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ACCELERATED COMMUNICATION

A Single Conservative Amino Acid Substitution in the Reverse Transcriptase of Human Immunodeficiency Virus-1 Confers Resistance to (+)-(5*S*)-4,5,6,7-Tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione (TIBO R82150)

JOHN W. MELLORS, GUANG-JIN IM, ENZO TRAMONTANO, SUSAN R. WINKLER, DANIEL J. MEDINA, GINGER E. DUTSCHMAN, HENGAMEH Z. BAZMI, GIOVANNA PIRAS, CHARLES J. GONZALEZ, and YUNG-CHI CHENG

Department of Internal Medicine, Division of Infectious Diseases, University of Pittsburgh Medical Center and the Veterans Administration Medical Center, Pittsburgh, Pennsylvania 15240 (J.W.M., H.Z.B.), Departments of Internal Medicine (S.R.W.) and Laboratory Medicine (D.J.M.), Veterans Administration Medical Center, West Haven, Connecticut 06516, and Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510 (G.-j.I., E.T., G.E.D., G.P., C.J.G., Y.-c.C.)

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SUMMARY

Tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives (*e.g.*, R82150) are potent, human immunodeficiency virus-1 (HIV-1)-specific, inhibitors of reverse transcriptase (RT) that are undergoing initial evaluation in clinical trials. Because HIV-1 has become resistant to other RT inhibitors, we investigated the potential for viral resistance to TIBO R82150 by serial *in vitro* passage of HIV-1_{IIIB} in the presence of drug. R82150-resistant variants (>100-fold increase in IC_{50}) dominated the replicating virus population after only three or four passages. R82150-resistant virus was partially cross-resistant to other HIV-1-specific RT inhibitors, including nevirapine (~10-fold increase in IC_{50}) and 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (~3.5-fold increase) but remained susceptible to 2′,3′-dideoxynucleosides and phosphonoformate. DNA sequencing of cloned resistant RT, combined with site-specific mutational analyses and construction of mutant recombinant proviruses, demonstrated that a single, conservative amino acid substitution (Leu^{100} to Ile) in HIV-1 RT is responsible for high level R82150 resistance and partial nevirapine resistance. These studies indicate that a subtle mutation in HIV-1 RT can dramatically affect viral susceptibility to an HIV-1-specific RT inhibitor. The clinical efficacy of TIBO derivatives and other HIV-1-specific RT inhibitors may be limited by the emergence of drug-resistant viral strains.

Several different chemical classes of non-nucleoside inhibitors of RT have been discovered and are being evaluated in clinical trials (1–6). Prototype compounds from each class include the dipyridodiazepinone derivative nevirapine (BI-RG-587) (1, 2), the TIBO derivative R82150 (3, 4), the pyridinone derivatives L-697,639 and L-697,661 (5), and the bis(heteroaryl)piperazine derivative U80493E (6). These structurally distinct compounds share several common characteristics; they are potent, highly specific, noncompetitive inhibitors of HIV-1 RT. They have no activity against HIV-2, simian immunodeficiency virus, feline leukemia virus, avian myeloblastosis virus, or murine retroviruses (1–6). HIV-1-specific RT inhibition is

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ABBREVIATIONS: RT, reverse transcriptase; TIBO R82150, (+)-(5*S*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione; nevirapine or BI-RG-587, 11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyridol[3,2-*b*:2′,3′-*e*]diazepin-6-one; HIV-1, human immunodeficiency virus type 1; L-697,639, 3-{[(4,7-dimethyl-1,3-benzoxazol-2-yl)methyl]amino}-5-ethyl-6-methylpyridin-2(1*H*)-one; L-697,661, 3-{[(4,7-dichloro-1,3-benzoxazol-2-yl)methyl]amino}-5-ethyl-6-methylpyridin-2(1*H*)-one; L-693,593, 5-ethyl-6-methyl-3-(2-phthalimidoethyl)pyridin-2(1*H*)-one; U80493E, *N*-ethyl-2-{4-[(4-methoxy-3,5-dimethylphenyl)methyl]-1-piperazinyl}-3-pyridinamine; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; $TCID_{50}$, 50% tissue culture infective dose; IC_{50}, 50% inhibitory concentration; D4T, 2′,3′-didehydro-3′-deoxythymidine; DDI, 2′,3′-dideoxyinosine; DDC, 2′,3′-dideoxycytidine; AZT, 3′-azido-3′-deoxythymidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dFBS, dialyzed fetal bovine serum; PBMC, peripheral blood mononuclear cells; PFA, phosphonoformic acid; PHA, phytohemagglutinin; kb, kilobase(s); PCR, polymerase chain reaction; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1*H*)-one or -thione.

also characteristic of the acyclic nucleoside HEPT derivatives (7, 8) and the pyridimine 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide)pyrimidine nucleoside analogs (9).

The narrow activity spectrum of these HIV-1-specific inhibitors indicates that they interact with sites on RT that are not conserved among retroviruses and thus could potentially be substituted without loss of polymerase function. Because of the genetic variability of HIV-1, concern was raised that viral variants resistant to HIV-1-specific inhibitors would exist in nature (10–13). Indeed, drug-resistant variants readily emerge in tissue culture after selection with the pyridinone L-693,593 (11) or nevirapine (12, 13). Cross-resistance of these variants to TIBO R82150 and HEPT derivatives suggested a common binding site and mechanism of action for these compounds. Genetic analysis confirmed that a single mutation in HIV-1 RT (Tyr^{181} to Cys) confers resistance to pyridinones, nevirapine, TIBO R82150, and HEPT derivates (11–13). A second resistance mutation in HIV-1 RT (Lys^{103} to Asn) has been detected in resistant variants that emerge after selection with L-693,593 (11) but not after selection with nevirapine (12, 13). This suggests that additional compound-specific mutations may play a role in resistance to HIV-1-specific inhibitors.

To provide additional insight into the molecular basis of resistance to HIV-1-specific RT inhibitors, we have selected and molecularly characterized a drug-resistant variant of HIV-1 that emerges in tissue culture after selection with TIBO R82150. These studies have identified a previously unrecognized, conservative amino acid substitution in RT (Leu^{100} to Ile) that is responsible for high level R82150 resistance and partial resistance to nevirapine and HEPT.

Materials and Methods

Cells. H9 cells (provided by R. C. Gallo, National Cancer Institute, Bethesda, MD) and MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health; contributed by D. Richman) were cultured in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, and 20% dFBS (JRH Biosciences, Lenexa, KS). HeLa-CD4 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health; contributed by B. Chesebro) were cultured in Dulbecco's modified Eagle's medium (Whittaker) supplemented with antibiotics, 2 mM L-glutamine, and 10% dFBS. PBMC isolated from healthy seronegative donors were activated with PHA (10 μg/ml; Difco Labs, Detroit, MI) for 3 days before HIV-1 infection. PBMC were maintained after infection in RPMI 1640 supplemented with 10% interleukin-2 (Cellular Products, Buffalo, NY), 20% dFBS, 2 mM L-glutamine, and antibiotics.

Virus. Stock preparations of HIV-1 (HTLV-IIIB strain; provided by R. C. Gallo) were harvested from infected H9 cells by the "shake-off" method (13). Stock virus infectivity was determined by end-point dilution in MT-2 cells as described (14).

Compounds. TIBO R82150 was synthesized by K. Parker, Brown University (Providence, RI). Nevirapine and HEPT were obtained from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). TIBO R82150, nevirapine, and HEPT were dissolved in 100% dimethylsulfoxide and stored at −20°. PMEA was obtained from A. Holy, Czechoslovakia Academy of Science (Praha, Czechoslovakia). DDC was purchased from Pharmacia Inc. (Piscataway, NJ). D4T and DDI were provided by Bristol-Myers Squibb (Wallingford, CT). AZT was obtained from Burroughs-Wellcome (Research Triangle Park, NC). PFA was purchased from Sigma Chemical Co. (St. Louis, MO). PMEA, DDC, D4T, DDI, AZT, and PFA were dissolved in sterile water and stored at −20°. AZTTP was kindly provided by R. Schinazi, Emory University (Atlanta, GA).

Selection of resistant virus. H9 target cells (1×10^7) were incubated for 2 hr with $HIV\text{-}1_{IIIB}$ (1×10^6 $TCID_{50}$) in the presence of 1.0 μM R82150. The cells were washed twice after infection and cultured in medium containing 1.0 μM R82150. On day 7 after infection, cell-free viral progeny were harvested by the shake-off method (13) and used to initiate a new cycle of infection in the presence of drug. Breakthrough virus was screened for resistance after each passage by determining viral infectivity in MT-2 cells (end-point dilution) in the presence and absence of 1.0 μM R82150. The $\log_{10}$ reduction in viral infectivity by 1.0 μM R82150 was calculated by subtracting the infectivity titer determined in the presence of drug from that in the absence of drug.

Drug susceptibility testing of HIV-1. Drug susceptibility assays were performed in MT-2 cells, HeLa CD4 cells, and PHA-activated PBMC. Assays in MT-2 (cytopathic effect inhibition) and HeLa CD4 cells (plaque reduction) were performed as published (13, 14). PHA-activated PBMC (5×10^5) were inoculated with 0.01 $TCID_{50}$ of virus/cell, washed after 2 hr, and added to medium containing serial 3-fold dilutions of drug. Culture supernatants were harvested 7 days after infection and assayed for HIV-1 p24 antigen with an enzyme immunoassay (Abbott Laboratories, North Chicago, IL). p24 antigen levels in no-drug control supernatants were consistently >300 ng/ml. The drug concentrations that inhibited viral replication by 50% (IC_{50}) were calculated from linear-$\log_{10}$ plots of percentage of inhibition versus $\log_{10}$ drug concentration.

Cloning and expression of HIV-1 RT. The full length 1.68-kb coding sequence of HIV-1 RT was amplified by PCR, cloned, and expressed in *Escherichi coli* JM 109 as published (13, 15). In brief, HIV-1 RT cDNA was synthesized from viral RNA with murine Moloney leukemia virus RT (Bethesda Research Laboratories, Gaithersburg, MD) and a 3′ primer (5′-CTTATCTATTCCATCTAGAAATAGT-3′) (16). The cDNA was then PCR amplified with the addition of a 5′ primer (5′-TTGCACTTTGAATTCTCCCATTAG-3′) (16). The PCR product was diluted 10,000–100,000-fold and used as template for a second PCR amplification with primers containing 5′ *Nco*I and *Hind*III restriction sites (5′ primer, 5′-TGCCATGGCCATTAGCCCTATTGAGACTGT-3′; 3′ primer, 5′-CGAAGCTTTATAGTATTTTCCTGATTCCAGCACTG-3′) (15). The second PCR product was digested with *Nco*I and *Hind*III, purified from agarose gels, ligated into pKK233–2, and used to transform *E. coli* JM 109 as described (15). Ampicillin-resistant transformants were grown to late logarithmic phase and RT expression was induced for 6 hr with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Soluble extracts of bacterial lysates were prepared and screened for RT activity as described (15). Clones expressing high level RT activity (~40% of clones) were selected for RT purification and further analysis.

Purification of recombinant RT. Recombinant RT was purified from bacterial lysates as follows. The crude bacterial extract was dialyzed for 6 hr in 50 mM Tris·HCl, 5 mM dithiothreitol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol (solution A), and passed sequentially through DE52, diethylaminoethyl-cellulose (Whatman Laboratory, Maidstone, England), and P11 cellulose phosphate (Whatman) columns. The P11 column was eluted with a linear gradient of 1 M NaCl dissolved in solution A. Fractions were collected and assayed for peak RT activity and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples used for studies of RT susceptibility to inhibitors contained a single 66-kDa band by silver staining (data not shown).

RT assays. The sensitivity of crude virion-associated RT to inhibition by drug was determined using detergent-disrupted (0.5% Triton X-100) preparations of stock virus. Reactions were carried out in a 50-μl volume containing 5 μl of viral lysate, 0.05% Triton X-100, 50 mM Tris·HCl (pH 8.5), 10 mM dithiothreitol, 6 mM $MgCl_2$, 80 mM KCl,

0.25 mg/ml heat-inactivated bovine serum albumin, 6.25 μM [^{3}H]dTTP (16 Ci/mmol; New England Nuclear, Wilmington, DE), 0.5 A_{260} units/ml poly(rA)-oligo(dT)$_{10}$, and serial dilutions of inhibitor. Reactions were incubated at 37° for 60 min. Incorporation of radiolabel was linear during this interval (data not shown). Trichloroacetic acid-precipitable counts were quantitated by liquid scintillation counting as described (17).

The inhibitor sensitivity of recombinant RT was assayed in the same manner except that reactions were carried out in a 50-μl volume containing ~1.3 pg of purified enzyme or 5 μl of crude bacterial lysate (diluted 50-fold), 50 mM Tris·HCl (pH 7.8), 50 mM KCl, 6 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 10 μM [^{3}H] dGTP (1.0 Ci/mmol; NEN), and 0.5 A_{260} units/ml poly(rC)-oligo(dG)$_{12-18}$ as the template-primer (Pharmacia).

Site-specific mutagenesis of RT. The desired mutations in the coding sequence of RT were introduced by oligonucleotide-directed mutagenesis, following the method of Kunkel *et al.* (18). To facilitate mutagenesis, RT was cloned into the *Nco*I/*Hind*III sites of the phagemid vector pSL1190 (Pharmacia). Mutants were screened for the desired mutation by nucleotide sequencing. The mutated RT was then recloned into pKK233-2 and the presence of the desired mutation was again confirmed by sequencing before induction of RT expression.

Nucleic acid sequencing. RT clones were sequenced by the chain-termination method of Sanger *et al.* (19), with Sequenase T7 DNA polymerase (United States Biochemical Corporation, Cleveland, OH). A set of six primers were used to sequence the entire RT gene, as described (13).

Production of mutant recombinant HIV-1. Plasmids encoding infectious molecular clones of HIV-1 with the desired point mutations in RT were constructed through a two-step cloning procedure. First, the mutated RT plasmid was digested with *Eco*RV/*Hind*III and the 1.25-kb *Eco*RV-*Hind*III fragment 3′ to the mutation was replaced with the 5.2-kb *Eco*RV-*Hind*III fragment from the BH10 HIV-1 clone (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health; contributed by B. Hahn and B. Shaw). This created a plasmid having a second *Bal*I restriction site 3′ to the RT coding region. This intermediate plasmid was then digested with *Bal*I and the 1.9-kb *Bal*I-*Bal*I *pol* fragment was purified and ligated into *Bal*I-digested pHIV-1$_{BRU}$ (an infectious proviral clone kindly provided by Keith Peden, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Infectious virus was produced by DEAE-dextran-mediated transfection of 5 × 10^6 MT-2 cells with 10 μg of plasmid DNA (20). Culture supernatants, harvested 5–7 days after transfection, contained an infectious virus titer of $10^{5.0}$-$10^{5.5}$ $TCID_{50}$/ml.

Results

Selection of TIBO R82150-resistant virus. Resistant virus was selected by sequential passage of cell-free HIV-1$_{IIIB}$ in the presence of 1 μM R82150. After each passage, breakthrough virus was screened for resistance by quantitating viral infectivity in the presence and absence of 1 μM R82150. Table 1 shows that viral susceptibility to R82150 decreased with each passage, with the virus becoming completely resistant to 1 μM drug after four or five passages. Detailed drug susceptibility studies of passage 4 virus in MT-2 cells (cytopathic effect inhibition), HeLa-CD4 cells (plaque reduction), and PHA-stimulated PBMC (p24 inhibition) demonstrated that the IC_{50} of R82150 had increased >100-fold compared with parental HIV-1$_{IIIB}$ (Fig. 1). The susceptibility of parental HIV-1$_{IIIB}$ to R82150 did not change with serial passage in the absence of drug (data not shown). R82150-resistant virus replicated to high titer (>10^6 $TCID_{50}$/ml; Table 1) and produced syncytia in MT-2 and HeLa-CD4 cells with kinetics and morphology similar to those of parental HIV-1$_{IIIB}$, indicating that the *in vitro* replicative and cytopathic properties of the resistant variants had not obviously changed.

TABLE 1
Altered drug sensitivity of HIV-1 passaged serially in the presence of TIBO R82150

Virus passage[a]	Viral infectivity[b]	Reduction in infectivity by 1 μM TIBO R82150[c]
	log_{10} $TCID_{50}$/ml	log_{10}
0	6.4	2.6
1	4.7	1.1
2	4.7	0.6
3	6.9	0.5
4	7.0	0.2
5	6.4	0.0

[a] Number of viral passages in the presence of 1.0 μM TIBO R82150.
[b] Virus was harvested 7 days after infection. Infectivity was determined by end-point dilution in MT-2 cells.
[c] Calculated by subtracting the viral infectivity titer in the presence of 1 μM TIBO R82150 from the titer in the absence of drug.

Susceptibility of R82150-resistant HIV-1 to other antiretroviral agents. The susceptibility of R82150-resistant HIV-1 to other non-nucleoside and nucleoside analogs was determined in MT-2 cells (Table 2). In previous reports, HIV-1 variants resistant to pyridinones or nevirapine have shown nearly equal cross-resistance to R82150 and HEPT derivatives (10–13). In contrast, Table 2 shows that R82150-resistant virus exhibited only limited cross-resistance to nevirapine (~10-fold increase in IC_{50}) and HEPT (~3.5-fold increase). This raised the possibility that the mutations in HIV-1 responsible for R82150 resistance were different from those responsible for nevirapine (Tyr^{181} to Cys) or pyridinone (Tyr^{181} to Cys and Lys^{103} to Asn) resisitance (10–13).

R82150-resistant virus remained susceptible to the nucleoside analogs AZT, D4T, DDC, DDI, and PMEA (21) and to the pyrophosphate analog PFA. Retained susceptibility to nucleoside analogs and PFA has also been noted for nevirapine- and pyridinone-resistant HIV-1 (10–13).

Phenotype of native RT from R82150-resistant HIV-1. The susceptibility of crude, virion-associated RT to inhibition by R82150 was studied in detergent-disrupted stock preparations of R82150-resistant and parental virus (HIV-1$_{IIIB}$). Fig. 2 demonstrates a clear difference in the R82150 susceptibility of the RTs from the two viruses. RT from HIV-1$_{IIIB}$ was inhibited in a concentration-dependent manner, whereas RT from resistant virus was not inhibited by R82150 at concentrations as high as 10 μM. These initial studies with crude enzyme preparations demonstrate a concordance between RT and viral susceptibility to R82150. This is not the case for RT from AZT-resistant strains of HIV-1, which remains as susceptible to AZTTP as is RT from AZT-sensitive virus (16, 22).

DNA sequence analysis of RT from R82150-resistant virus. To define the genetic and biochemical basis for R82150 resistance, the full length 1.68-kb RT coding sequences from parental (HIV-1$_{IIIB}$) and R82150-resistant virus populations were amplified by PCR, cloned, and expressed in *E. coli*. About 40% of transformants expressed high level RT activity with isopropyl-β-D-thiogalactopyranoside induction. Extracts from these clones were screened for susceptibility to 600 nM R82150. The majority of clones (nine of 13) derived from resistant virus expressed RT with reduced susceptibility to R82150 (<40% inhibition), and in seven clones RT was inhibited <20% by 600

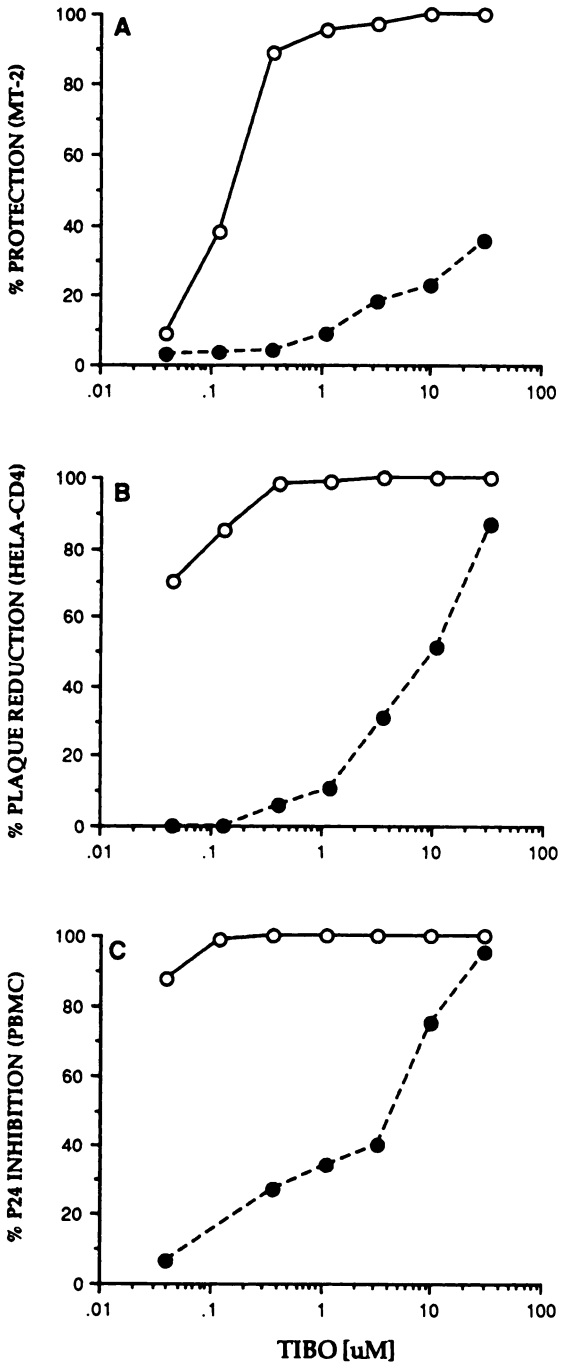

Fig. 1. Sensitivity of parental (O) and resistant (●) HIV-1 to TIBO R82150 assayed in MT-2 cells (A), HeLa-CD4 cells (B), and PHA-stimulated PBMC (C). Mean values of triplicate determinations are shown. Assays were performed as described in Materials and Methods.

nM R82150 (data not shown). In contrast, none of the 13 clones derived from parental HIV-1_{IIIB} exhibited this degree of resistance. Cloned RTs from resistant and parental virus were equally sensitive to AZTTP and PFA (data not shown).

Complete DNA sequencing of eight R82150-resistant and seven control RT clones identified two nucleotide changes common to resistant clones. These changes altered the predicted amino acids in RT at positions 83 (arginine to lysine) and 100 (leucine to isoleucine). The Tyr^{181} to Cys mutation responsible for nevirapine and pyridinone resistance was not

TABLE 2

Sensitivity of R82150-resistant HIV-1 to other antiretroviral agents

Drug sensitivities were determined in MT-2 cells as described in Materials and Methods. IC_{50} values were calculated from linear-log_{10} plots of percentage of protection versus drug concentration. Mean values from triplicate cultures are shown.

Compound	IC_{50}		Fold increase in IC_{50}
	HIV-1_{IIIB}	R82150-resistant HIV-1	
		μM	
TIBO R82150	0.20	>30.0	>150
BI-RG-587	0.03	0.30	10
HEPT	5.60	19.50	3.5
AZT	0.07	0.06	<1
D4T	2.20	1.80	<1
DDC	0.14	0.11	<1
DDI	3.10	2.50	<1
PMEA	2.85	2.25	<1
PFA	19.90	22.70	1.1

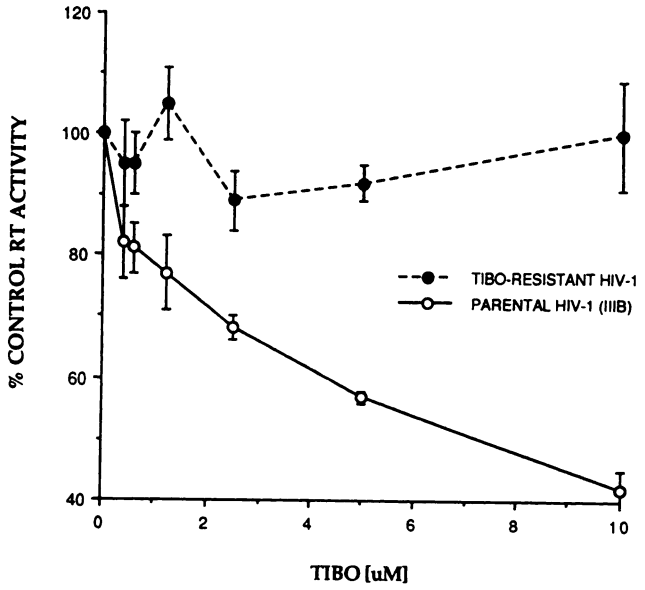

Fig. 2. Sensitivity of crude virion-associated RT from parental (O) and resistant (●) HIV-1 to TIBO R82150. RT assays were performed as described in Materials and Methods. Mean ± standard deviation values are shown.

detected in any of the R82150-resistant clones. The Leu^{100} residue lies within the polymerase domain of RT and is absolutely conserved in all previously reported HIV-1 isolates, strongly implicating its substitution in R82150 resistance (23). In contrast, the Arg^{83} to Lys variation exists in the the common HIV-1_{MN} strain (23) and was present in only seven of eight R82150-resistant clones, suggesting a less important role in R82150 resistance.

Role of specific mutations in R82150 resistance. To evaluate the role of these mutations in R82150 resistance, site-specific mutagenesis was performed to generate RT plasmids containing either the Arg^{83} to Lys mutation (AGA to AAA), the Leu^{100} to Ile mutation (TTA to ATA), or both mutations. Highly purified RT from these plasmids was tested for sensitivity to R82150 in comparison with wild-type RT cloned from parental HIV-1_{IIIB}. Fig. 3 shows that the Ile^{100} mutation conferred resistance to R82150, whereas the Lys^{83} mutation had no effect on R82150 sensitivity. The degree of R82150 resistance resulting from the Ile^{100} mutation alone was equal to that

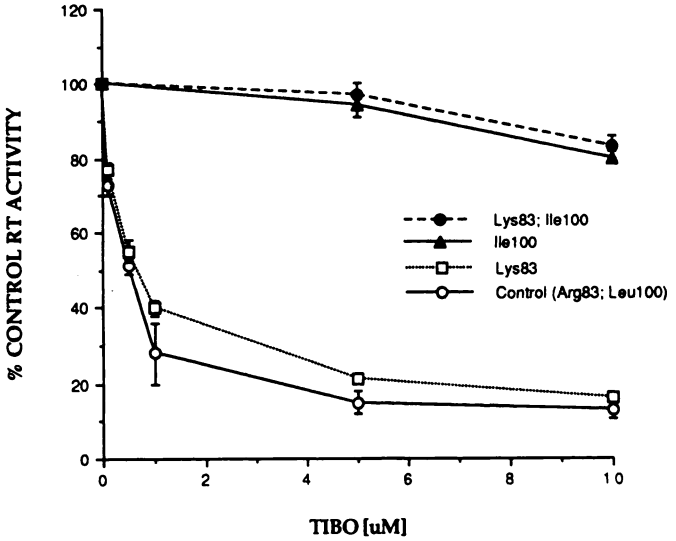

Fig. 3. Sensitivity of purified cloned RTs to TIBO R82150. Control RT was cloned from parental HIV-1_{IIIB}. The amino acid substitutions in the mutated RT clones were introduced by oligonucleotide-directed mutagenesis.

TABLE 3
Sensitivity of mutated RTs to non-nucleoside inhibitors

Recombinant RT clone[a]	IC_{50}[b]	
	TIBO R82150	BI-RG-587
	μM	
Control (Arg^{83}, Leu^{100})	0.64 ± 0.09	0.62 ± 0.09
T1 (Ile^{100})[c]	>20.0	2.08 ± 0.20
A2 (Lys^{83})	0.64 ± 0.02	0.56 ± 0.13
A3 (Lys^{83}, Ile^{100})	>20.0	2.12 ± 0.01

[a] Control RT was cloned from HIV-1_{IIIB}.
[b] Drug concentrations that inhibited RT activity to 50% of control; mean ± standard deviation values are shown.
[c] The mutations shown in parentheses were introduced by oligonucleotide-directed mutagenesis and confirmed by DNA sequencing.

with both mutations, indicating that the Lys^{83} mutation did not confer additional R82150 resistance even in the context of the Ile^{100} mutation. The mutated RTs were also studied for cross-resistance to nevirapine. Table 3 shows that the Ile^{100} mutation produced minimal cross-resistance to nevirapine (~3-fold) and that the Lys^{83} mutation had no effect on nevirapine sensitivity.

R82150 susceptibility of mutant recombinant proviruses. Infectious proviral clones of HIV-1 containing either the Lys^{83} mutation, the Ile^{100} mutation, both mutations, or the wild-type RT genotype (Arg^{83} and Leu^{100}) were constructed to determine the effects of these mutations on viral susceptibility to R82150. Infectious virus was produced by transfection of MT-2 cells and drug susceptibility was assayed in MT-2 cells after infection with an equal inoculum of mutant or wild-type control virus (0.1 $TCID_{50}$/cell). Table 4 shows that the Ile^{100} mutation alone conferred high level viral resistance to R82150 (>150-fold). The Lys^{83} mutation had no effect on susceptibility to R82150 either alone or in the context of the Ile^{100} mutation.

The stability of the R82150-resistant viral phenotype was assesed by serial passage of virus containing the Ile^{100} mutation in MT-2 cells in the absence of drug. Virus remained highly resistant to R82150 (>150-fold) after 10 passages, indicating that the resistant phenotype was stable in the absence of selective pressure (data not shown).

TABLE 4
Susceptibility of mutant recombinant HIV-1 to TIBO R82150

Recombinant HIV-1 genotype	R82150 susceptibility, IC_{50}
	μM
Control (Arg^{83}, Leu^{100})	0.16
Ile^{100}	>30.0
Lys^{83}	0.15
Lys^{83}, Ile^{100}	>30.0

Discussion

These studies demonstrate that variants of HIV-1 that are resistant to TIBO R82150 rapidly dominant the replicating virus population in tissue culture when drug selective pressure is applied. The genetic basis for the R82150-resistant phenotype is a single nucleotide change (TTA to ATA) in the 1.68-kb coding sequence of HIV-1 RT. This change produces a single, conservative, amino acid substitution in RT, from Leu^{100} to Ile. The role of this substitution in R82150 resistance was unequivocally demonstrated by site-specific mutagenesis of RT and by introduction of the Ile^{100} mutation into an infectious proviral clone of HIV-1. A second amino acid variation (Arg^{83} to Lys) was seen in the majority of resistant RT clones, but this variation did not appear to contribute to RT or viral resistance to R81250. It remains possible that the Lys^{83} mutation confers a subtle replicative advantage in the presence of drug that is not detected by standard *in vitro* assays of RT or viral susceptibility.

The importance of Ile^{100} mutation in R82150 resistance was not suggested by previously published work. Several investigators (11–13, 24, 25) have reported that the Tyr^{181} and Tyr^{188} residues in RT are critical for TIBO inhibitory activity. Substitution at either of these tyrosines yields TIBO-resistant RT and virus. Thus, it might be predicted that one or both of these tyrosines would be substituted in resistant strains that emerge after selection with R82150. This prediction, however, is based on data derived from site-specific mutational analysis of RT (24, 25) and from resistant viral variants that have been isolated after selection with pyridinones (11) or nevirapine (12, 13) but not with TIBO derivatives. In the present study, selection with TIBO R82150 yielded resistant mutants without substitution of Tyr^{181} or Tyr^{188}. The reason for the dominance of the Ile^{100} mutation in R82150-selected virus is not clear, but the conservative leucine to isoleucine substitution may preserve RT function and thus viral replicative efficiency to a greater extent than the nonconservative tyrosine to cysteine substitution (11–13). Alternatively, structural differences between TIBO R82150 and nevirapine may alter the relative importance of specific residues on RT that are required for inhibitor binding and activity.

In summary, the collective evidence to date indicates that at least three amino acid residues in HIV-1 RT, *i.e.*, Leu^{100}, Tyr^{181}, and Tyr^{188}, are critical for TIBO R82150 activity. The recently published three-dimensional structure of HIV-1 RT (26) helps explain the relationship of these residues to each other and to the catalytic site of the enzyme. The Leu^{100}, Tyr^{181}, and Tyr^{188} residues lie within the "palm" subdomain of the polymerase cleft of RT near the putative catalytic site, which is composed of the three carboxylates of Asp^{110}, Asp^{185}, and Asp^{186}. Nevirapine is known to bind to a deep pocket at the junction between the "palm" and the "thumb" subdomains of RT and to be in

contact with the side chains of Tyr^{181} and Tyr^{188}. The partial cross-resistance of RT containing the Ile^{100} mutation to nevirapine (Table 3) suggests that nevirapine also interacts with the Leu^{100} residue. It is probable that TIBO R82150 binds in the same region as nevirapine, but interaction with Leu^{100} appears more critical. How binding of either of these compounds to RT blocks polymerase activity is currently unknown (26).

Although the R82150-resistant variants reported here were identified in an *in vitro* system, the conservative nature of the mutations required for resistance raises the possibility that similar variants will emerge in HIV-infected patients treated with TIBO derivatives. This possibility should be closely monitored in clinical trials of these compounds. Factors such as disease stage, viral burden, and the dose and duration of drug therapy will undoubtedly contribute to the frequency with which resistance develops (27).

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Send reprint requests to: John W. Mellors, M.D., Montefiore University Hospital, Infectious Diseases Unit, A-West, 3459 Fifth Avenue, Pittsburgh, PA 15213.