type RT occupies a distinct volume of the binding site and possesses an electrostatic property allowing interaction with D192, which may be important for the stabilization of the K101 loop. The K101 loop is in close contact with nevirapine and delavirdine, and any alteration in this region (i.e., the K103N mutation) can cause weaker binding resulting in RT resistance to these drugs.

Table 1. Inhibitory activity of **HI-236** and **HI-240** on p24 production in peripheral blood mononuclear cells infected with HIV strains $HTLV_{IIIB}$, RT-MDR, A17, and A17 variant.



HI-236	0.1	<0.001	0.005	0.1	11
HI-240	0.6	<0.001	0.005	0.2	41
HI-241	0.7	<0.001	0.02	N.D.	N.D.
HI-242	6.4	N.D.	N.D.	N.D.	N.D.
HI-253	0.7	<0.001	0.004	N.D.	N.D.
HI-445	3.7	0.003	N.D.	N.D.	N.D.
HI-172	5.8	<0.001	>1	N.D.	N.D.
HI-276	>10.	3.8	>1	N.D.	N.D.
HI-280	5.6	<0.001	28	>100	>100.
HI-281	7.0	0.016	7	38	55
Delavirdine	1.5	0.009	0.4	50	>100
Nevirapine	23	0.034	5	>100	>100
MKC-442	0.8	0.004	0.3	N.D.	N.D.
Trovirdine	0.8	0.007	0.02	N.D.	N.D.
AZT	N.D.	0.004	0.15	0.006	0.004

*Purified recombinant HIV RT assay[4] (cell-free Quan-T-RT system, Amersham, Arlington Heights, IL), which utilizes the scintillation proximity assay principle.[6] A DNA/RNA template is bound to SPA beads via a biotin/strepavidin linkage. The primer DNA is a 16-mer oligo(T) which has been annealed to a poly(rA) template. The primer/template is bound to a strepavidin-coated SPA bead. ^{3}H-TTP is incorporated into the primer by reverse transcription and then quantitated relative to an untreated sample.
N.D., not determined; WT, wild-type. The synthesis of **HI-172, HI-240, HI-241, HI-253, HI-280,** and **HI-281** and their activities against WT RT expressing the $HTLV_{IIIB}$ strain of HIV-1 were recently reported.[3,4,7]

We postulated that our recently reported lead compound N-[2-(2-fluorophenethyl)]-N'-[2-(5-bromo-pyridyl)]-thiourea (**HI-240**)[3] would be effective against HIV RT mutants. **HI-240** was predicted to interact more favorably with RT mutants than other compounds such as nevirapine or delavirdine. The activity of **HI-240**, which contains a 2'-fluoro group (Figure 1C), against wild-type HIV RT was compared with compounds that contain fluoro substitutions at other positions on the phenyl ring (3'-F and 4'-F substituted compounds). **HI-240** was shown to be the most active in enzyme assays measuring inhibition of recombinant RT (IC_{50}rRT = 0.6 μM), followed by the 3'-F compound (**HI-241**, IC_{50}rRT = 0.7 μM), and lastly 4'-F (**HI-242**, IC_{50}rRT = 6.0 μM).[3] The HIV replication assays using peripheral blood mononuclear cells infected with the NNI-sensitive HIV strain $HTLV_{IIIB}$ showed a similar trend, with IC_{50}[p24] values < 1 nM for 2'-F (**HI-240**) and 10 nM for the 4'-F compound.[3] **HI-240** was more potent than phenethylthiazolylthiourea (PETT[8–11]) derivatives **HI-253**, **HI-445**, **HI-172**, and **HI-276**, and more potent than dihydroalkylthiobenzyloxopyrimidine (S-DABO[12–16]) compounds **HI-280** and **HI-281** against both wild-type RT and the multidrug resistant (MDR) strain of RT (see IC_{50} rRT and IC_{50} p24 RT-MDR data, Table 1). The inhibition trend for compounds tested against recombinant wild-type RT was consistent with the inhibition trend for MDR RT. Modeling analysis further revealed that the extensive contact of the V106 residue with the alkylthio group of S-DABO derivatives (**HI-280** and **HI-281**) constitutes additional VDW contact which is lost upon mutation to MDR RT. Because the VDW contact loss is more pronounced for S-DABO derivatives than for the PETT compounds (**HI-240**, **HI-241** and **HI-253**), **HI-280** and **HI-281** would be predicted to have lower activity against MDR RT. The molecular modeling is consistent with the measured IC_{50} values showing the relatively poor performance of **HI-280** and **HI-281** against MDR RT. Furthermore, our lead compound **HI-240** was also more active than trovirdine (see IC_{50} rRT and IC_{50} p24 for wild-type RT, Table 1). Trovirdine was also shown to be three times less potent than **HI-240** against the multiple-drug-resistant mutant strain of HIV RT (Table 1), which may reflect the fact that trovirdine lacks a polar ring substituent which can provide more favorable interactions with binding site residues. As for drug resistance against **HI-240**, the polar character of the Wing 2 residues of RT that can interact favorably with the 2'-F group of **HI-240** in the binding site would be unaffected by the clinically observed mutants K103N, V106A, Y181C (Figure 1D). Therefore, the favorable interaction with the 2'-F group of **HI-240** would not be lost in these RT mutants. In addition, since **HI-240** was 100- to 1000-fold more potent than delavirdine or nevirapine against wild-type RT,[3] a decrease in **HI-240** potency against an RT mutant could still leave a relatively potent inhibitor, which may not be the case for the inherently less potent compounds delavirdine and nevirapine.

The results of our modeling analysis showed that the Y181 residue stacks in a favorable herringbone orientation with the aromatic residue of NNI compounds including **HI-240**. The Y181 stacking interaction is lost in the Y181C mutant, which is predictive of some degree of resistance against **HI-240**, delavirdine, and nevirapine. When we tested **HI-240** against the Y181C mutant we found that it was 200-times better (IC_{50}: 0.2 μM) than delavirdine (IC_{50}: 50 μM) and more than 500-times better than nevirapine (IC_{50} > 100 μM). The MDR mutant contains one mutation, V106A, which is located the in NNI binding pocket. Because delavirdine,[17] nevirapine,[18,19] and **HI-240** all have a central portion of the molecule which is in contact with RT residue 106, they appear to show comparably lower activities against this mutant. However, the degree of resistance differed

for the three compounds; **HI-240** showed a 100-fold better activity (IC_{50}: 0.006 μM) against MDR HIV than delavirdine (0.4 μM) and an 800-fold better activity than nevirapine (5 μM).

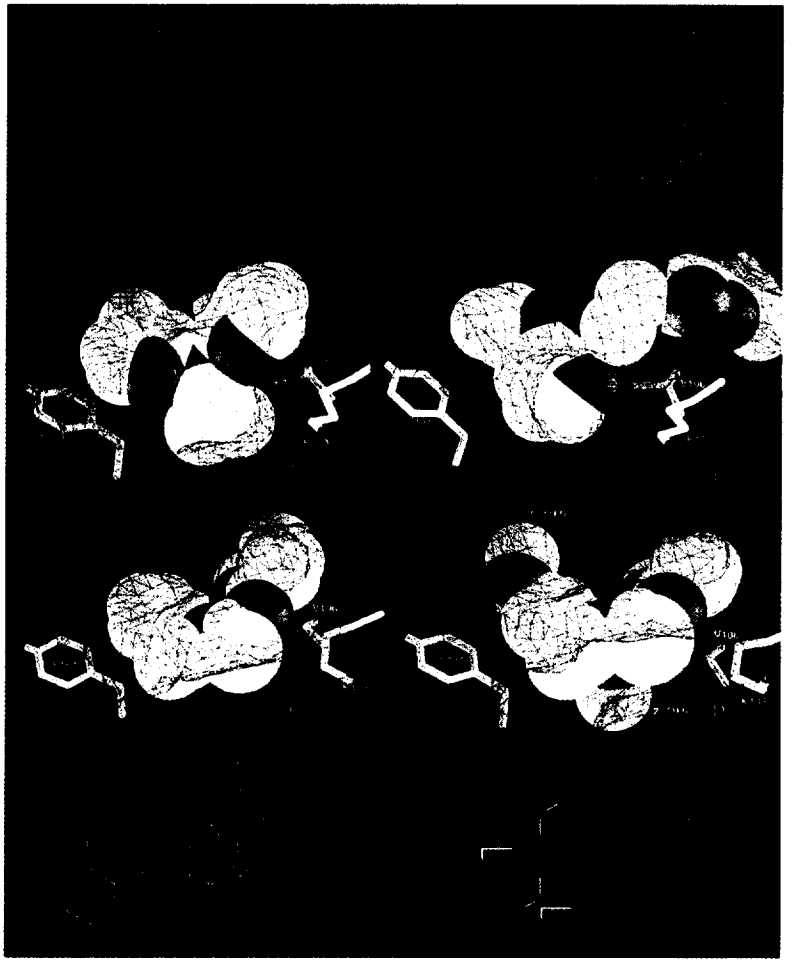


Figure 1. Space filling models of NNI molecules which bind to the NNI binding pocket of HIV RT mutants Y181C, V106A and K103N. Binding pocket shown is from crystal structures of RT which were modified to show RT mutations (GRASP program[20]). (**A**) Nevirapine (white and blue space-filling atoms), based on the crystal structure of RT complexed with nevirapine (protein databank [PDB] access code: 1vrt).[19] (**B**) Delavirdine (red, white, and blue space-filling atoms), based on the crystal structure of RT complexed with delavirdine (PDB access code: 1klm).[17] (**C**) **HI-240** (white and blue) and (**D**) lead compound **HI-236** (red, white, and blue) docked into the NNI binding pocket based on crystal structure of RT/9-Cl-TIBO (PDB code: 1rev).[21] Y181 can π-stack with aromatic rings of nevirapine, **HI-240**, and **HI-236**, and can form edge-to-face contact with the pyridyl ring of delavirdine in the Wing 2 region of the binding site. (**D**) Schematic drawing of **HI-236** molecule illustrates predicted interactions with RT. Red box defines the Wing 2 region of the binding site.

Based on our composite binding pocket model, we previously recognized that the Wing 2 region has a substantial molecular volume (approximately 160 $Å^3$) surrounding the phenyl ring at the Wing 2 region that defines space that can potentially be more efficiently occupied by a larger functional group.[2] We next decided to design a novel thiourea compound with optimized van der Waal's (VDW) contact with the binding pocket. We predicted that this strategy would improve the potency against wild-type RT and improve the inhibition profile against Wing 2 mutants of RT. This prompted us to synthesize **HI-236** which contains one methoxy group at the 2' position (same position as the fluoro atom of **HI-240**) and another methoxy group at the 5' position of the phenyl ring, which can contact the Wing 2 region (see Figure 1B) better than **HI-240**. Docking results showed

that the unoccupied volume surrounding **HI-236** in the NNI binding site of RT was 135Å^3, a decrease of 25Å^3 relative to the unoccupied volume surrounding **HI-240** (160 Å^3) (Figure 1C-D). These docking results are consistent with activity data showing an improved potency for **HI-236**, which can contact RT residues better than **HI-240** (Table 1).

Design and Activity of HI-236

HI-236 was docked into the NNI binding site of RT using a molecular docking procedure in the Affinity module with the Insight II program.[22] Docking of the compound into the NNI binding site required the use of X-ray coordinates of an RT/NNI complex (in this case the RT/9-Cl-TIBO complex[21] was used). The docking studies indicated that the 2-methoxy group of **HI-236** was situated beneath the ethyl linker and fit favorably into a cavity of the binding pocket, providing additional contact with protein residues that cannot be achieved by **HI-240**. Likewise, the 5-methoxy group of **HI-236** provides close contact with residues P95 and W229 and is near Y181, leaving 25 Å^3 less unoccupied space at Wing 2 relative to **HI-240**. Inhibitor interactions with P95 and W229 are especially desirable because no RT mutations have been reported for these residues (a mutation of W229 would be unlikely since it is important for polymerase function[5]). Based on our established procedure using a modified LUDI score function,[23,24] the estimated K_i value of **HI-236** (0.1 μM) was better than that of **HI-240** (0.7 μM).[3] **HI-236** was synthesized using literature procedures[3] and tested for anti-HIV activity. Like **HI-240**, **HI-236** elicited potent anti-HIV activity with IC_{50} values less than 0.001 μM in 3 of 3 independent experiments that was consistently lower than the IC_{50} values for AZT (0.004 μM). Furthermore, the IC_{90} value of **HI-236** (9 nM) was 10-fold better than that of AZT (100 nM). Neither **HI-240** nor **HI-236** were cytotoxic at concentrations up to 100 μM. Therefore, the calculated selectivity index (IC_{50}[MTA] / IC_{50}[p24]) of **HI-236** was $> 10^5$.

The same modeling procedure was applied for the analysis of RT mutants (106A, Y181C) (Table 1). **HI-236** was predicted to have a better K_i value than trovirdine against the RT mutants because it contains a larger functional group which has more contact with the Wing 2 region of RT when Y181 or Y188 of RT is mutated to a smaller residue Cys (see Figure 2D). **HI-236** demonstrated an IC_{50} value of 0.005 μM against MDR RT (slightly better than that for **HI-240**), 0.1 μM against Y181C mutant (two times better than that for **HI-240**), and 11 μM against Y181C+K103N (four times better than that for **HI-240**). These results are consistent with our predictions that **HI-236** would be a more potent anti-HIV compound than **HI-240**. Compounds **HI-236** and **HI-240** are 20–30 times less active against Y181C RT mutant than the RT wild-type (Table 1), yet maintained a useful activity level (IC_{50} values of 0.1 μM and 0.2 μM, respectively). **HI-236**, like **HI-240**, maintained promising activity against the V106A mutant (Table 1). This result is consistent with our model, which shows that residue changes in the Wing 1 region (V106A, K103N mutants) should not affect **HI-236**, which contains functional group modifications only in the Wing 2 region, distant from Wing 1. In conclusion, **HI-236** is predicted to not only have significant potency against wild-type RT but also to show high potency against many RT mutants such as 106A (four times better) and Y181C, the latter of which is resistant to many examined NNI compounds currently in clinical use.

Based on our study, we conclude that for an NNI of HIV RT to be active against the Wing 2 mutants, such as the especially problematic Y181C RT mutant, (a) the inhibitor should be highly potent against wild-type

RT and therefore could afford a considerable activity loss against mutants (i.e., a picomolar-level inhibitor against wild-type RT may still be effective against RT mutants at nanomolar concentrations) (**b**) the inhibitor should maximize the occupancy in the Wing 2 region of the NNI binding site of RT, and (**c**) the inhibitor should contain functional groups which provide favorable chemical interactions with Wing 2 residues of RT.

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