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Comprehensive Mutant Enzyme and Viral Variant Assessment of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Resistance to Nonnucleoside Inhibitors

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The nonnucleoside reverse transcriptase (RT) inhibitors comprise a class of structurally diverse compounds that are functionally related and specific for the human immunodeficiency virus type 1 RT. Viral variants resistant to these compounds arise readily in cell culture and in treated, infected humans. Therefore, the eventual clinical usefulness of the nonnucleoside inhibitors will rely on a thorough understanding of the genetic and biochemical bases for resistance. A study was performed to assess the effects of substitutions at each RT amino acid residue that influences the enzyme's susceptibility to the various nonnucleoside compounds. Single substitutions were introduced into both purified enzyme and virus. The resulting patterns of resistance were markedly distinct for each of the tested inhibitors. For instance, a >50-fold loss of enzyme susceptibility to BI-RG-587 was engendered by any of four individual substitutions, while the same level of relative resistance to the pyridinone derivatives was mediated only by substitution at residue 181. Similarly, substitution at residue 106 had a noted effect on virus resistance to BI-RG-587 but not to the pyridinones. The opposite effect was mediated by a substitution at residue 179. Such knowledge of nonnucleoside inhibitor resistance profiles may help in understanding the basis for resistant virus selection during clinical studies of these compounds.

An essential step in the replicative cycle of human immunodeficiency virus type 1 (HIV-1) is the synthesis, catalyzed by the virally encoded reverse transcriptase (RT), of a DNA copy of the viral RNA. Accordingly, the development of RT inhibitors has been the central focus of numerous anti-HIV-1 therapeutic research programs. Over the past several years, a chemically diverse class of RT inhibitors has been described. These compounds have been designated the nonnucleoside RT inhibitors to distinguish them from the nucleoside analogs. The class includes the pyridinone derivatives L-697,661 and L-696,229 as well as BI-RG-587 and the TIBO derivative R82913 (7, 8, 14, 16, 18, 22). These compounds are potent inhibitors of HIV-1 infection in cell culture. Inhibition is noncompetitive with respect to either template-primer or nucleotide binding and is specific for the HIV-1 RT (7, 16, 23). None inhibits the enzyme from HIV-2.

Viral variants with reduced susceptibilities to the nonnucleoside inhibitors have been derived by cell culture selection. Analysis of RT from variants resistant to either the pyridinone compounds or BI-RG-587 identified a substitution of Cys for Tyr at position 181 that confers greater than a 100-fold loss of susceptibility (15, 17). In addition, substitution of Asn for Lys at position 103 mediates approximately 10-fold resistance to the pyridinones (15). Substitution at position 100 engenders resistance to a TIBO derivative (13). Mutational analysis of recombinantly expressed RT also identified the Tyr residue at position 188 as important in mediating enzyme susceptibility (3, 5, 19, 20).

The data from these initial studies suggested that the structurally different members of the nonnucleoside inhibitor class uniquely interact with the RT. The resistance effects of various amino acid substitutions at positions 103, 181, and 188 are specific to individual inhibitors (19). Given this observation and the continuing clinical interest in the nonnucleoside compounds, a study was performed to assess further the effects of substitutions at additional amino acid residues that influence RT susceptibility. The residues and the specific substitutions chosen for study were selected following analysis of resistant viral variants derived in cell culture and from infected persons treated with L-697,661 in ongoing clinical trials. A series of recombinant mutant RT enzymes and viral variants was constructed; each mutant enzyme or virus contained a single amino acid substitution. The susceptibilities of the mutants to a panel of diverse nonnucleoside inhibitors were fully characterized.

MATERIALS AND METHODS

Construction and expression of recombinant RT. The cloning and expression vector was pRT-lacI (5). Recombinant RT genes were constructed by introduction of point mutations by using either gapped-duplex or polymerase chain reaction-mediated mutagenesis (4, 10). RT expression was induced in *Escherichia coli* AB1899 as described by Condra et al. (5). Expression was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

Purification of recombinant RT. Induced bacterial cells from a 500-ml culture were harvested by centrifugation and were stored at −70°C until use. RT was purified by the two-step scheme described by Sardana et al. (19). Only

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TABLE 1. Inhibition of substituted RT enzymes by nonnucleoside RT inhibitors[a]

Enzyme[b]	L-697,661		L-696,229		BI-RG-587		R82913	
	IC_{50} (nM)	F.D.	IC_{50} (nM)	F.D.	IC_{50} (nM)	F.D.	IC_{50} (nM)	F.D.
Wild type	35 ± 7	1.0	35 ± 5	1.0	108 ± 9	1.0	388 ± 52	1.0
98 (Ala→Gly)	180 ± 50	4.6 ± 0.8	125 ± 12	3.9 ± 0.3	319 ± 74	2.7 ± 0.0	3,410 ± 800	6.5 ± 1.6
100 (Leu→Ile)	511 ± 20	17.5 ± 0.3	186 ± 31	5.8 ± 0.8	923 ± 109	9.0 ± 1.2	47,290 ± 2,950	107.0 ± 28.0
101 (Lys→Glu)	41 ± 1	1.2 ± 0.2	64 ± 7	2.0 ± 0.1	196 ± 31	1.6 ± 0.1	2,270 ± 20	4.3 ± 0.0
103 (Lys→Asn)[c]	648 ± 142	10.4 ± 1.7	1,105 ± 37	26.0 ± 4.0	7760 ± 65	64.5 ± 3.8	24,662 ± 9,460	79.0 ± 33.0
106 (Val→Ala)	10 ± 0	0.3 ± 0.0	62 ± 0	1.9 ± 0.1	10,327 ± 2,600	99.4 ± 20.6	2,700 ± 710	6.6 ± 0.1
108 (Val→Ile)	77 ± 11	2.8 ± 0.1	133 ± 4	2.8 ± 0.4	288 ± 45	2.4 ± 0.3	838 ± 11	2.7 ± 0.2
179 (Val→Asp)	100 ± 24	2.0 ± 0.3	74 ± 2	2.3 ± 0.4	191 ± 30	1.6 ± 0.3	1,900 ± 180	6.1 ± 0.0
179 (Val→Glu)	265 ± 76	7.1 ± 0.5	169 ± 5	4.6 ± 0.1	221 ± 53	2.2 ± 0.4	2,664 ± 410	8.6 ± 0.2
181 (Tyr→Cys)[c]	>10,000	>200	2,901 ± 840	223.0 ± 60.0	22,719 ± 5,105	113.0 ± 25.0	5,502 ± 175	14.0 ± 4.0
188 (Tyr→Cys)[c]	105 ± 14	2.2 ± 0.4	1,317 ± 287	31.0 ± 6.1	20,636 ± 6,695	81.1 ± 9.9	9,790 ± 1,103	22.5 ± 5.0

[a] IC_{50}s represent 50% inhibitory concentrations in the RT inhibition assay (see text). F.D. values represent fold differences in IC_{50} compared with that for wild-type enzyme. Fold differences were calculated by using simultaneous mutant and wild-type enzyme IC_{50} determinations obtained in the same assay. Mean values and deviations were calculated from the results of three independent assays.

[b] Each mutant enzyme expressed the noted amino acid substitution at the indicated RT residue position.

[c] The data for the RT variants at positions 103, 181, and 188 have been published previously (19) and are included here for purposes of comparison. The preincubation step was not included in these RT assays (see text). This did not significantly affect the calculated IC_{50}s for these mutants.

enzymes that were minimally 75 to 90% pure were used in the inhibition assay.

RT inhibition assay. The RT inhibition assay was performed, with minor modifications, as described previously (7, 19). A sufficient quantity of recombinant RT was added to each assay reaction to mediate the incorporation of 10 to 20 pmol of nucleotide. The enzyme was preincubated, in the presence or absence of inhibitor, for 15 min at 30°C in buffer containing 55 mM Tris (pH 8.2), 80 mM KCl, 12 mM $MgCl_2$, 1.0 mM dithiothreitol, 1.0 mg of bovine serum albumin per ml, 20 μg of rC · dG_{12-18} per ml, 50 μM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 0.01% (vol/vol) Triton X-100. The reaction mixture was then cooled to 0°C and 18.5 μM [^{3}H]dGTP was added. The reaction mixture was incubated at 37°C for 45 min. The acid-precipitable material was subsequently collected on glass fiber filters by using a Skatron semiautomatic cell harvester. The incorporated radioactivity was measured by liquid scintillation counting. The test inhibitors were dissolved in dimethyl sulfoxide (DMSO) and were added to the assay mixture to give the desired concentration of inhibitor. The final assay reactions contained no more than 5.0% DMSO.

Construction and production of variant HIV-1. A 1,009-bp *Msc*I to *Bsp*1286I fragment from the recombinant RT expression plasmid was subcloned into NL4-3, an infectious proviral clone of HIV-1 (1). The fragment encodes amino acid residues 25 to 359 of RT. Virus was produced by transfecting either SW480 or HeLa cells with 10 μg of the constructed plasmid DNA by the calcium phosphate technique described previously (9). The resulting virus was amplified by cocultivation of the transfected cells with H9 human T-lymphoid cells. The RT genes of the variant virus stocks were sequenced to confirm the presence of the appropriate mutations.

Virus inhibition assay. The virus inhibition assay was performed as described by Nunberg et al. (15) by using MT-4 human T-lymphoid cells. Virus was used to infect cells 24 h prior to the addition of test inhibitor. Each variant virus stock had been titrated prior to use, and the virus inocula were individually adjusted so that all variants yielded an identical level of virus production by the assay's end. Twenty-four hours after infection, the cells were seeded into 96-well cell culture plates at 5.0×10^4 cells per well. The test compounds were then added in serial twofold dilutions. Following an additional 72 h of incubation, virus production was assessed by viral core p24 antigen assay (Coulter Immunology, Hialeah, Fla.).

RESULTS

Susceptibilities of mutant RT enzymes to the nonnucleoside inhibitors. The RT residues and specific amino acid substitutions chosen for study were identified in resistant viral variants selected by L-697,661 in cell culture or in treated, infected humans (unpublished data). The substitutions at residues 98, 101, and 179 were noted in patient-derived variants, while that at residue 100 was seen only in a cell culture-derived variant. The substitutions at 103, 108, and 181 were observed in resistant virus obtained from both sources. In addition, the substitutions at residues 100 and 106 were previously reported in resistant virus selected by other nonnucleoside inhibitors in cell culture (12, 13, 21). Residue 188 was implicated in mediating susceptibility to the nonnucleoside compounds by biochemical studies of mutant enzyme (3, 5, 19, 20).

Each recombinant RT contained a single substitution. The mutant enzymes were expressed and purified and were then tested in an in vitro RT inhibition assay with four nonnucleoside inhibitors. The results (Table 1) show that the substitutions at residues 98, 101, 108, and 179 mediated a two- to eightfold loss of susceptibility to the test compounds. In contrast, the substitutions at residues 100, 103, 106, 181, and 188 exhibited noted differential effects on inhibition by the various compounds. The enzyme expressing a substitution at residue 100 (Leu→Ile) was much less susceptible to the TIBO derivative R82913 than it was to the other inhibitors, while a substitution at residue 106 (Val→Ala) or 188 (Tyr→Cys) engendered a striking loss of susceptibility specifically for BI-RG-587. As reported previously (19), the substitution at position 103 (Lys→Asn) was responsible for greater resistance to BI-RG-587 and R82913 than to the two pyridinone derivatives. Similarly, the substitutions at residue 181 (Tyr→Cys) resulted in greater resistance for the pyridinones and BI-RG-587 than for R82913 (19).

Susceptibilities to the nonnucleoside inhibitors of viral vari-

TABLE 2. Inhibition of HIV-1 variants by nonnucleoside RT inhibitors[a]

Virus[b]	L-697,661		L-696,229		BI-RG-587		R83913	
	IC_{95} (nM)	F.D.	IC_{95} (nM)	F.D.	IC_{95} (nM)	F.D.	IC_{95} (nM)	F.D.
Wild type	100	1.0	100	1.0	400	1.0	800	1.0
98 (Ala→Gly)	800	8.0	400	4.0	800	2.0	>3,000	>3.8
100 (Leu→Ile)	200	2.0	200	2.0	400	1.0	>3,000	>3.8
101 (Lys→Glu)	800	8.0	800	8.0	3,000	7.5	>3,000	>3.8
103 (Lys→Asn)	800	8.0	800	8.0	>3,000	>7.5	>3,000	>3.8
106 (Val→Ala)	100	1.0	200	2.0	>3,000	>7.5	>3,000	>3.8
108 (Val→Ile)	400	4.0	200	2.0	400	1.0	3,000	3.8
179 (Val→Asp)	400	4.0	100	1.0	400	1.0	>3,000	>3.8
179 (Val→Glu)	800	8.0	400	4.0	400	1.0	>3,000	>3.8
181 (Tyr→Cys)	>3,000	>30.0	>3,000	>30.0	>3,000	>7.5	>3,000	>3.8
188 (Tyr→Cys)	800	8.0	1,500	15.0	>3,000	>7.5	>3,000	>3.8

[a] IC_{95}s represent the concentrations of the test compounds that inhibited by at least 95% the spread of virus infection in the virus inhibition assay (see text). F.D. values represent fold differences in IC_{95} compared with that for wild-type virus. The values were derived from two or three independent assays. No variance was noted among the assays.

[b] Each viral variant expressed the noted amino acid substitution at the indicated RT residue position.

ants expressing RT substitutions. HIV-1 variants containing each of the studied RT substitutions were prepared by proviral mutagenesis. The variants were tested in a cell culture inhibition assay, and the 95% inhibitory concentrations for each compound were determined. The results (Table 2) were in general agreement with the data obtained by in vitro assay of the substituted RT enzymes. There were, however, two noted exceptions. The enzyme data predicted a minimal effect of the substitution at residue 101 on the susceptibility to the pyridinone derivatives. Yet virus that expressed this substitution exhibited an eightfold loss of susceptibility to these inhibitors. In addition, the effect of the substitution at residue 100 on resistance to the pyridinones was striking in the enzyme but minimal in the virus.

Analysis of variant virus susceptibility to the chemically different nonnucleoside compounds showed that each of the substitutions at residues 98, 101, 103, and 181 had approximately equivalent effects on the loss of virus susceptibility to all four of the tested inhibitors (see also Table 1). In contrast, the substitution at residue 106 had a noted effect on virus resistance to BI-RG-587 and R82913, but it had no effect on virus susceptibility to the pyridinones. The opposite effect was mediated by the substitution at residue 179. A mutation at residue 100 engendered resistance only against the TIBO derivative. All of the viruses were tested for inhibition by zidovudine and were found to be equally susceptible (data not shown).

DISCUSSION

The eventual clinical usefulness of the nonnucleoside RT inhibitors will largely be defined by the genetic basis for viral resistance to these compounds. Viral variants that exhibit decreased susceptibilities to the nonnucleosides have been readily isolated in cell culture (13, 15, 17) and have been observed in HIV-1-infected humans undergoing experimental therapy (unpublished data). The RT amino acid substitutions associated with the resistance phenotype cluster with the RT regions that appear to physically interact with the inhibitors, as demonstrated by both low-resolution crystal structure and competitive binding studies (6, 7, 11, 23). Also, while some of the responsible RT residue positions can accept a number of different amino acid substitutions with various effects on enzyme susceptibility (19), there appears to be a preference for the selection of characteristic substitutions at each residue. In the present study, these substitutions were introduced at the appropriate RT residue positions within both the enzyme and the virus. The effects of the individual alterations on the susceptibilities to each of four nonnucleoside inhibitors were assessed.

Resistance was generally equivalently manifested by both mutant RT and virus. The noted exceptions were seen with substitutions at residues 100 and 101. The effects of these substitutions on virus susceptibility, particularly with the pyridinone derivatives, were quite different from their effects on enzyme susceptibility. The differences were not resolved by the determination of K_i instead of 50% inhibitory concentrations (data not shown). This is the first report of such a discrepancy for the nonnucleoside inhibitors, and its basis remains unclear. These results caution against the direct extrapolation of enzyme susceptibility data to the virus. Results of genetic and phenotypic analyses of resistant viruses obtained during a clinical study of L-697,661 were more accurately predicted by the resistance assessments of mutant virus than those of mutant enzyme (unpublished data).

As seen in previous studies (5, 19), various individual amino acid substitutions mediated profoundly different susceptibility effects on the different nonnucleoside compounds. This provides further support for the view that each structurally different inhibitor interacts in a unique way with the enzyme's inhibitor-binding site. Accordingly, the resistance profiles established for each individual inhibitor may help in understanding the basis for the selection of resistant virus during clinical study of that inhibitor and may assist in study design. For instance, if therapy with a given dose of compound results in selection of a virus population that expresses an amino acid substitution known to mediate a 10-fold loss of virus susceptibility, then the dose may be adjusted in an attempt to suppress the resistant variant. In addition, combination therapeutic regimens may be designed to exploit the differences in resistance profiles among the inhibitors. The profiles of novel nonnucleoside inhibitors can be determined rapidly by using the panels of mutant RT enzyme and virus reported here.

Finally, the data show that resistance to the nonnucleosides can be mediated by many independent substitutions. Recently, Chow et al. (2) reported that mutant virus expressing substitutions that mediate resistance for both didanosine and zidovudine is not viable if the Asn-for-Lys substitution

at residue 103 is also expressed. The authors suggest that simultaneous therapy with didanosine, zidovudine, and a nonnucleoside inhibitor may not allow for selection of virus resistant to all three compounds. However, the probability of such an event can be assessed only following the construction and phenotypic analysis of virus variants that express all possible combinations of resistance-engendering substitutions. Those studies are in progress.

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