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Article

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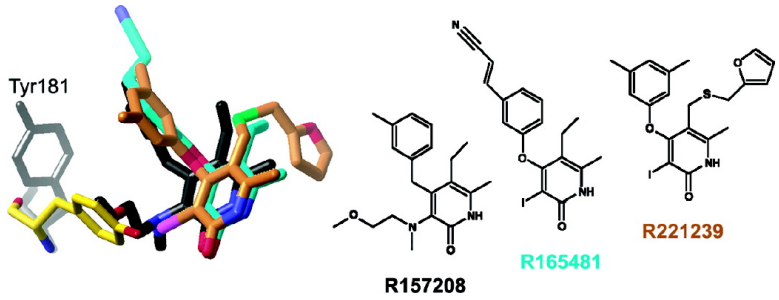


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# Crystal Structures for HIV-1 Reverse Transcriptase in Complexes with Three Pyridinone Derivatives: A New Class of Non-Nucleoside Inhibitors Effective against a Broad Range of Drug-Resistant Strains

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In the treatment of AIDS, the efficacy of all drugs, including non-nucleoside inhibitors (NNRTIs) of HIV-1 reverse transcriptase (RT), has been limited by the rapid appearance of drug-resistant viruses. Lys103Asn, Tyr181Cys, and Tyr188Leu are some of the most common RT mutations that cause resistance to NNRTIs in the clinic. We report X-ray crystal structures for RT complexed with three different pyridinone derivatives, R157208, R165481, and R221239, at 2.95, 2.9, and 2.43 Å resolution, respectively. All three ligands exhibit nanomolar or subnanomolar inhibitory activity against wild-type RT, but varying activities against drug-resistant mutants. R165481 and R221239 differ from most NNRTIs in that binding does not involve significant contacts with Tyr181. These compounds strongly inhibit wild-type HIV-1 RT and drug-resistant variants, including Tyr181Cys and Lys103Asn RT. These properties result in part from an iodine atom on the pyridinone ring of both inhibitors that interacts with the main-chain carbonyl oxygen of Tyr188. An acrylonitrile substituent on R165481 substantially improves the activity of the compound against wild-type RT (and several mutants) and provides a way to generate novel inhibitors that could interact with conserved elements of HIV-1 RT at the polymerase catalytic site. In R221239, there is a flexible linker to a furan ring that permits interactions with Val106, Phe227, and Pro236. These contacts appear to enhance the inhibitory activity of R221239 against the HIV-1 strains that carry the Val106Ala, Tyr188Leu, and Phe227Cys mutations.

## Introduction

Because the enzyme reverse transcriptase (RT) catalyzes several steps in the replication of human immunodeficiency virus (HIV),[1] the causative agent of AIDS, RT is an attractive target for anti-AIDS drug development. RT converts the single-stranded viral genomic RNA into double-stranded DNA. The enzyme has two enzymatic activities: a DNA polymerase, which synthesizes a growing DNA strand using either an RNA or DNA template, and an RNase H activity, which digests RNA when it is present as part of an RNA/DNA duplex. RT is a heterodimer consisting of 66 kDa (p66) and 51 kDa (p51) subunits. Each of these polypeptide chains contains the same 440 N-terminal amino acid residues that comprise the four polymerase subdomains: thumb, palm, fingers, and connection. The C-terminus of p66 contains an additional 120 amino acids that include the RNase H domain.[2,3] Despite folding into similar subdomain structures, the two subunits differ dramatically in their overall conformation. The p66 subunit is shaped like a right-hand and contains a large cleft formed by the fingers, palm, and thumb subdomains that accommodates double-stranded nucleic acid template-primers.

There are two classes of anti-RT drugs: nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs). The NRTIs, including such drugs as AZT (3′-azido-3′-deoxythymidine) and 3TC (lamivudine, 2′,3′-dideoxy-3′-thiacytidine), are converted to triphosphates by the host and act as dNTP mimics. NRTIs lack a normal 3′-hydroxyl and, when incorporated in viral DNA, terminate DNA synthesis. The NNRTIs, including the drugs nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one), delavirdine (U-90152, 1-[(5-methanesulfonamido-1*H*-indol-2-yl)carbonyl]-4-[3-[(1-methylethyl)amino]pyridinyl]piperazine), and efavirenz (EFV, DMP-266, (*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2(*H*)-3,1-benzoxazin-2-one), bind to a hydrophobic pocket (the non-nucleoside inhibitor binding pocket, NNIBP) in the p66 palm subdomain near the polymerase active site. Crystallographic studies showed

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**Table 1.** X-ray Diffraction Data and Refinement Statistics[a]

| | HIV-1 RT/ATP + R157208 | HIV-1 RT/R165481 | HIV-1 RT/R221239 |
|---|---|---|---|
| | X-ray Diffraction Data[a] | | |
| PDB ID | 2BAV | 2B5J | 2BE2 |
| X-ray source | APS (Feb 2001) | APS (Feb 2001) | CHESS (Jan 2002) |
| resolution (in Å) | 40−2.95 | 40−2.9 | 23−2.43 |
| no. of frames used (1.5° oscillation each) | 152 | 110 | 133 |
| unit cell parameters | All crystallized in monoclinic *C*2 crystal form. | | |
| *a* (in Å) | 226.24 | 224.59 | 223.85 |
| *b* (in Å) | 69.43 | 69.30 | 68.80 |
| *c* (in Å) | 104.45 | 105.02 | 104.43 |
| $\beta$ (in deg) | 106.49 | 106.47 | 107.22 |
| completeness (in last shell) | 95.5% (77.4%) | 95.4% (83.2%) | 91.7% (54.9%) |
| no. of reflections (no. of observations) | 31 625 (137 038) | 33 078 (103 273) | 54 672 (154 077) |
| average $I/\sigma(I)$ | 13.0 | 15.6 | 14.2 |
| $\sigma$-cutoff in scaling | −1.0 | −1.0 | −1.0 |
| *R*-merge (in last shell) | 0.063 (0.40) | 0.046 (0.32) | 0.056 (0.57) |
| | Refinement Statistics | | |
| resolution (in Å) | 20−2.95 | 20−2.9 | 23−2.43 |
| *R*-working | 0.243 | 0.248 | 0.235 |
| *R*-free | 0.305 | 0.304 | 0.265 |
| no. of reflections used (completeness) | 30 359 (91.9%) | 31 761 (91.8%) | 50 774 (88.4%) |
| no. of reflections in R-free set | 1499 | 1576 | 2483 |
| rmsd bonds (in Å) | 0.009 | 0.009 | 0.009 |
| rmsd angles (in deg) | 1.70 | 1.68 | 1.81 |
| no. of protein atoms | 7898 | 7900 | 8025 |
| no. of inhibitor and solvent atoms | 26 | 24 | 238 |

[a] All these data were collected at a wavelength of 1.0 Å from flash-cooled crystals either at APS (beam line 14-BM-C), or at CHESS (beam line F1).

that the binding of an NNRTI causes a large conformational change in HIV-1 RT, in particular, a displacement of the $\beta12-\beta13-\beta14$ sheet that contains the DNA primer grip.[4−7] Binding of an NNRTI may also influence the geometry of the polymerase catalytic site.[8,9]

The therapeutic efficacy of NNRTIs, like other types of anti-AIDS drugs, has been limited by the emergence of drug-resistant viral variants.[10−16] There is a need to develop new NNRTIs that are effective against the existing drug-resistant viral strains. A novel pyridinone class of NNRTIs has been prepared that has the ability to inhibit wild-type and drug-resistant HIV-1 strains[17−19] (and D. S. Grierson, personal communication). We here report crystal structures of HIV-1 RT in complexes with three of these pyridinone derivatives. Of these inhibitors, R157208 (5-ethyl-3-[(2-methoxyethyl)methylamino]-6-methyl-4-(3-methylbenzyl)pyridin-2(1*H*)-one) is a member of the benzylpyridinone subclass of NNRTIs (compound **62** in this series).[19] The other two, R165481 (*E*-3-[3-(5-ethyl-3-iodo-6-methyl-2-oxo-1,2-dihydropyridin-4-yloxy)phenyl]acrylonitrile) and R221239 (4-(3,5-dimethylphenoxy)-5-(furan-2-ylmethylsulfanylmethyl)-3-iodo-6-methylpyridin-2(1*H*)-one), belong to the 3-iodo-4-aryloxypyridinone (IOPY) subclass (ref 17 and D. S. Grierson, personal communication). On the basis of these structures, we assess how the major chemical substitutions on the pyridinone backbone affect NNRTI binding and the activity of these inhibitors against RT variants containing common drug-resistance mutations.

## Results and Discussion

**Overall Protein Conformation.** Structures of RT/R157208, RT/R165481, and RT/R221239 were determined at 2.95, 2.90, and 2.43 Å resolution, respectively (Table 1). In each case, the overall conformation of the protein is characteristic of RT/NNRTI complexes, in which the cleft between the fingers and the thumb of p66 is wider than it is in RT complexed with either duplex DNA[20,21] or RNA:DNA.[22] The pyridinone compounds R157208, R165481, and R221239 are chemically related to the HEPT (1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine) series of NNRTIs,[23] but the central pyrimidinone ring of HEPT is replaced with a pyridin-2-one ring in the pyridinone compounds, and the substituents on the ring are different (Figure 1). When bound to RT, the two aromatic rings of R157208, R165481, and R221239 are in a butterfly-like conformation, which has been seen in other RT/NNRTI complexes,[24] although the ring orientations are different. The substituted benzyl or phenoxyl ring corresponds to "wing I" of the butterfly and the pyridinone ring to "wing II" (Figure 2). R157208, R165481, and R221239 have many of the same interactions with RT seen in other RT/NNRTI complexes; however, there are some notable differences.

**R157208 in a Complex with RT.** A distinguishing feature of R157208 is the (2-methoxyethyl)methylamino substituent at the 3-position of the pyridinone ring (Figure 1). Additionally, an ethyl group is substituted at the 5-position and a methyl group is substituted at the 6-position. The location of the terminal methyl group of the 5-ethyl group is not well-defined in the electron density (the same ambiguity occurs in the electron density for this substituent in the structure of the RT/R165481 complex described below). The 3′-methyl-substituted benzyl ring at the 4-position of the pyridinone ring of R157208 makes extensive hydrophobic contacts with Trp229. This highly conserved residue is a part of the primer grip and appears to be essential for normal polymerase activity.[3,25−28] The 4′- and 5′-carbon atoms and the 3′-methyl group of R157208 interact with Trp229. Additionally, hydrophobic interactions are observed between the 5′-carbon and Leu234 as well as between the 3′-methyl group and Pro95 (Figure 1). The RT/R157208 structure is similar to most RT/NNRTI complexes in that the side chain of Tyr181

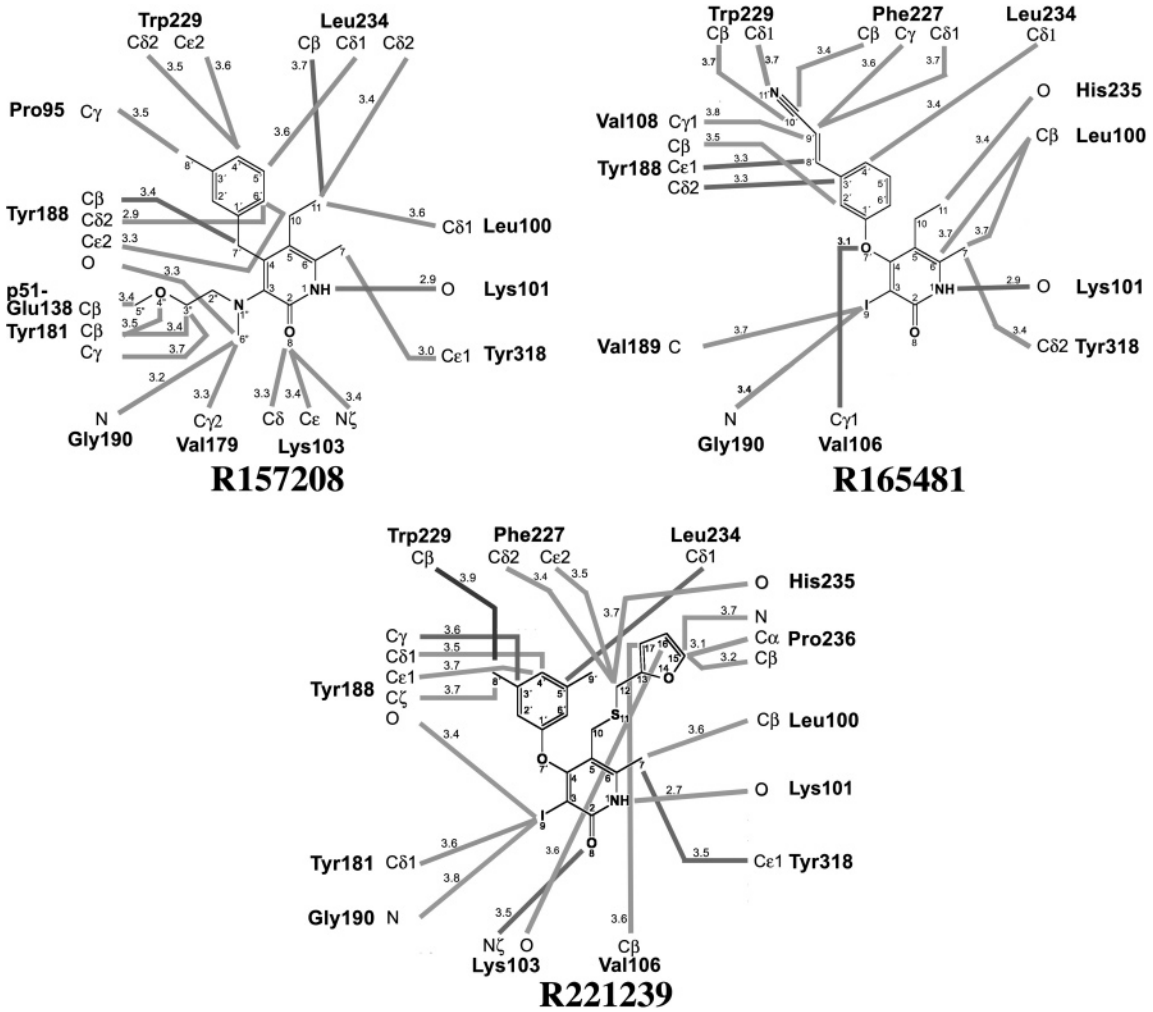



**Figure 1.** Ligand structures and protein−inhibitor contacts. R157208, R165481, and R221239 are shown with their atomic numbering schemes. Selected protein−inhibitor interactions are indicated with distances (Å) to accompany discussion in the text. For a more comprehensive list of protein−inhibitor contacts up to 4.0 Å, see Supporting Information, Tables S-1−3.

flips (rotates) about 100° around the $\chi 1$ dihedral (the side chain of Tyr188 also rotates a similar amount) with respect to apo-RT to form the NNIBP and points toward the polymerase active site.[5,7,8] In RT structures that do not have a bound NNRTI, by contrast, the Tyr181 side chain points away from the active site toward the putative entrance to the NNIBP. When the aromatic side chain of Tyr181 assumes the flipped conformation, it makes hydrophobic stacking interactions with wing I of an NNRTI that binds in the butterfly-like conformation. NNRTIs with which the Tyr181 side chain stacks are sensitive to the Tyr181Cys mutation.[6,23] In RT complexes with either R157208 or the chemically related HEPT-like compounds MKC-442 (6-benzyl-1-(ethoxymethyl)-5-isopropyluracil) and TNK-651 (6-benzyl-1-[(benzyloxy)methyl]-5-isopropyluracil),[23] the side chain of Tyr181 occupies the flipped position (Figure 2) and interacts primarily with the substituent at the 3-position of the central ring (the pyridinone ring in R157208, Figure 1). In the HEPT series, derivatives that interact with Tyr181 are substantially more potent against wild-type RT than those that do not make these contacts.[23,29] In the RT/R157208 complex, the (2-methoxyethyl)-methylamino substituent at the 3-position is a flexible extension that forms part of wing I (Figure 3) and interacts primarily with the side chain of Tyr181 (Figure 1; Supporting Information, Table S-1). The 3′-methyl-benzyl ring, a part of wing I, has extensive hydrophobic interactions with Tyr188, which is positioned so that its hydroxyl group makes a hydrogen bond with the side chain of the active site residue Asp186. The extensive interactions between R157208 and these two tyrosine residues make this compound relatively susceptible to the mutations Tyr181Cys and Tyr188Leu (Table 2).[19] The (2-methoxyethyl)methylamino substituent of R157208 is within 3.2 Å of Gly190. A Gly190Ala/Ser mutation would be expected to cause a steric conflict with the C6″ methyl group of this substituent; as expected, R157208 is sensitive to this mutation (Table 2). This is consistent with the previous observation that a side chain on residue 190 causes resistance toward NNRTIs by steric hindrance.[30]

The NH-group at the 1-position of the pyridinone ring of R157208 (Figure 1) forms a hydrogen bond with the carbonyl oxygen of Lys101 (Supporting Information, Table S-1). This interaction is present in the complexes of the other two pyridinone compounds and occurs when most potent NNRTIs bind to RT.[16,31]

Almost all NNRTIs are vulnerable to the Lys103Asn mutation, including R157208 (Table 2). It has previously been reported that the Lys103Asn mutation introduces a network of hydrogen bonds that stabilize the conformation of RT that interferes with the binding of an NNRTI.[32] Although the Lys103Asn mutation usually

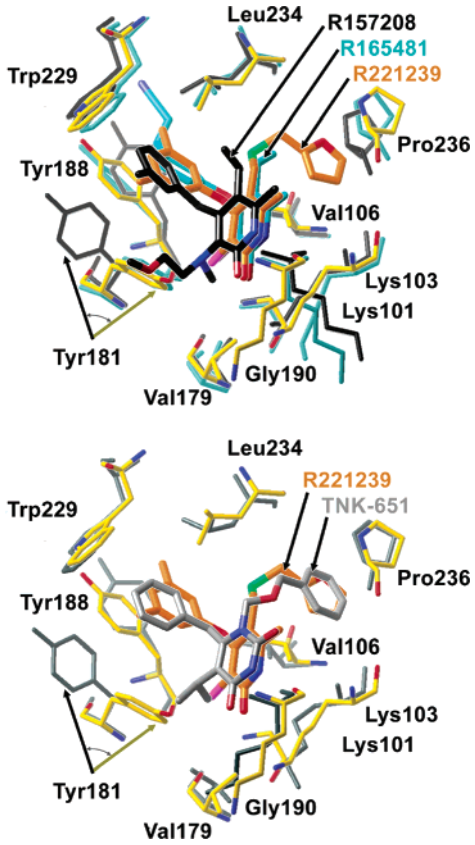



**Figure 2.** Inhibitor conformations in the NNIBP. The figure was generated using the program Maestro.[51] (a) (Upper panel) The superposition of the structures of the three RT/NNRTI complexes is shown (R157208 carbons in black, R165481 carbons in cyan, R221239 carbons in orange, RT/R221239 protein carbons in yellow). For clarity, Pro95, Leu100, Phe227, and Tyr318 are not shown. The benzyl/phenoxyl ring (left) is in the wing I position (using the butterfly-like analogy[24,52]), and the pyridinone ring (right) occupies the wing II position. To accommodate the (2-methoxyethyl)methylamino substituent of R157208, the Tyr181 side chain is rotated (upward) toward Trp229 in the RT/R157208 complex compared to the other two complexes. (b) (Lower panel) Superposition of RT/R221239 and RT complexed with HEPT-like TNK-651[23] (gray). If the isopropylpyrimidine ring of TNK-651 is replaced with the iodopyridinone ring of R221239, the Tyr181 side chain is not flipped.

causes resistance, there are NNRTIs, like TMC125−R165335 (etravirine, 4-((6-amino-5-bromo-2-((4-cyanophenyl)amino)pyrimidin-4-yl)oxy)-3,5-dimethylbenzonitrile), that interact specifically with Asn103[33,34] and are therefore effective against the Lys103Asn mutant. The O8 atom of the pyridinone ring of R157208 has the potential to interact with the side chain of Asn103. However, this interaction may require repositioning of the inhibitor.

**R165481 in a Complex with RT.** In R165481, the bulky (2-methoxyethyl)methylamino substituent on the 3-position of the pyridinone ring of R157208 is replaced with an iodine atom. The one-carbon methylene linker between the two rings of R157208 is replaced by an oxygen atom in R165481, so that the benzyl ring of R157208 becomes a phenoxyl ring in R165481. In addition, an acrylonitrile group is substituted for the methyl substituent at the 3′-position of that ring (Figure 1). These modifications substantially improve the interactions of this inhibitor with the protein.

The phenoxyl ring of R165481 makes extensive hydrophobic interactions with Tyr188 that are similar to those made by the benzyl ring of R157208. The 3′-acrylonitrile substituent on the phenoxyl ring of R165481 extends into a "tunnel" (Figure 3) that leads out of the NNIBP toward the catalytic site, suggesting that structure-based modifications at this location might produce an inhibitor that can interact with the conserved amino acids of the polymerase active site.[35,36] The amino acid residues Val108, Tyr188, Phe227, Leu228, and Trp229 form the walls of this tunnel and interact extensively with the acrylonitrile group (Figure 1, Table S-2). The extensive interactions between the protein and the acrylonitrile substituent contribute to the inhibitor's subnanomolar activity against wild-type RT. These interactions may help to stabilize the position of the inhibitor in the pocket when NNRTI resistance mutations elsewhere in the NNIBP alter the interactions of the protein with the inhibitor (Table 2). An analogous substitution of an acrylonitrile group at the para-position of the wing I anilino ring in the prototype compound TMC120−R147681 (dapivirine, 4-[4-(2,4,6-trimethylphenylamino)pyrimidin-2-ylamino]benzonitrile), in the diarylpyrimidine (DAPY) series of NNRTIs,[37] led to the development of the promising drug candidate R278474 (rilpivirine, 4-[[4-[[4-[(1*E*)-2-cyanoetenyl]-2,6-dimethylphenyl]amino]2-pyrimidinyl]amino]-benzonitrile),[38,39] which is effective against a wide range of NNRTI-resistant HIV-1 strains.

The side chains of Tyr188 and Phe227 interact extensively with the acrylonitrile substituent, and mutations at residues 188 and 227 impair the inhibitory activity of R165481 (Table 2). Although there are hydrophobic contacts with the C$\beta$ atoms of Phe227 and the conserved residue Trp229 (Figure 1; Supporting Information, Table S-2), these are not sufficient for the inhibitor to retain strong inhibitory activity against HIV-1 RTs that carry the Tyr188Leu and Phe227Cys mutations. This suggests that the binding and inhibitory activity of R165481 depends critically on intercalation of the acrylonitrile group between the aromatic rings of Tyr188 and Phe227, as well as on the shape and hydrophobicity of the tunnel linking the NNIBP and the polymerase active site (Figure 3).

Binding of R165481 does not flip the orientation of the Tyr181 side chain, which distinguishes this inhibitor from most NNRTIs, including R157208. In the RT/R165481 complex, the side chain of Tyr181 points away from the polymerase active site, just as observed in the structures of unliganded RT[7] and HEPT-bound RT.[23] However, HEPT is a 17 $\mu$M inhibitor,[23] whereas R165481 is a subnanomolar inhibitor (Table 2). The removal of the bulky (2-methoxyethyl)methylamino substituent from the 3-position of the pyridinone ring eliminates all the significant interactions between R165481 and Tyr181, and a Tyr181Cys mutation does not lead to significant RT resistance against the inhibitor (Table 2).

The wing II component of the inhibitor makes most of its contacts with Leu100, Lys101, and Tyr318. R165481 makes more extensive contacts with the Tyr318 side chain than R157208 and has hydrophobic contacts with the C$\beta$ atom of Leu100 (Figure 1; Supporting Information, Table S-2). Modeling suggests that a Leu100Ile mutation would maintain these contacts and

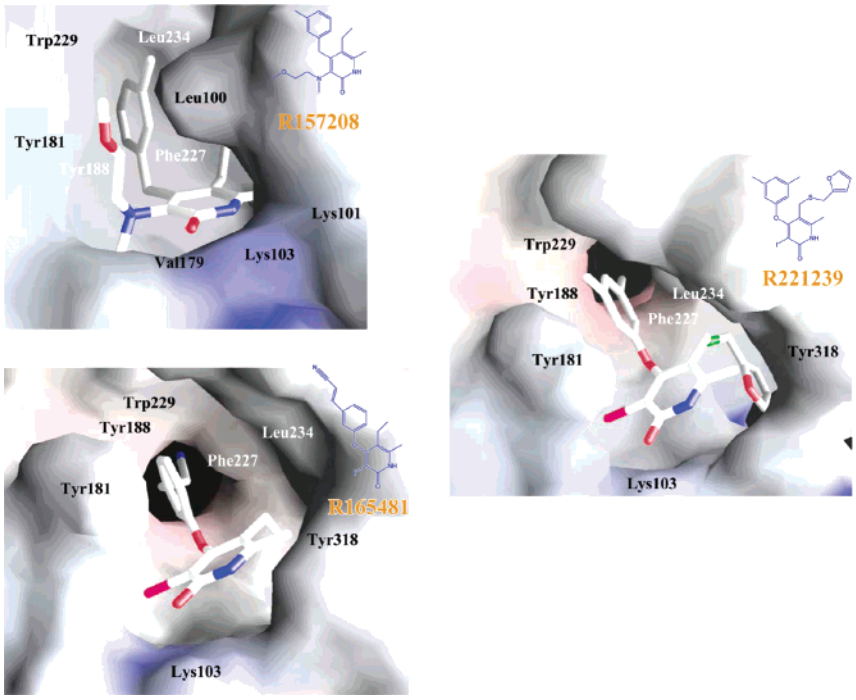



**Figure 3.** Binding modes of R157208, R165481, and R221239 to HIV-1 RT. Electrostatic potential surfaces were computed using the program GRASP.[53] For clarity, Pro95 was omitted in calculating the surface for each structure. For the RT/R165481 and RT/R221239 structures, Gly99, Leu100, and Lys101 were also omitted from the calculations. In the RT/R157208 complex, the (2-methoxyethyl)methylamino substituent aligns itself with the *m*-methylbenzyl group and points toward the hydrophobic core of the NNIBP. In the RT/R221239 complex, one of the methyl groups on the phenoxyl ring points toward a tunnel leading out to the catalytic site, whereas in the RT/R165481 complex the acrylonitrile substituent on the phenoxyl ring partially enters this tunnel and enhances interactions with Trp229 and other residues.

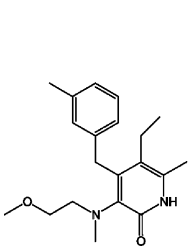
would not cause major steric conflicts with the inhibitor. These observations are consistent with the data that show that a Leu100Ile mutation confers to RT only low-level resistance against R165481 (Table 2). In contrast, the inhibitory activity of delavirdine is impaired by steric hindrance caused by this mutation.[40] A recent structural study suggests that the Leu100Ile mutation causes resistance by steric hindrance. Certain NNRTIs can adapt to the pocket changes in the vicinity of the Leu100Ile mutation. If an NNRTI cannot appropriately alter its binding in response to the Leu100Ile mutation, key inhibitor interactions with Tyr181 and Try188 are reduced.[41] It is likely that acrylonitrile interactions allow R165481 to adjust its position appropriately in response to a Leu100Ile mutation. The hydrogen bond between the Lys101 main-chain carbonyl oxygen and the pyridinone nitrogen should be relatively insensitive to side-chain substitutions, although the Leu100Ile mutation could potentially perturb the geometry of this interaction.[31] R165481 is a better RT inhibitor than R157208 and has more wing I and wing II interactions with the protein; moreover, Tyr181 does not make significant contacts with this R165481, which makes the inhibitor effective against HIV strains carrying the Tyr181Cys mutation.

**R221239 in Complex with RT.** R221239 differs from R165481 in two of its substituents. On the phenoxyl ring, the acrylonitrile substituent is replaced by methyl groups substituted at the 3′- and 5′-positions, and the 5-ethyl substituent on the pyridinone ring is replaced by a furfuryl methyl thioether (Figure 1). As in R165481, the phenoxyl ring of R221239 makes extensive hydrophobic interactions with Tyr188 (Figure 1; Figure 4). In the RT/R221239 complex, like the RT/R165481 complex, the Tyr181 side chain points away from the polymerase active site instead of stacking against the inhibitor, which explains why R221239 is effective against viruses that carry the Tyr181Cys mutation (Table 2).

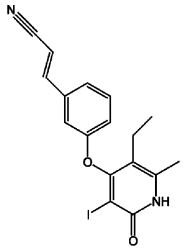
The *m*-methyl groups of the dimethylphenoxyl ring of R221239 present a large surface area for contact with Trp229, similar to what has previously been seen in a structure of RT complexed with GCA-186 (6-(3′,5′-dimethybenzyl)-1-ethoxymethyl-5-isopropyluracil), a HEPT derivative that is an analogue of emivirine.[29] The dimethylphenoxyl ring of R221239, however, makes fewer contacts with Trp229 than the acrylonitrile phenoxyl ring of R165481 (Figure 1; Supporting Information, Tables S-2 and S-3). The position of the dimethyl-substituted phenoxyl ring is partially stabilized by interactions with the terminal methyl groups of Leu100 (Supporting Information, Table S-3). As in the RT/R165481 complex, it is possible to model a Leu100Ile mutation without causing major steric hindrance, and this mutation is observed to have a negligible effect on the activity of R221239 (Table 2). The C9′ methyl group of R221239 also interacts with Pro95, similar to the interaction seen in the RT/R157208 structure (Figure 1; Supporting Information, Tables S-1 and S-3).

SAR data suggest that the furfuryl methyl thioether substantially increases the inhibitory activity of this compound both against wild-type and some NNRTI resistant mutants, compared to derivatives with a variety of alternative substituents (ref 17 and D. S. Grierson, personal communication). The furfuryl methyl thioether is a primary characteristic of R221239, occupying part of the wing II position. The furan ring of this substituent is sandwiched between the Val106/

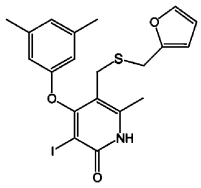
**Table 2.** Structure−Activity Relationships for Three Pyridinone NNRTIs[a]



R157208



R165481



R221239

| | R157208 | R165481 | R221239 |
|---|---|---|---|
| wild-type | 0.001 | 0.0008 | 0.002 |
| Leu100Ile | 0.008 | 0.002 | 0.006 |
| Lys101Glu | 0.016 | nd | 0.006 |
| Lys103Asn | 0.063 | 0.001 | 0.001 |
| Val106Ala | 0.063 | nd | 0.002 |
| Glu138Lys | 0.008 | nd | 0.002 |
| Val179Glu | 0.004 | nd | 0.001 |
| Tyr181Cys | 0.794 | 0.004 | 0.005 |
| Tyr188Leu | 1.000 | 0.200 | 0.032 |
| Gly190Ala | 0.100 | nd | 0.003 |
| Gly190Ser | 0.398 | nd | 0.002 |
| Phe227Cys | 3.16 | 0.200 | 0.039 |
| Leu100Ile + Lys103Asn | 0.794 | 0.008 | 0.032 |
| Lys101Glu + Lys103Asn | 1.000 | nd | 0.004 |
| Lys103Asn + Tyr181Cys | >10 | 0.004 | 0.005 |
| Phe227Leu + Val106Ala | 3.98 | 0.800 | 0.125 |

[a] The $IC_{50}$ ($\mu$M) is shown for each inhibitor complexed with either wild-type RT or RT containing the indicated point mutation(s) (see also refs 17−19) (nd = not done).

Phe227 pair and Pro236. This interaction is similar to the stacking interactions seen in complexes of RT with the HEPT-like compound TNK-651[23] and the BHAP [bis(heteroaryl)piperazine] class of NNRTIs.[40] Unlike the BHAP derivatives, however, the furfuryl methyl thioether makes several additional contacts with Lys103 and Leu234; TNK-651 and R221239 make similar contacts with Lys103.[23] The interaction of R221239 with the main chain of Lys103 (Figure 1; Supporting Information, Table S-3) may help to account for the strong activity of this compound against the Lys103Asn mutant (Table 2). The Pro236Leu mutation induces considerable RT resistance against the BHAP inhibitors.[42] The Pro236Leu mutant may also alter the hydrophobic interactions between the mutated amino acid and R221239. However, we do not yet know how the Pro236Leu mutation affects the inhibitory activity of the pyridinone compounds. Nonetheless, the flexibility of the furfuryl methyl thioether and the additional contacts it makes with RT should make R221239 more effective against both wild-type and mutant RTs than the BHAPs. A Phe227Cys mutation would change the shape of the NNIBP and eliminate the hydrophobic side-chain interactions with the furfuryl methyl thioether and the dimethylphenoxyl ring; this mutation causes a ~20-fold decrease in the inhibitory activity of R221239 (Table 2). A Val106Ala mutation does not affect the activity, probably because this mutation would preserve the C$\beta$ contact with the furan ring, and the interactions of Phe227 with the furfuryl methyl thioether could compensate for the loss of other Val106 side-chain contacts (Figure 1; Supporting Information, Table S-3). However, when the Val106Ala mutation is coupled with a Phe227Leu mutation, the sandwich between this pair of residues on one side and Pro236 on the other side is lost, leading to a 60-fold drop in inhibition (Table 2). Val106 interacts with both the pyridinone ring and the furan ring. It has recently been reported that a Val106Ala mutation can lead to a significant shift in the position of some NNRTIs in the NNIBP, which could affect other protein−inhibitor interactions.[41] Val106 makes more contacts with R221239 than R157208. However, the Val106Ala mutation causes resistance only against R157208 (Table 2) and not to R221239, probably because R157208 is unable to make compensatory interactions such as those between the furfuryl methyl thioether of R221239 and the NNIBP.

The protein−inhibitor interactions involving the furfuryl methyl thioether of R221239 may also help explain why R221239 retains inhibitory activity against viruses carrying the Tyr188Leu mutation (Table 2). When Tyr188 is mutated to a less bulky amino acid, the shape of the NNIBP changes. This results in the repositioning of the inhibitor in the NNIBP, as well as the side chains of nearby amino acid residues, and critical protein−inhibitor interactions can be jeopardized.[30] One way to compensate for the loss of interactions when Tyr188 is mutated is to create additional protein interactions with a flexible extension of the NNRTI that allows the rest of the inhibitor to adjust its position and maintain important contacts with the NNIBP. The furan ring is linked to the pyridinone ring by flexible bridging atoms, so that the furfuryl methyl thioether may serve as an anchor-and-tether that provides additional protein−inhibitor contacts and allows other portions of the inhibitor to form productive contacts with the NNIBP in the absence of interactions with Tyr188.

As in the RT/R165481 complex, the iodine atom in the RT/R221239 structure interacts with several main-chain and side-chain protein atoms (Figures 1 and 4; Supporting Information, Table S-3). In both RT/inhibitor complexes, the carbonyl oxygen of Tyr188 appears to interact with the iodine, but in the higher resolution RT/221239 structure, this carbonyl oxygen is observed to bend slightly toward the iodine, although not enough to abolish a $\beta$-sheet hydrogen bond with the Tyr181 nitrogen. The iodine also has a favorable interaction with the main-chain nitrogen of Gly190 (Figure 4). The importance of iodine interactions is highlighted by the observation that replacement of the iodine with a hydrogen greatly diminishes the activity of the IOPY inhibitors.[17] This position of iodine in the NNIBP produces main-chain interactions and prevents the Tyr181 side chain from interacting with the inhibitor.

**Resistance Mutations and NNRTI Design.** Crystal structures provide information that can be used to

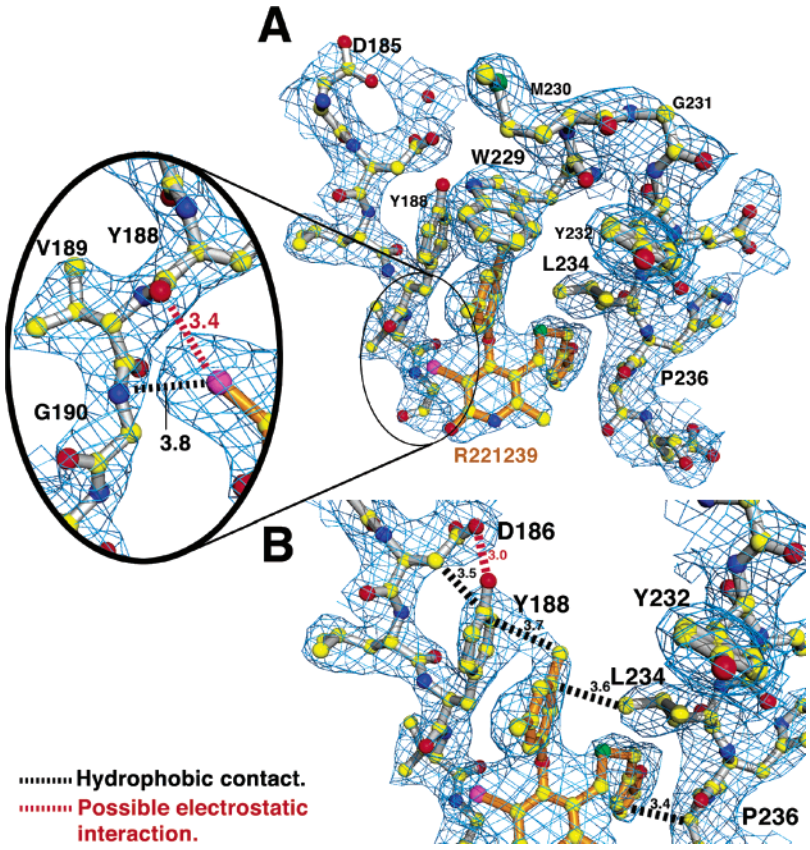



**Figure 4.** Key interactions of R221239 in the NNRTI pocket. The figure was generated using the programs MolScript[54,55] and POV-Ray.[56] (A) The inhibitor R221239 in the NNRTI hydrophobic pocket is superposed on a 2.43 Å resolution $3F_o - 2F_c$ electron density difference map (light blue), contoured at 1$\sigma$. The inhibitor's 3′,5′-dimethylphenoxyl ring interacts with Trp229, which limits motion of the primer grip and prevents the RT thumb from moving in toward the palm and fingers. Left inset: Iodine makes good main-chain contacts with residues Tyr188−Gly190, including a possible electrostatic interaction with the Tyr188 carbonyl. (B) Trp229 has been removed to show the hydrophobic stacking interactions between the protein and the inhibitor extending from the NNIBP to Asp186, part of the active site. A few representative contacts are depicted as dashed lines (for other protein−inhibitor stacking contacts, see Figure 1 and Supporting Information, Table S-3). These interactions may influence the catalytic activity of the enzyme. In addition, the furan ring (lower right) has extensive contacts with Val106 (not shown) and Pro236.

design better inhibitors. However, the NNIBP is flexible and adjusts to accommodate NNRTIs with different structures and shapes, making it difficult to predict accurately how the shape of the binding pocket might change in response to either an inhibitor modification or a resistance mutation. With this in mind, we propose modifications to R221239, based on the current crystal structures, that might lead to greater inhibitory activity against a broader range of mutant RTs.

Several features of the current structures may be useful in developing improved IOPY inhibitors that are more effective against common resistance mutations. Tyr181 does not play a significant role in protein−inhibitor interactions for the binding of R165481 or R221239. This is in sharp contrast to the chemically related HEPT class of NNRTIs, in which the most potent inhibitors interact extensively with Tyr181. As a result, potent HEPT-like inhibitors are sensitive to Tyr181Cys.[29] IOPYs provide an alternative whose inhibitory activity does not depend on interactions with Tyr181. R165481 and R221239 are optimized against the most common NNRTI-resistance mutations, including Tyr181Cys and Lys103Asn (Table 2). The RT/R165481 and RT/R221239 crystal structures suggest that both the acrylonitrile and furfuryl methyl thioether substitutions are advantageous and improve the effectiveness of the iodopyridinone inhibitors against wild-type and several resistant mutant strains. However, R165481 is vulnerable to mutations that affect the interactions of the acrylonitrile group, such as Tyr188Leu and Phe227Cys/Leu. The furfuryl methyl thioether substituent on R221239 may provide a means for enhancing the activity of IOPY derivatives that contain an acrylonitrile substituent. Finally, interactions between RT and the iodine atom of R165481 and R221239 are critical to the activity of those inhibitors and may contribute to their effectiveness against a broad range of drug-resistant RT variants.

The inhibitory activity of R221239 could be improved by replacing one of the methyl substituents of the dimethylphenoxyl ring with an acrylonitrile group. As shown in Figure 3, one of the methyl carbons of R221239 is positioned similarly to C8′ of the acrylonitrile substituent of R165481 (Figure 1), so that this modification would create additional hydrophobic interactions with the protein. Retention of the C9′ methyl group of R221239 (Figure 1) would help the inhibitor make good contacts with Trp229 in the Phe227Cys mutant. Changing the C9′ methyl substituent to an alternative such

as a nitrile group might strengthen its interaction with Trp229.

Since the advantage that the furfuryl methyl thioether imparts to R221239 may be reduced in resistant RT strains containing mutations at residues 106, 227, and 236, chemical modifications to this substituent that would generate additional interactions with main-chain atoms should improve the effectiveness of this inhibitor against RTs carrying these mutations. For example, a hydroxyl on the 16-position of the furan ring (Figure 1) might be able to form a hydrogen bond with the main-chain carbonyl of Lys103. Although 3-hydroxyfurans are intrinsically unstable, the corresponding 3-hydroxyisoxazole analogue could be a viable alternative.

The iodine atom of R221239 is only 3.4 Å away from the Tyr188 carbonyl oxygen, which is less than the van der Waals contact distance of 3.55 Å. Unusual interactions between a halogen and an electronegative atom such as a carbonyl or water oxygen have been observed in other protein structures.[43,44] A recent quantum mechanical study explained hundreds of such examples in the Protein Data Bank (PDB). The results suggested that chlorine, bromine, and especially iodine in small organic molecules have anisotropic surface charge distributions, including significant electropositive patches that make it possible for these halogens to serve as Lewis acids for certain geometries. If the halogen was on an aromatic ring, then electron-withdrawing substituents on that ring were calculated to increase the electropositive surface area on the halogen. The ideal C−X···O angle (where X is a halogen) was predicted to be 165°.[45] In the RT/R221239 structure, this angle is 161.6°. It is therefore likely that the interaction seen between the iodine atom and the Tyr188 carbonyl oxygen in the RT/R221239 structure is electrostatic in nature (Figure 4). Introduction of electron-withdrawing substituents on the pyridinone ring might induce or strengthen an interaction in which the iodine atom serves as an electron acceptor for the Tyr188 carbonyl oxygen. For example, a halogen or nitrile substituent could replace the methyl group at the 6-position opposite to the iodine. It is possible that a nitrile substituent might have the added advantage of enhancing the hydrophobic interaction with the phenyl ring of residue Tyr318. Comprehensive discussion of the synthesis, chemistry, and structure−activity relationships for the 3-iodo-4-aryloxypyridinones and related NNRTIs are available.[17−19]

**Conclusions.** R157208, R165481, and R221239 all bind to HIV-1 RT in a manner which, in many respects, is similar to many other NNRTIs. However, the crystal structures presented here show that R165481 and R221239 have specific interactions with RT not seen in complexes with most other NNRTIs, including those already in clinical use. These novel interactions, some of which involve main-chain or C$\beta$ atoms of the protein, could be useful in the development of NNRTIs that are less susceptible to many of the common drug-resistant RT mutations. The IOPY series of NNRTIs has the advantage that the inhibitory activity does not depend on interaction of the inhibitor with Tyr181, a frequent site of drug-resistance mutations. R165481 and R221239 are not sensitive to Lys100Ile and Lys103Asn mutations. On the basis of observations from the three crystal structures presented here, it should be possible to modify R221239 and increase its effectiveness against a broader range of mutant RTs. Additionally, the acrylonitrile group on R165481 may provide a site for synthesis of a substituent that has access to the polymerase active site and can interact with the conserved amino acids in this region.

## Experimental Section

**Protein Preparation and Purification.** HIV-1 RT was prepared as described.[46] The synthesis of R157208 has been described,[19] and syntheses of R165481 and R221239 will be published elsewhere (D. S. Grierson, personal communication). To form each RT/inhibitor complex, 1.1 $\mu$L of a 20 mM inhibitor stock solution in dimethyl sulfoxide was combined with 0.4 $\mu$L of 20% $\beta$-octyl glucopyranoside and 31.3 $\mu$L of 40 mg/mL RT (in 10 mM Tris pH 8.0, 75 mM NaCl) on ice.

**Crystallization and Data Collection.** RT/R157208, RT/R165481, and RT/R221239 complexes were crystallized by vapor diffusion in microseeded hanging drops as described earlier.[46] Crystals of RT/R157208 and RT/R165481 were cryoprotected in mother liquor containing 25% ethylene glycol and were flash-cooled in a gaseous $N_2$ stream prior to data collection as previously described.[33] An RT/R221239 crystal was cryopreserved in liquid $N_2$ after soaking in mother liquor containing 10 mM $MnCl_2$, 15% PEG 8000, and 25% sucrose. X-ray data for the three RT/NNRTI complexes were collected at a temperature of 100 K at Argonne National Laboratories and the Cornell High Energy Synchrotron Source. The data were processed using HKL-DENZO-SCALEPACK[47] (Table 1).

**Structure Determination and Refinement.** Initial phases were determined by molecular replacement with the program AMoRe[48] using the RT/R100943 structure (PDB accession number 1S6P) as a search model.[33] Cycles of model building and refinement were conducted using the graphics package O[49] and the structure refinement package CNS 1.0[50] with bulk solvent correction. The atomic coordinates and structure factors for the refined structures have been deposited in the Protein Data Bank.

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**Supporting Information Available:** Three tables listing all protein−inhibitor contacts (distances ≤4.0 Å) for the RT/R157208, RT/R165481, and RT/R221239 crystal structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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