Mutational Analysis of Tyr-501 of HIV-1 Reverse Transcriptase

EFFECTS ON RIBONUCLEASE H ACTIVITY AND INHIBITION OF THIS ACTIVITY BY N-ACYLHYDRAZONES*

Received for publication, October 24, 2001
Published, JBC Papers in Press, October 29, 2001, DOI 10.1074/jbc.M110254200

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N-(4-tert-Butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH) is a potent inhibitor of the ribonuclease H (RNase H) activity of human immunodeficiency virus (HIV)-1 reverse transcriptase (RT). Molecular modeling predicted that BBNH binds to the HIV-1 RT RNase H active site via two major interactions, coordination to the metal ion cofactor (Mg²⁺ or Mn²⁺) in the enzyme active site and aromatic ring-stacking interaction between the naphthyl ring of BBNH and amino acid Tyr-501. The latter residue equivalent is conserved in virtually all RNases H, suggesting the need for an aromatic or p-stacking interaction in this region. To assess the importance of Tyr-501 in the binding of BBNH for the inhibition of RT RNase H activity, we used site-specific mutagenesis to generate RT with a variety of substitutions at this position. Most substitutions resulted virtually in a complete loss of RNase H activity. However, three mutants, Y501F, Y501W, and Y501R, possessed RNase H activities comparable with wild-type enzyme. Whereas BBNH inhibited Y501F RT RNase H activity with potency equivalent to wild-type RT, the Y501W mutant showed a 6-fold resistance to inhibition by BBNH, and the Y501R mutant was completely resistant to inhibition by BBNH. The replication “fitness” of HIV molecular clones with the Y501W or Y501R mutation was significantly compromised compared with wild-type virus. Importantly, BBNH was an effective inhibitor of the DNA polymerase activity of all Y501X mutants tested. Our results highlight the importance of Tyr-501 in RT RNase H activity and in N-acylhydrazone inhibitor binding and suggest that drugs that target critical residues in HIV-1 proteins may be a useful approach in new antiviral development.

The conversion of retroviral genomic RNA into the double-stranded viral DNA intermediate is catalyzed entirely by the viral enzyme, reverse transcriptase (RT).¹ To carry out this complex conversion, RT must be multifunctional, possessing RNA-dependent DNA polymerase activity to synthesize DNA complementary to the viral genomic RNA, ribonuclease H (RNase H), activity in order to degrade the RNA component of the resultant RNA-DNA duplex, thereby releasing the newly formed DNA as a template to allow the DNA-dependent DNA polymerase activity to complete the synthesis of double-stranded viral DNA. RT RNase H is also crucial for the strand transfer reactions that are essential for the synthesis of viral DNA. Because of the essential role of HIV-1 RT in virus replication, numerous anti-chemotherapeutic agents have been developed against this enzyme (for recent reviews, see Refs. 1 and 2). Virtually all of these inhibitors are directed against RT DNA polymerase activity with very few inhibitors of RT RNase H so far identified.

The few inhibitors of HIV RT RNase H include illimaquinone (3), azidothymidylate (4, 5), certain naphthalenesulfonic acid derivatives (6), N-ethylmaleimide (7), poly(1-methyl-6-thioinosinic acid) (8), and certain plant or stem-bark extracts (9, 10). None of these is especially potent inhibitors. To date, the only compounds shown to inhibit HIV-1 RT RNase H with reasonable potency are N-acylhydrazones (11) and phenylhydrazones such as PD029687 (12).

We were the first group to describe a reasonably potent inhibitor of HIV-1 RT RNase H activity (11). This inhibitor, N-(4-tert-butylbenzoyl)-2-hydroxynaphthaldehyde hydrazone (BBNH, Fig. 1), is one of a family of metal-binding hydrazones, which were developed primarily as antitumor agents and antioxidants (13). BBNH inhibits both the HIV-1 RT DNA polymerase and RNase H domains (11). BBNH also inhibits the RNase H activity of HIV-2 RT, Moloney murine leukemia virus RT, and Escherichia coli RNase HI (11). We have suggested that this multifunctional inhibition is the result of the binding of two molecules of BBNH/molecule of HIV-1 RT with one molecule interacting close to the non-nucleoside RT inhibitor-binding pocket (NNIBP) in the polymerase domain of the enzyme and the second molecule binding in the spatially distinct RNase H domain of the enzyme (11).

Molecular modeling suggested that BBNH binds to the RT RNase H domain close to the active site, and that residue Tyr-501 is integral in the binding interaction. We therefore altered Tyr-501 by site-directed mutagenesis to better define this potential interaction. Our results indicate that this residue appears to be essential for RT RNase H activity, because most substitutions abrogate enzyme activity. The Y501F, Y501W, and Y501R mutants retained appreciable RNase H activity, and only the Y501F mutant remained sensitive to BBNH inhibition.
bition, an observation consistent with the molecular model for BBNH binding to the RNase H domain. Importantly, the introduction of the Y501W and Y501R mutations in molecular clones of HIV-1 resulted in virus that was significantly attenuated in replication fitness.

**EXPERIMENTAL PROCEDURES**

**Materials**—BBNH and N-(4-tert-butylbenzoyl)-2-hydroxy-1-salicylyl-hydrazone (BBSH) were synthesized by standard methods (14). 1H NMR and elemental analyses of these compounds were entirely consistent with the expected chemical structures. [3H]dATP, [3H]dTTP, [3H]dGTP, [3H]dCTP, [3H]dITP, and the homopolymeric template/primer poly(rA)-oligo(dT)12-18 were purchased from Amersham Biosciences, Inc. All other reagents were of the highest quality available and were used without further purification.

**Molecular Modeling**—A model for the interaction of BBNH with HIV-1 RT was constructed using the x-ray crystallographic coordinates for HIV-1 RT complexed with nevirapine (15) (Protein Data Bank accession number 3HTV). This structure was chosen because of the presence of magnesium atoms bound in the RNase H active site. The initial conformation of BBNH was derived from its x-ray crystal structure (16). Docking and energy minimization experiments were carried out with the anneal function of Sybyl 6.5 (Tripos Inc., St Louis, MO). Charges were calculated by the Gasteiger-Huckel method, and iterative minimization was carried out with the Tripos force field until the energy difference between iterations was <0.01 kcal/mol.

**Cloning, Site-directed Mutagenesis, and Purification of WT and Tyr-501 Mutant HIV-1 RT**—The p66- and p51-kDa subunits of HIV-1 RT were cloned into a pBAD/HisB prokaryotic expression vector (InvitroGen) between the Hinfl and HindIII restriction endonuclease cleavage sites. Mutations at position 501 were introduced into the gene encoding the RNase H domain and BBNH (dotted surface). B, other potential hydrogen bond interactions between residues in the HIV-1 RT RNase H domain and BBNH.

**Assay of RT-catalyzed RNA-dependent DNA Polymerase Activity**—The specific activities of WT and mutant RT were determined by measuring the RNA-dependent DNA polymerase activity of each enzyme using 0.2 units/ml of poly(rA)-oligo(dT)12-18, 20 μM [3H]dTTP, and 8.5 mM purified RT p66/p51 heterodimer in 50 mM Tris-HCl, pH 7.9, 60 mM KCl, 10 mM MgCl2. Samples were incubated for 10 min at 37°C and then quenched by the addition of ice-cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. After a 20-min incubation on ice, samples were filtered using a 1.2-μm glass fiber type C filter multi-well plate (Millipore) and washed sequentially with 10% trichloroacetic acid and ethanol. The extent of radionucleotide incorporation was then determined by liquid scintillation spectrometry. Assays for the inhibition of RT DNA polymerase activity by BBNH were carried out as described previously (11).

More detailed inhibition kinetic analysis of RT RNA-dependent DNA polymerase activity employed a heteropolymeric template/primer prepared as previously described (18) using the T7 polymerase RNA transcript from AccI-linearized plasmid pHIV-PBS as template and a synthetic 18-nucleotide deoxyoligonucleotide as DNA synthesis initiation primer. Reaction assays comprised 0.5 units/ml of heteropolymeric template/primer, variable concentrations of a mixture of equimolar [3H]dATP, [3H]dCTP, [3H]dTTP, [3H]dGTP, [3H]dITP (ranging from 1–20 μM total dNTP concentration), and 10 mM purified RT p66/p51 heterodimer in 50 mM Tris-HCl, pH 7.9, 60 mM KCl, 10 mM MgCl2. Samples were incubated and processed as described above. Inhibition data were analyzed by Dixon plot analysis.

**Assay of RT-catalyzed RNase H Activity**—RNase H activity was assayed using an 18-nucleotide 5′-32P-heteropolymeric RNA template (5′-GAUCUGAGCUUGGGAGCU-3′) annealed to a complementary 18-nucleotide DNA oligomer (5′-AGCTCCAGGCTAGCAGTAC-3′). 14 mM 5′-32P-RNA-DNA duplex was incubated with 5 nM RT heterodimer in 50 mM Tris-HCl, pH 7.9, 60 mM KCl, 2.5 mM MgCl2 in 10 μl of final volume. Samples were incubated for 10 min at 37°C and then quenched with an equal volume of gel loading dye (98% deionized formamide, 10 mM EDTA, 1 mg/ml bromphenol blue, and 1 mg/ml xylene cyanol). RNase H-generated degradation products were resolved by electrophoresis using 14% acrylamide/7 M urea sequencing gels visualized by autoradiography and quantitated by densitometry.
RESULTS

Molecular Model for the Interaction of BBNH with HIV-1 RT RNase H—The model for the binding of BBNH to the RNase H domain of HIV-1 RT is shown in Fig. 2. Crystallographic analysis shows BBNH to be a flat planar molecule (16). Our docking studies indicate a good fit between the inhibitor and a region close to the RNase H active site that is open and accessible to ligand binding (Fig. 2A). Residues within 4 Å of the bound inhibitor include Asn-474, Gln-475, Arg-448, Glu-478, Ser-499, His-539, and Tyr-501 (Fig. 2B).

Two major binding determinants were identified. The first involves the coordination of one of the hydrazone-linkage nitrogen atoms and the hydrazone carbonyl oxygen to the RNase H active site metal. This interaction is consistent with our model BBSH, was an exceptionally weak inhibitor of RT RNase H activity (activity (11)). Nevertheless, BBSH retained significant inhibitory potency against RT DNA polymerase activity.

Characterization of HIV-1 RT Y501X Substitution Mutants—To test the interaction between Tyr-501 and BBNH, we generated a number of mutant enzymes with various substitutions at position 501, including Y501F, Y501W, Y501R, Y501E, Y501Q, Y501G, Y501A, Y501L, Y501S, and Y501H. Most of these mutant enzymes had significantly impaired RNase H activity (Fig. 3, Table II), and some also showed attenuated inhibitor activity (11).

Inhibition parameters for RNase H activity of Y501X mutant HIV-1 RT

| Enzyme | Relative activity | % wild-type enzyme |
|--------|------------------|-------------------|
| RDDP   |                  |                   |
| Y501F  | 104 ± 28         | 85                |
| Y501W  | 85 ± 15          | 100               |
| Y501R  | 105 ± 18         | 90                |
| Y501Q  | 135 ± 18         | inactive          |
| Y501G  | 65 ± 9           | inactive          |
| Y501L  | 56 ± 11          | inactive          |
| Y501S  | 45 ± 6           | inactive          |
| Y501E  | 17 ± 6           | 25                |
| Y501A  | <5               | inactive          |
| Y501H  | <5               | inactive          |

*9 = The specific activity of wild-type p66/p51 RT was 460 pmol/min/μg determined with poly(rA)·oligo(dT)12-18 as template/primer as described under "Experimental Procedures."
Experimental Procedures.

Interestingly, the DNA polymerase activity of all Y501 mutant enzyme was completely resistant to BBNH inhibition. The molecular clones of HIV-1 with various substitutions at RT residue 501 were constructed and examined for their ability to infect and replicate in lymphoblastoid MT2 cells. Only the Y501F and Y501W mutant viruses were able to replicate as the WT virus despite binding only to the NNIBP (21).

Molecular modeling showed that BBNH can be readily docked into the RT RNAse H domain in a manner consistent with previously reported data (Fig. 2A). For example, a major interaction involves the coordination of the metal-chelating moieties of BBNH with the active site metal in the RNAse H domain. This is consistent with the metal dependence for BBNH inhibition of RT RNAse H activity as well as the competitive nature of this inhibition (11). Despite considerable effort, very few potent small molecule inhibitors of HIV-1 RT RNAse H have been identified. This may partly be attributed to the fact that the RNAse H active site has a rather open structure (as might be expected for an active site that must accommodate the large RNA-DNA duplex substrate), and initially it appears that the region possesses insufficient potential contacts to allow for reasonable binding interactions with small molecules. However, BBNH is a flat molecule with an extended planar structure (16) and possesses metal-binding properties that allow interaction with the active site metal. Interestingly, the only other potent inhibitor of HIV-1 RT RNase H, the phenylhydrazone PD029687, was recently shown to also be capable of binding to a site at or near the NNIBP in the polymerase domain of RT resulting in the inhibition of the DNA polymerase activity of the enzyme, whereas binding to the second site in the RNAse H domain provides inhibition of the RNAse H activity of the enzyme. However, it was still possible, although unlikely, that the inhibition of both activities might arise from single site binding of the inhibitor. Indeed, certain non-nucleoside reverse transcriptase inhibitors have been shown to affect RT RNAse H activity despite binding only to the NNIBP (21).

重要性与 Tyr-501 在活性和抑制 HIV-1 RT RNAse H

### 表 III

BBNH 抑制的 RDDP 和 RNAse H 活性针对 Y501X 突变体的 HIV-1 RT

| Enzyme     | IC<sub>50</sub> μM | K<sub>i</sub> μM |
|------------|-------------------|-----------------|
| WT         | 0.9 ± 0.1         | 2.2 ± 0.5       |
| Y501F      | 1.1 ± 0.2         | 2.0 ± 0.5       |
| Y501W      | 2.3 ± 0.1         | 10.0 ± 1.3      |
| Y501R      | 1.3 ± 0.1         | 4.4 ± 0.3       |
| Y501Q      | 1.2 ± 0.2         | n.d.            |
| Y501L      | 1.1 ± 0.3         | n.d.            |

a Values are the means ± S.D. from three independent experiments, each carried out in duplicate. IC<sub>50</sub> determinations were carried out using poly(rA)·oligo(dT)<sub>12-18</sub> as template/primer, and K<sub>i</sub> determinations were conducted using a heteropolymeric template/primer as described under "Experimental Procedures."

b Values are the means ± S.D. from three independent experiments, each carried out in duplicate.

### 表 IV

HIV-1 模分子克隆中的突变体 RT Tyr-501 突变体活性

| Mutant        | TCID<sub>50</sub>/ng p24 | Infectivity % wild-type |
|---------------|---------------------------|-------------------------|
| Wild-type (RT Y501) | 830 ± 50                  | 100                     |
| RT Y501F      | 580 ± 65                  | 70                      |
| RT Y501W      | 165 ± 30                  | 20                      |
| RT Y501R      | -                         | n.a.                    |
| RT Y501Q      | -                         | n.a.                    |

a Values are means ± S.D. from six determinations.

DISCUSSION

We previously suggested that the ability of BBNH to inhibit multiple RT activities might be because of the binding of the inhibitor to two different sites on the enzyme based on biochemical and kinetic data (11). Our hypothesis was that binding interactions between the phenol ring of Tyr-501 and the...
second ring of the naphthyl moiety of BBNH is important for the non-metal binding interactions of the inhibitor with the RT RNase H active site. The observation that BBSH, a BBNH analog with a reduced ring system, is unable to inhibit RT RNase H is also consistent with this model. Furthermore, BBSH remains a potent inhibitor of RT DNA polymerase activity, implying that this analog can still bind to the RT polymerase domain presumably at or near the NNIBP. This observation provides further evidence that the inhibition of multiple RT activities by BBNH arises from the binding of the inhibitor to multiple sites on RT.

Mutational analyses of Tyr-501 also support the prediction that this residue is crucial for the binding of BBNH. In the absence of bound nucleic acid duplex substrate, Tyr-501 is completely exposed to solvent, and the phenol ring is free to “flip” around its C$_p$–C$_s$ bond. This conformational flexibility enables the phenol side chain of Tyr-501 to readily assume an orientation to interact with the naphthyl ring system of BBNH, thereby providing the crucial stacking interaction. The Y501F substitution would not affect the side chain conformational flexibility. Thus, the RNase H activity of Y501F RT is fully sensitive to BBNH inhibition. The increased side chain bulk of the Y501W mutant could decrease the rotational freedom about the C$_p$–C$_s$ bond. This decreased mobility would restrict the side chain from adopting the appropriate orientation to facilitate BBNH binding, consistent with the decreased sensitivity of the Y501W mutant to BBNH inhibition. Whereas arginine is capable of π-bonding interactions, the size and non-planar nature of the Y501R mutation is such that interactions with BBNH would be difficult. Indeed, the RNase H activity of the Y501R mutant is completely insensitive to BBNH inhibition. Importantly, the DNA polymerase activity of the Y501F, Y501W, and Y501R mutants remains fully sensitive to inhibition by BBNH, additional evidence that the inhibition of multiple RT activities of WT enzyme arises from multiple site binding of the inhibitor.

Our mutational analyses of Tyr-501 also provided the surprising observation that most substitutions at this position resulted in significant losses of RNase H activity, suggesting an essential role for Tyr-501 in RT RNase H activity. In this context, it is interesting to note that virtually all RNases H for which sequence information is available have a tyrosine residue in the position corresponding to that of Tyr-501 in the HIV-1 RT RNase H domain (22, 23). The functional role of Tyr-501 is unclear at present, however, it is interesting to note that each of the amino acid substitutions at Tyr-501, which allowed retention of enzymatic activity, namely phenylalanine, tryptophan, and arginine, are capable of participating in π-stacking interactions. One possibility is that Tyr-501 participates in stacking interactions with base moieties of the RNA-DNA nucleic acid duplex substrate, thereby assisting in positioning the substrate in the RNase H active site. Indeed, crystal structures of the binary and ternary complexes of HIV-1 RT bound with double-stranded DNA suggest that Tyr-501 contacts the bound nucleic acid duplex (24, 25). However, further structural information is needed to clarify the role of Tyr-501 in RT RNase H and the equivalent residue in other RNases H.

Although the Y501F, Y501W, and Y501R mutant RT retained WT levels of DNA polymerase and RNase H activity in in vitro assays, molecular clones of HIV-1 containing these same mutations showed varying decreases in viral replication fitness. Whereas the Y501F mutant virus was only slightly decreased in replication efficiency, the Y501W mutant virus showed a 5-fold decrease in replication compared with WT virus, and the Y501R virus was unable to replicate. These observations provide additional evidence of the essential nature of Tyr-501 and also suggest that Tyr-501 may be “immutable” in the context of viral fitness. The development of antiviral drug resistance is a major problem in the therapy of HIV infection, and it may be advantageous to identify drugs that “target” essential protein residues such as Tyr-501. Although it is certainly likely that HIV-1 will develop resistance to such compounds, the development of this resistance may be delayed relative to some other therapeutics, thereby providing prolonged clinical utility.

Whereas BBNH is one of the few relatively potent inhibitors of HIV-1 RT RNase H so far identified, its inhibitory potency is significantly less than other antivirals in clinical use, and as such it should be considered mainly as an interesting lead compound for the development of more potent RNase H inhibitors. Our molecular model suggests that the bound BBNH may be within contact distance of several other RT residues, namely Arg-448, Asn-474, Gln-475, Ser-499, and His-539 (Fig. 2B). BBNH analogs that possess structural moieties, which can take advantage of these potential additional contacts, might demonstrate significant improvements in inhibitory potency. Such studies are underway in our laboratory.
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J. Biol. Chem. 2002, 277:1370-1374.
doi: 10.1074/jbc.M110254200 originally published online October 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110254200

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