Mutational Analysis of Tyr-318 within the Non-nucleoside Reverse Transcriptase Inhibitor Binding Pocket of Human Immunodeficiency Virus Type I Reverse Transcriptase*

Heidi Pelemans, Robert M. Esnouf‡, Heidi Jonckheere, Erik De Clercq, and Jan Balzarini§

From the Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

The highly conserved Tyr-318 is part of the non-nucleoside reverse transcriptase inhibitor (NNRTI)-specific lipophilic pocket of human immunodeficiency virus type I reverse transcriptase (RT) and makes contact within 4 Å with the NNRTIs in all reported RT/NNRTI complexes. Using site-directed mutagenesis, six mutant RTs were constructed bearing the mutations Y318H, Y318R, Y318L, Y318C, Y318W, and Y318F. We found that only the Y318W and Y318F mutant RTs retained substantial RT activity, whereas the catalytic activities of the Y318K, Y318C, Y318H, and Y318L RT mutants were less than 5% of the wild-type activity. The Y318F mutant RT retained substantial sensitivity to the majority of NNRTIs tested, whereas the Y318W mutant RT showed varying degrees of resistance to NNRTIs. Subunit-specific site-directed mutagenesis revealed that there was no difference in the catalytic activity or resistance/sensitivity spectrum toward NNRTIs regardless of whether the Tyr-318 mutation was introduced in both subunits or only in the p66 subunit of RT. Recombinant viruses harboring the Y318F or Y318W mutation in the RT showed a similar resistance/sensitivity pattern to NNRTIs as their corresponding 318 mutant recombinant RTs. Our findings stress a functional or structural role for Tyr-318 in wild-type RT and argue for the design of novel NNRTIs that interact more closely with this amino acid in the NNRTI-specific pocket of human immunodeficiency virus type I RT.

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) form a large and chemically diverse group of compounds. Although the NNRTIs are very potent and selective inhibitors of human immunodeficiency virus type I (HIV-1) with low toxicity, their use for anti-AIDS therapy is severely limited by the resistance spectrum toward NNRTIs regardless of whether the NNRTIs bind in a common internal lipophilic pocket within the HIV-I RT and cause inhibition of the enzyme activity by distortion of the polymerase active site (4). Virtually all mutations conferring resistance to NNRTIs (for an overview, see Ref. 5) correspond to amino acid residues lining this internal pocket (Fig. 1). Mutations have been reported for almost all of the pocket-lining residues, with the only exceptions being Leu-234 and Tyr-318.

Tyr-318 makes contact (within 4 Å, mainly involving the Cε, Cη, and Oη atoms) with the NNRTIs in all reported RT/NNRTI complexes, and the most extensive contacts are with delavirdine (BHAP U-90152; Fig. 2; Ref. 3). This implies that mutations at Tyr-318 might confer resistance to NNRTIs. When searching for mutations in RTs, sequencing is generally limited to the first 750 base pairs of the RT gene corresponding to residues 1–250 of the RT. Thus, it cannot be excluded that Tyr-318 mutant RTs may have been selected under NNRTI pressure but may not have been properly identified.

To examine the potential role of Tyr-318 in resistance development and HIV-I RT activity, Tyr-318 mutant RTs were created by site-directed mutagenesis and evaluated for both catalytic activity and resistance/sensitivity toward a variety of NNRTIs including delavirdine, a potent NNRTI for which structural studies have suggested a significant interaction with Tyr-318 (Fig. 1; Ref. 3). We have also constructed a number of recombinant viruses harboring mutations at position 318 in their RT gene and examined their replication competence and resistance/sensitivity spectrum toward NNRTIs.

MATERIALS AND METHODS

Test Compounds—[2′,5′-Bis-O-(tert-butylidimethylsilyl)-β-D-ribofuranosyl]-3′-sulpho-5′-(4′-amino-1′,2′-oxathiole-2′,2′-dioxide) derivatives of N9-methylthymine (TSNO-m9T) were obtained from Dr. M.-J. Camaras (Consejo Superior de Investigaciones Cientificas, Madrid, Spain). Nevirapine (Boehringer Ingelheim, Ridgefield, CT), Tivirapine (8-chloro-TB01) and loviride (α-anilino phenyl acetamide; R9439) were provided by Dr. P. Ganong (Boehringer Ingelheim, Ridgefield, CT). Tivantoin (8-chloro-TB01) and loviride (α-anilino phenyl acetamide; R9439) were provided by Dr. R. Andries (Janssen Pharmaceutica, Beerse, Belgium). Bisheteroarylpiperazine U-90152 (delavirdine) and quinoxaline HBY 097 (S)-4-isoproxyoxycarbonyl-6-methoxy-3-(methylmethyl)3,4-dihydrohydroquinoline-2(1H)-thione were provided by Dr. R. Kirsch (Hoechst AG, Frankfurt, Germany). The 1-[2-hydroxyethoxy]methyl-6-(phenylthio)thymine derivative MKC-442 was kindly provided by Dr. M. Baba (Fukushima Medical College, Fukushima, Japan). The thioxo-carboxanolide derivative UC-781 (N-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furan-carboxoamide) was obtained from Unichemical Limited (Middlebury, CT; Guelph, Ontario, Canada). Zidovudine (AZT), 2′,3′-dideoxyguanosine, and 2′,3′-dideoxyinosine-5′-triphosphate (ddGTP) were from Sigma (St. Louis, MO). AZT-TP and dTTP-TP were provided by Dr. P. Herdewijn (Rega Institute, Leuven, Belgium).

Cells—CEM cells were obtained from the American Type Culture Collection (Rockville, MD). MT4 cells were provided by Dr. N. Yamamoto (Tokyo Medical School and Dental University School of Medicine, Tokyo, Japan).

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‡ A fellow of the “Onderzoeksfonds” of the Katholieke Universiteit Leuven, Belgium.
§ To whom correspondence should be addressed: Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium. E-mail: Jan.Balzarini@rega.kuleuven.ac.be.

1 The abbreviations used are: NNRTI, non-nucleoside reverse transcriptase inhibitor; RT, reverse transcriptase; HIV, human immunodeficiency virus; TSAO-m3T, 3-methylthymine; TIBO, tetrahydroimidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione; AZT, zidovudine; TP, triphosphate.
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Activity Assay for the Various Test Compounds against Wild-type and Mutant Recombinant HIV-I Strains in CEM Cell Cultures—CEM cells were suspended at approximately 200,000 cells/ml culture medium and infected with wild-type recombinant HIV-I or position 318 mutated recombinant HIV-I strains. 100 μl of the infected cell suspensions were then added to 200-μl microtiter plate wells containing 100 μl of an appropriate dilution of the test compounds. After 4 days of incubation at 37 °C, the cell cultures were microscopically examined for syncytium formation site were used. The two primers, each of which was complementary to opposite strands of the vector, were extended during temperature cycling by means of Pfu DNA polymerase, leading to a mutated plasmid template and to select for mutation-containing synthesized DNA. The nicked vector DNA containing the desired mutations was then transformed into Escherichia coli XL1-Blue. The presence of the desired mutations was determined by restriction with DraIII and confirmed by sequencing the complete RT gene on an ABI Prism 310 sequencer (Perkin Elmer) using the dRhodamine terminator cycle sequencing ready reaction kit (Perkin Elmer).

Expression and Purification of Mutant Recombinant HIV-I RT—For the purpose of easy purification, a (His)_6 tag was introduced at the amino terminus of the p66 subunit of the RT. An EcoRI-NcoI fragment located just before the RT coding region of pKRT2 was amplified by polymerase chain reaction with ULM00 DNA polymerase and primers HJ20 (5’-GGATCCGGAATTCTCATGTTTGACAGC-3’) and HJ22 (5’-CTGATCCATGGCGTGATGGTGATGGAGCATGGTCTGTTTCTCTGTTGTTG-3’). In this way, the NcoI restriction site containing the start codon of the RT sequence was altered, and a new NcoI site was created after the (His)_6 residues, which were in frame with the RT gene. The polymerase chain reaction product was digested with EcoRI and NcoI and ligated into pKRT2 digested with EcoRI and NcoI to create pKRT2His. The Tyr-318 mutations created in pKRT2 were cloned into pKRT2His by exchanging the NcoI-HindIII fragment that contained the RT gene.

Recombinant HIV-I RT enzymes were expressed from a two-plasmid co-expression system, which was described earlier by Jonckheere et al. (7). The p66 subunit of RT is expressed from pACYC66His, and the p51 subunit is expressed from pKRT51. To construct wild-type or position 318 mutated pKRT2His, wild-type or position 318 mutated pACYC66His, and EcoRI, and the RT-containing fragment was ligated into pKRT2His digested with EcoRI and NcoI to create pKRT2His. The Tyr-318 mutations created in pKRT2 were cloned into pKRT2His by exchanging the NcoI-HindIII fragment that contained the RT gene.

Site-directed Mutagenesis of HIV-I RT—All mutant enzymes used in this study were derived from the RT sequence cloned in pKRT2 (6).

Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Westburg, Leusden, Netherlands). Briefly, supercoiled double-stranded pKRT2 DNA and two synthetic oligonucleotide primers containing the desired mutation at position 318 and a silent mutation that creates a unique DraIII restriction site were used. The two primers, each of which was complementary to opposite strands of the vector, were extended during temperature cycling by means of Pfu DNA polymerase, leading to a mutated plasmid containing staggered nicks. After temperature cycling, the product was treated with DpnI. The DpnI endonuclease is specific for methylated and hemihemethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA containing the desired mutations was then transformed into Escherichia coli XL1-Blue. The presence of the desired mutations was determined by restriction with DraIII and confirmed by sequencing the complete RT gene on an ABI Prism 310 sequencer (Perkin Elmer) using the dRhodamine terminator cycle sequencing ready reaction kit (Perkin Elmer).

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20 ml of wash buffer (50 mM sodium phosphate buffer, 0.5 mM NaCl, 25 mM imidazole, 1% 2-mercaptoethanol, and 10% glycerol). The RT was eluted with 10 ml of the phosphate buffer containing 125 mM imidazole. The imidazole-containing buffer was exchanged by the heparin buffer (20 mM Tris-HCl, pH 7.8, 0.05 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol, and 10% glycerol), and the eluate was concentrated to 2 ml using Ultrafree-15 centrifugal filtration devices (Millipore, Brussels, Belgium). The (His)6-tagged RT was further purified to about 90% purity over a heparin column (Hitrap Heparin; Pharmacia Amersham). After the binding of the RT to the heparin column, elution was accomplished by a linear salt gradient of 0.05–1 M NaCl. Heterodimer RT eluted at approximately 0.3 M NaCl, as determined by SDS-polyacrylamide gel electrophoresis of the eluted peak fractions. All fractions containing heterodimeric RT were pooled and stored in a 50% glycerol buffer at –20 °C. Protein concentrations in the stock solutions were determined with the Bio-Rad Protein Assay using bovine serum albumin (Bio-Rad) as a standard.

Preparation of E. coli Extracts—25 ml of LB containing 100 μg/ml ampicillin were inoculated with an overnight culture of E. coli XL1-Blue transformed with wild-type or position 318 mutated pRT2His at an A600 of 0.1. The culture was grown at 37 °C, induced with isopropyl-β-D-thiogalactopyranoside for the expression of the p66 form of RT, and stored as described in the previous section. The cell pellet was resuspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 5 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 1 mg/ml lysozyme, and 10% glycerol) and sonicated. The lysate was centrifuged (12,000 rpm, 20 min), and the supernatant was stored at –80 °C in aliquots of 100 μl.

RT Assay—For determination of the IC50 of the test compounds, the RT assay was performed as described previously (8). A fixed concentration of the labeled substrate [2,8-3H]dGTP (specific radioactivity, 3.6 Ci/mmol), or [methyl-3H]dTTP (specific radioactivity, 51 Ci/mmol; 5.6 Ci/mg; 1 μCi) or [methyl-3H]dTTP (specific radioactivity, 51 Ci/mmol; 5.6 Ci/mg; 1 μCi) was used. A fixed concentration of the template DNA (20 mM oligo(dG)12–18 (0.1 mM), poly(rA)oligo(dG) template, or 486 pmol of [3H]dGTP as the template/primer, or 0.1 mM poly(rC)oligo(dG) template) was used. The IC50 of the test compounds was determined as the compound concentration that inhibited recombinant RT activity by 50%.

Recombinant Virus Assay—Recombinant viruses were obtained as described by Kellam and Larder (9). Briefly, recombinant viruses were obtained through homologous recombination of RT with RT-deleted proviral DNA that was propagated in pHVARTBstEI DEII. The RT used in the recombination was the NcoI-HindIII fragment from pKRT2. MT2 cells were electroporated with –2 μg of RT DNA and –10 μg of pHIVARTBstEI DNA. Upon successful homologous recombination, viable recombinant virus could be recovered from the cell culture. The presence of the Tyr-318 mutations was verified by sequencing of the virus DNA samples in an automated laser fluorescent DNA sequencer using the Automated T7 Sequencing kit from Pharmacia as described previously (10, 11).

RESULTS

RNA and DNA-dependent DNA Polymerase Activities of HIV-1 RTs Mutated at Position 318—Using site-directed mutagenesis, we constructed six recombinant RTs replacing the wild-type amino acid residue Tyr-318 with Phe, Trp, His, Leu, Cys, and Lys. Phe, Trp, and His have aromatic side chains, but Cys, and Lys. Phe, Trp, and His have aromatic side chains, but Cys has a small polar side chain. Phe is a neutral residue, whereas His is a positively charged function. The analysis of the RNA-dependent DNA polymerase activity revealed severely impaired RNA-dependent DNA polymerase activities for four of the six recombinant RTs mutated at position 318 (Fig. 3). Indeed, the Y318H, Y318K, Y318L, and Y318C mutant RTs displayed enzymatic activities ranging from 1.6 to 4.4% of wild-type RT. The Y318W mutant RT retained 73% of wild-type polymerase activity, whereas the Y318F mutant retained wild-type activity.

The DNA-dependent DNA polymerase activities showed a pattern similar to that of the RNA-dependent DNA polymerase activities; the Y318F and Y318W mutant RTs retained substantial DNA-dependent DNA polymerase activity (54 and 95% of the wild-type activity, respectively), whereas the Y318H, Y318K, Y318L, and Y318C mutant RTs showed severely impaired DNA-dependent DNA polymerase activities (Fig. 3).

Inhibitory Activities of NNRTIs and Nucleoside RT Inhibitors against Wild-type, Y318F, and Y318W Mutant Recombinant HIV-I RTs—The two most active mutant RTs, Y318F and Y318W, were evaluated for their sensitivities to a variety of NNRTIs, to ddGTP (with [3H]dGTP as the radiolabeled substrate and poly(rC)oligo(dG) as the template/primer), and to AZT-TP and d4T-TP (with [3H]dTTP as the radiolabeled substrate and poly(rA)oligo(dT) as the template/primer) (Table I). The Y318F mutant RT retained substantial sensitivity to all of the NNRTIs tested. Of these NNRTIs, quinoxaline HBY 097 and UC-781 were the most inhibitory to both the wild-type and the Y318F mutant. MKC-442 and delavirdine were 6- to 9-fold less active against the Y318F mutant than against the wild-type enzyme (Table I). The nucleoside RT inhibitors ddGTP, AZT-TP, and d4T-TP retained full inhibitory activity against both the Y318F and Y318W mutant RTs. In contrast to the Y318F RT, the Y318W mutant RT showed a high level of resistance against tivirapine (73-fold) and loviride (35-fold). The thiocarboxanilide derivative UC-781, nevirapine, and MKC-442 were 6- to 16-fold less inhibitory against the Y318W mutant RT than against the wild-type RT. Quinoxaline HBY 097, TS-602-m7, and delavirdine retained full inhibitory activity against Y318W RT (Table I).

The Role of the p51 Subunit of RT in the Eventual Activity of the NNRTIs against the Mutated Recombinant HIV-I RTs—In the two-plasmid co-expression system, the sequence of the p66 subunit of RT is cloned into pACYC184, whereas the sequence of the smaller p51 subunit is located on pKRT51 (7). To investigate whether Tyr-318 in the p51 subunit has a role in resistance development, the position 318 mutations were introduced only in the p66 subunit of the p66/p51 heterodimer RT by cloning the MstI-EcoRI fragment of the position 318 mutated pKRT2His in pACYC184, whereas the p51 subunit was expressed by the pKRT51 plasmid containing wild-type Tyr-318. The catalytic activities of all single-mutated p66/p51 RTs (containing the mutations solely in p66) and the double-mutated p66/p66 RTs (containing the mutations in both p66 subunits) were then determined (data not shown). The same pattern of polymerase activity was seen for the double-mutated p66/p66
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Poly(rA)z oligo(dT) contain the mutations in both subunits of the enzyme. The recombinant RTs used correspond to the p66/p66 homodimer and E. coli using recovered from the transfected cell cultures. Several attempts yielded the Y318W and the Y318F mutant recombinant viruses could be recovered from the transfected cell cultures. Interestingly, only the viruses harboring the Y318W, Y318F, Y318H, Y318L, Y318C, or Y318K mutations in their RT gene. An attempt was made to construct recombinant viruses harboring the Y318W, Y318F, Y318H, Y318L, Y318C, or Y318K mutations in their RT gene. Interestingly, only the Y318W and the Y318F mutant recombinant viruses could be recovered from the transfected cell cultures. Several attempts to construct viable recombinant viruses that contained Y318H, Y318C, Y318L, or Y318K mutant RT failed.

The resistance profile of the Y318W and Y318F mutant recombinant viruses was determined against the same variety of NNRTIs as used above for the Y318W and Y318F mutant recombinant RTs (Table II). For the Y318W recombinant virus, the highest levels of resistance were noted for nevirapine (54-fold), lovivride (26-fold), MKC-442 (20-fold), and tivirapine (18-fold). UC-781 and HBY 097 were only 5.5- and 3-fold less effective, respectively, and TSAO-mT retained its full activity against the Y318W mutant virus (Table II). The Y318F mutant virus was, in general, less resistant to the NNRTIs than the Y318W mutant virus. MKC-442 showed the highest degree of resistance (30-fold), followed by delavirdine (17-fold), nevirapine (8-fold), and HBY 097 (7-fold). UC-781, tivirapine, and lovivride remained highly active against the Y318F mutant virus and lost less than 4-fold of their activity. Thus, in agreement with our findings for mutant RTs, the resistance of the Y318F mutant virus against the NNRTIs was much less pronounced than that of the Y318W mutant virus.

### Table I

| Template/primer | IC₅₀ (µM) |
|-----------------|----------|
| Poly(rC)oligo(dG) |          |
| UC-781          | 0.022 ± 0.001 | 0.028 ± 0.002 | 0.23 ± 0.02 |
| TSAO-mT         | 2.5 ± 2.2  | 4.6 ± 1.3  | 1.0 ± 0.3  |
| Nevirapine      | 6.1 ± 0.3  | 8.1 ± 0.3  | 95 ± 6     |
| Delavirdine     | 0.66 ± 0.04 | 5.9 ± 0.9  | 0.28 ± 0.11|
| HBY 097         | 0.014 ± 0.002 | 0.020 ± 0.003 | 0.015 ± 0.0003 |
| Lovivride       | 0.60 ± 0.2  | 0.40 ± 0.2  | 22 ± 1.4   |
| MKC-442         | 0.122 ± 0.007 | 0.70 ± 0.1  | 0.8 ± 0.1  |
| Tivirapine      | 0.122 ± 0.006 | 0.12 ± 0.02  | 8.9 ± 0.3  |
| ddGTP           | 0.079      | 0.089      | 0.074 ± 0.006|
| Poly(rA)oligo(dT) |          |
| dT-TP           | 0.062 ± 0.001 | 0.037 ± 0.001 | 0.059 ± 0.005 |
| AZT-TP          | 0.030 ± 0.002 | 0.039 ± 0.003 | 0.056 ± 0.001 |

*The IC₅₀ of the compounds against the HIV-I RTs was determined using E. coli extracts as the source of the wild-type and mutant RTs. The recombinant RTs used correspond to the p66/p66 homodimer and contain the mutations in both subunits of the enzyme.*

### Discussion

X-ray crystallographic analyses of HIV-I RTs complexed with NNRTIs and computer-assisted modeling studies of NNRTIs in the HIV-I RT have identified a number of amino acids that line the NNRTI-specific lipophilic pocket and consequently may play a role in the interaction with NNRTIs (Fig. 1). One of these amino acids is Tyr-318, whose functional hydroxyphenyl group points into the pocket, forming a hydrogen bond to the main chain of either His-235 or Lys-238. Tyr-318 is highly conserved among all HIV-I, HIV-II, and simian immunodeficiency virus strains that have been characterized thus far. There is only one HIV-I strain (i.e. ETH2220) reported that had a Phe instead of a Tyr at position 318 (12). No functional and/or structural role has yet been identified for Tyr-318. Several NNRTIs interact relatively closely with Tyr-318 (3, 13, 14). However, to the best of our knowledge, no mutations at this amino acid position have ever been detected in NNRTI-treated HIV-I-infected cell cultures or HIV-I-infected individuals treated with NNRTIs.

Our site-directed mutagenesis studies revealed that only the
Table II

| Compound     | IC50 (µM) for recombinant mutant HIV-I strains* | dG | dG |
|--------------|-----------------------------------------------|----|----|
|              | HIV-Y318F                                   |    |    |
| UC-781       | 0.003 ± 0.0                                  | 0.010 ± 0.004 | 0.016 ± 0.007 |
| TSAO-mT      | 0.005 ± 0.003                                | 0.021 ± 0.002 | 0.032 ± 0.002 |
| Nevirapine   | 0.027 ± 0.0                                  | 0.21 ± 0.2    | 1.45 ± 0.8    |
| Delavirdine  | 0.041 ± 0.02                                 | 0.71 ± 0.47   | 0.011 ± 0.001 |
| HBY 097      | 0.004 ± 0.001                                | 0.01 ± 0.001  | 0.0048 ± 0.00 |
| Loviride     | 0.010 ± 0.004                                | 0.02 ± 0.004  | 0.26 ± 0.13   |
| MKC-442      | 0.012 ± 0.002                                | 0.091 ± 0.005 | 0.06 ± 0.03   |
| Tivirapine   | 0.018 ± 0.004                                | 0.05 ± 0.003  | 0.32 ± 0.01   |
| dG           | 8 ± 10                                       | 16 ± 6        | 7 ± 5         |

* EC50: 50% effective concentration or the compound concentration required to inhibit HIV-I-induced cytopathicity in CEM cell cultures by 50%.

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Y318W RT may instead contain a hydrogen bond between the Nε1 atom of Y318W and the main chain oxygen atom of His-235, affecting the ability of Pro-236 to act as a “trap door” (14) that closes in behind the inhibitors once they are in the NNRTI binding pocket. This effect may, in turn, lead to a decreased sensitivity of NNRTIs against the mutant RT. Changes affecting residue Pro-236 might be expected to have an anomalous effect on delavirdine (3), which is in agreement with our present data.

To assess the impact of a mutational change at position 318 in HIV-I, we attempted to construct recombinant viruses bearing mutations at residue 318 in their RT genes. We were repeatedly unsuccessful in making recombinant viruses harboring mutations Y318H, Y318L, Y318K, and Y318C. We demonstrated that the corresponding RTs have unusually low DNA polymerase activities, and this may explain the failure to construct mutant HIV-I strains containing mutations at position 318 that severely compromise the catalytic activity of the RT. Only the Y318F and Y318W RT mutant viruses could be constructed, with their corresponding RT possessing wild-type (Y318F) or moderately decreased (Y318W) catalytic activities. When CEM cell cultures were infected with wild-type and the Y318F and Y318W RT recombinant virus strains at an identical p24 input, we could not find striking differences in virus replication based on the appearance of p24 in the cell culture supernatants (data not shown). Obviously, the (moderately) lower catalytic RT activity found for the Y318W RT enzyme was not rate limiting and was not low enough to affect the replication capacity of the mutant virus strain in CEM cells.

The position 318 mutated recombinant viruses showed a resistance/sensitivity profile similar to that of the corresponding position 318 mutated RTs for the majority of the compounds. When all of the compounds listed in Tables I and II were taken together, strong correlations were found between the EC50s of the NNRTIs for the mutant HIV-I strains and the IC50s of the NNRTIs for the corresponding mutant RTs (correlation coefficient r = 0.88 for wild-type virus and enzyme, correlation coefficient r = 0.92 for Y318F mutant virus and enzyme, and correlation coefficient r = 0.98 for Y318W mutant virus and enzyme). However, in a few cases, differences were observed between the RT and the virus essays with regard to the sensitivity/resistance spectrum of the drugs (e.g., tivirapine).

The antiviral activity of tivirapine against Y318W mutated virus was much less pronounced than expected from the RT sensitivity data. We do not have a clear-cut understanding of this phenomenon. A plausible explanation, however, is that the RT experiments were performed under artificial conditions using poly(rC)-oligo(dG) as the template/primer instead of the endogenous natural template of the virus. It is likely that the conformation of the NNRTI pocket and the interaction of the mutant enzyme with the template/primer may differ, depending on the nature of the mutation in the enzyme. Therefore, the interaction of the drug (e.g., tivirapine) with the mutant enzyme (e.g., Y318W) in the presence of the artificial template/primer may not be as optimal as that for the other drugs. Thus, it cannot be excluded that the RT assay system using poly(rC)-oligo(dG) as the template/primer is suboptimal for some compounds under our experimental conditions, resulting in an underestimation or overestimation of their anti-RT activity.

The lower RT catalytic activity of the Y318W RT mutant virus most likely has no major impact on the sensitivity of the mutant virus to the NNRTIs. We found that a 5-fold difference in multiplicity of infection did not significantly affect the sensitivity of the virus to the NNRTIs HBY 097, UC-781, MKC-442, and nevirapine. The EC50 values for high and low multi-
plicity of infection were 0.003 and 0.002 μM for HBV97, 0.003 and 0.001 μM for UC-781, 0.007 and 0.004 μM for MKC-442, and 0.061 and 0.034 μM for nevirapine, respectively. Thus, it is unlikely that – if the lower catalytic activity of the Y318W RT enzyme should result in lower replication efficiency - this would markedly affect the sensitivity of the mutant viruses for the NNRTIs.

Although interactions between several NNRTIs and residue 318 are observed in RT/NNRTI complexes, optimizing these interactions has not been a primary goal for drug design. By identifying the importance of Tyr-318 for enzyme viability, this study now suggests that targeting Tyr-318, particularly the aromatic ring, should be a good strategy. With this in mind, we have reexamined the crystal structures of RT/NNRTI complexes. The RT/delavirdine complex (3) shows the closest interactions involving Tyr-318 (Fig. 2), leaving very little room for alterations. However, attaching a hydroxymethyl group to position 3 of the indole ring may facilitate hydrogen bonding with Tyr-318, and a methyl group added at position 2 of the piperazine ring may interact well with the aromatic ring of residue 318. The RT/9-chloro-TIBO complex (15) shows that substituents on position 10 of the TIBO ring would be well positioned to interact with the face of the Tyr-318 ring. Whereas a hydroxyl substituent may give the optimum interaction, it is interesting to note that 9,10-chloro-TIBO (R85255) has been found to be similarly active (IC₅₀ = 25 nM; Ref. 16) to 9-chloro-TIBO (IC₅₀ = 33 nM). With other NNRTIs, the geometry of the binding site makes it difficult to fully exploit Tyr-318. Nevirapine (and close analogues) may benefit from a 7-hydroxyl group, which might make it difficult to fully exploit Tyr-318. Nevirapine (and close analogues) may benefit from a 7-hydroxyl group, which might be able to hydrogen bond to Tyr-318. For the 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives, the oxygen at position 2 comes very close to the ring of Tyr-318, suggesting that a replacement with sulfur or more drastic changes to the thymine ring to allow an amine or hydroxy group in the equivalent position are worth exploring. The RT/loviride complex structure (13) offers little hope for targeting Tyr-318.

In conclusion, Tyr-318 represents a highly conserved amino acid among all known HIV-I, HIV-II, and simian immunodeficiency virus RTs. Its functionality is severely compromised upon mutation to other nonaromatic amino acids. Mutations at this amino acid site of RT either lead to a virtually inactive enzyme (i.e. Y318L, Y318K, Y318C, and Y318H) or to a viable enzyme (i.e. Y318F and Y318W) that shows little, if any, resistance against several NNRTIs, including HBV 97 and UC-781. Therefore, it is unlikely that mutations at position 318 will appear under selective pressure of these drugs in cell culture or in HIV-I-infected individuals, because they virtually suppress the mutant viruses and RT enzymes to a similar extent as the wild-type virus and RT enzyme. However, it cannot be excluded that Y318F or Y318W RT mutant HIV-I might emerge under selective pressure of other NNRTIs that showed a more pronounced loss of antiviral activity (i.e. delavirdine and MKC-442 for Y318F RT mutant virus and nevirapine, loviride, MKC-442, and tivirapine for Y318W RT mutant virus). Thus, our site-directed mutagenesis data and recombinant mutant virus constructs indicate that the amino acid residue Tyr-318 could be a suitable target for novel NNRTIs with improved potency and increased ability to suppress virus drug resistance development.

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Mutational Analysis of Tyr-318 within the Non-nucleoside Reverse Transcriptase Inhibitor Binding Pocket of Human Immunodeficiency Virus Type I Reverse Transcriptase

Heidi Pelemans, Robert M. Esnouf, Heidi Jonckheere, Erik De Clercq and Jan Balzarini

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