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ACCELERATED COMMUNICATION

In Vitro Selection and Molecular Characterization of Human Immunodeficiency Virus-1 Resistant to Non-nucleoside Inhibitors of Reverse Transcriptase

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SUMMARY

Several newly discovered potent and selective non-nucleoside inhibitors of human immunodeficiency virus-1 reverse transcriptase (RT) are undergoing evaluation in clinical trials. We studied the potential for development of viral resistance to one of the prototype compounds, BI-RG-587, a dipyridodiazepinone derivative. Human immunodeficiency virus-1 resistant to BI-RG-587 emerged after only one cycle of *in vitro* infection in the presence of the drug. Resistant virus was cross-resistant to the non-nucleoside tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione derivative R82150 but remained susceptible to 2′,3′-dideoxynucleosides and phosphonoformate. Both native (virion-associated) and recombinant RT derived from resistant virus were insensitive to BI-RG-587 and R82150. Nucleotide sequence analysis of multiple drug-resistant and -sensitive recombinant RT clones identified a single predicted amino acid change common to all resistant clones (tyrosine-181 → cysteine). These studies suggest that the viral resistance to non-nucleoside RT inhibitors may develop *in vivo*. This possibility should be carefully monitored in clinical trials of these compounds.

Recently, several promising classes of non-nucleoside inhibitors of HIV-1 RT (EC 2.7.7.49) have been discovered. The most active members of each class include the dipyridodiazepinone derivative BI-RG-587 (1, 2), the TIBO derivative R82150 (3, 4), and the pyridinone derivatives L-697,639 and L-697,661 (5). These compounds are potent and highly specific inhibitors of HIV-1 RT; they are not active against HIV-2, simian immunodeficiency virus, feline leukemia virus, avian myeloblastosis virus, or Moloney sarcoma virus (1–5). This highly specific activity against HIV-1 RT and not other retroviral RTs has also been noted for the acyclic nucleoside analogue HEPT and its derivatives (6, 7). This narrow spectrum of activity differs from the broad range of activity of AZT and other 2′,3′-dideoxynucleosides against mammalian retroviruses (8).

The development of resistance to antimicrobial drugs is a significant obstacle to the successful treatment of many infectious diseases. The reported experience with therapy of HIV-1 infection has been no exception to this theme. Strains of HIV-1 with reduced susceptibility to AZT have been frequently recovered from patients receiving long term AZT therapy (9, 10). Although the pathogenic significance of these isolates has not been proven, breakthrough of drug-resistant virus is a likely explanation for progressive HIV-1 disease on AZT therapy (11).

The potential for viral resistance must, therefore, be considered in the evaluation of new antiretroviral compounds. We

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ABBREVIATIONS: RT, reverse transcriptase; AZT, 3′-azido-3′-deoxythymidine; HEPT, 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine; sDDC, (±)-2′-deoxy-3′-thiacytidine; DDC, 2′,3′-dideoxycytidine; D4T, 2′,3′-didehydro-3′-deoxythymidine; DDI, 2′,3′-dideoxyinosine; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PFA, phosphonoformic acid; $TCID_{50}$, 50% tissue culture-infective dose; IC_{50}, 50% inhibitory concentration; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; ITPG, isopropyl-β-D-thiogalactopyranoside; TIBO, tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-thione; PCR, polymerase chain reaction; BI-RG-587, 11-cyclopropyl-5,-11-dihydro-4-methyl-6*H*-dipyridol[3,2-*b*:2′,3′-*e*]diazepin-6-one; 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; 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; MTT, 3-[4,5-diamethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

reasoned that resistance to BI-RG-587 or other non-nucleosides might readily develop because their specificity for HIV-1 RT, but not other retroviral RTs, indicates that they bind to a site on the enzyme that is neither conserved among retroviruses nor essential for RT activity. This report describes the detection and characterization of HIV-1 with reduced sensitivity to BI-RG-587 and TIBO R82150.

Materials and Methods

Cells. H9 cells (R. C. Gallo, National Cancer Institute, Bethesda, MD) and MT-2 cells (AIDS Research and Reference Reagent Program, NIAID, National Institutes of Health; contributed by D. Richman) were cultured in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, MD) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, and 20% dialyzed fetal bovine serum (JRH Biosciences, Lenexa, KS). HeLa-CD4 cells (AIDS Research and Reference Program, National Institute of Allergy and Infectious Disease, 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% dialyzed fetal bovine serum.

Virus. Stock preparations of HIV-1 (HTLV-IIIB strain; R. C. Gallo) were harvested from infected H9 cells by the "shake-off" method, as follows. Fifty milliliters of a virus-infected H9 culture were centrifuged at 400 × *g* for 10 min, to pellet the cells. The supernatant was discarded, and the cell pellet was resuspended in 5 ml of fresh medium and agitated vigorously by hand for 20 sec. The cells were pelleted at 600 × *g* for 10 min, and the supernatant containing virus was collected, clarified at 800 × *g* for 10 min, filtered (0.45 μm), aliquoted, and stored at −70°. The infectious titer of stock virus preparations was determined by triplicate end-point dilution in MT-2 cells (12). Virus-induced cytopathic effect (giant syncytium formation) was scored 5 days after infection. The $TCID_{50}$ was calculated with the Reed and Muench equation (13).

Compounds. BI-RG-587 and HEPT were obtained from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). The TIBO derivative R82150 was synthesized by K. Parker, Brown University (Providence, RI). BI-RG-587, HEPT, and TIBO R82150 were dissolved in 100% dimethyl sulfoxide and stored at −20°. PMEA was obtained from A. Holy, Czechoslovakia Academy of Science. sDDC was obtained from IAF Biochemical International (Montreal, Canada). DDC was purchased from Pharmacia Inc. (Piscataway, NJ). D4T and DDI were obtained from 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, sDDC, DDC, D4T, DDI, AZT, and PFA were dissolved in sterile water and stored at −20°. AZTTP was obtained from R. Schinazi, Emory University (Atlanta, GA).

RT assays. Assays of virion-associated RT were performed on 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, 250 μg/ml heat-activated bovine serum albumin, 6.25 μM [^{3}H]dTTP (16 Ci/mmol; New England Nuclear, Wilmington, DE), and 0.5 A_{260} units/ml poly(rA)-oligo$(dT)_{10}$ as the template-primer (Pharmacia). Recombinant RT activity in soluble extracts of *Escherichia coli* JM 109 was assayed in a 50-μl volume containing 5 μl of extract (diluted 10–500-fold), 50 mM Tris·HCl (pH 7.8), 50 mM KCl, 6 mM $MgCl_2$, 0.5 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). Reactions were incubated at 37° for 60 min. Incorporation of radiolabel was linear during this interval. Trichloroacetic acid-precipitable counts were quantitated by liquid scintillation counting, as described (14). RT activity was determined in the presence and absence of serial dilutions of inhibitor.

Drug susceptibility assays in MT-2 and HeLa-CD4 cells. Drug-mediated inhibition of virus-induced cytotoxicity was assayed in MT-2 cells as described, with modification (12). Triplicate wells of 96-well plates containing 1 × 10^4 MT-2 cells were infected with HIV-1 at a multiplicity of 0.5 $TCID_{50}$/cell. Serial dilutions of drug were added immediately after infection. Cell viability was quantitated 7 days after infection with the MTT-dye reduction method (12). The percentage of protection was calculated with the formula $[(a - b/c - b) \times 100]$, in which a = the A_{570} of drug-treated, virus-infected wells, b = A_{570} of no-drug, infected wells, and c = the A_{570} of no-drug, uninfected wells. The drug IC_{50} was calculated from linear-log_{10} plots of percentage of protection versus inhibitor concentration.

Plaque reduction assays in HeLa-CD4 cells were performed as described, with slight modification (12). Triplicate wells of 24-well plates containing 3.5 × 10^4 cells/well were treated with serial drug dilutions, starting 1 hr before inoculation with HIV-1. The viral inoculum was adjusted to produce ~100 plaques (multinucleated giant cells) in no-drug control wells. Plates were fixed with 10% formalin, 4 days after infection, and were stained with Diff-Quick (Baxter Scientific Products, McGaw Park, IL). Plaques were counted at 40× magnification, with the aid of an ocular grid (Olympus Corp., Lake Success, NY). The percentage plaque reduction was calculated with the equation $[(a - b/a) \times 100]$, in which a = the plaque number in no-drug control wells and b = the plaque number in drug-treated wells.

Cloning and expression of HIV-1 RT. A diagram of the scheme used to PCR amplify and molecularly clone HIV-1 RT genes from drug-resistant and -sensitive viruses is shown in Fig. 1. Total RNA was extracted from virus-infected H9 cells with the guanidinium method (15) and was used for cDNA synthesis with Moloney murine leukemia virus RT (Bethesda Research Laboratories, Gaithersburg, MD) and a 3′ primer (primer B, 5′CTTATCTATTCCATCTAGAAATAGT-3′) (16). cDNA was synthesized in a reaction mixture (20 μl) of 50 mM KCl, 4.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 10 μg/ml gelatin, 1 mM each of dATP, dGTP, dCTP, and dTTP, 20 units of placental ribonuclease inhibitor, 50 pmol of primer B, 2 μg of total RNA, and 200 units of Moloney murine leukemia virus RT. Reactions were incubated for

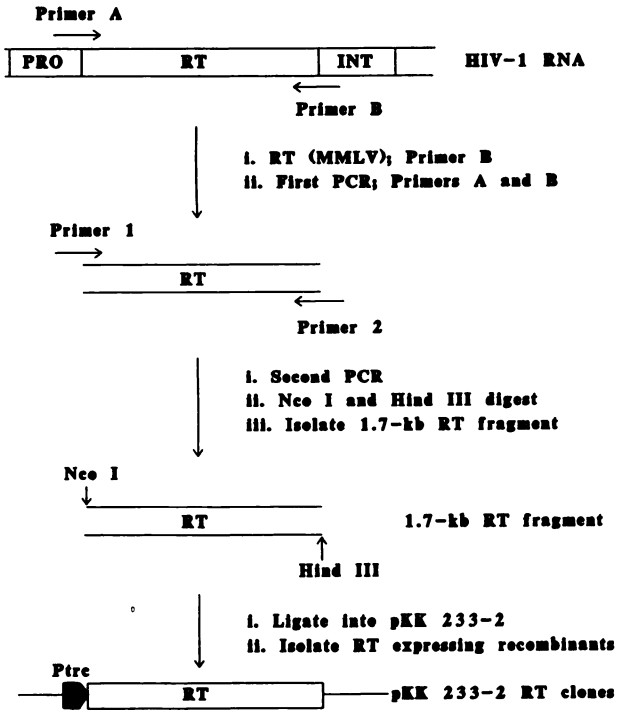

Fig. 1. Diagram of the scheme used to PCR amplify and molecularly clone HIV-1 RT. See Materials and Methods for description. *Ptrc*, *trc* promotor; *PRO*, protease; *INT*, integrase; *MMLV*, Moloney murine leukemia virus.

60 min at 37°. The 1.7-kilobase RT cDNA was then PCR amplified with the addition of a 5′ primer (primer A, 5′-TTGCACTTTGAATTCTCCCATTAG-3′) (16). PCR amplifications were carried out in a 100-μl mixture of 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 2 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 10 μg/ml gelatin, 50 pmol of primers A and B, and 2.5 units of Taq-1 DNA polymerase (Perkins-Elmer Cetus, Norwalk, CT). Reaction mixtures were heated at 94° before addition of Taq-1 polymerase, overlaid with 100 μl of mineral oil, and subjected to 30 amplification cycles, consisting of a denaturation step (1 min, 94°), a primer annealing step (1 min, 50°), and a DNA synthesis step (5 min, 72°), in a programmable thermal cycler (Perkin-Elmer Cetus). Amplified DNA from the first PCR reaction was diluted 10,000–100,000-fold and used as template for a second PCR amplification with primers 1 and 2, which contain the *Nco*I and *Hind*III restriction sites, respectively (primer 1, 5′-TGCCATGGCCATTAGCCCTATGAGACTGT-3′; primer 2, 5′-CGAAGCTTTATAGTATTTTCCTGATTCCAGCACTG-3′) (17). The second PCR product was digested with *Nco*I and *Hind*III, purified from agarose gels, ligated into the plasmid expression vector pKK 233-2 (digested with *Nco*I and *Hind*III), and used to transform *E. coli* JM 109, as described (17, 18). Ampicillin-resistant transformants were grown to late logarithmic phase, and RT expression was induced for 6 hr with 0.5 mM ITPG. Soluble extracts of bacterial lysates were prepared (17) and screened for RT activity.

Nucleotide sequencing. HIV-1 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). PCR primers 1 and 2 (described above) and four internal primers were used for the sequencing reactions [primer 3 (+ strand), 5′-TCAGTAACAGTACTGGATGTGGGTG-3′; primer 4 (+ strand), 5′-ATCTGTTGAGGTGGGGACTTACCAC-3′; primer 5 (+ strand), 5′-AGAAAACAGAGAGATTCTAAAAGAA-3′; primer 6 (− strand), 5′-CTATTAGTAACATATCCTGCTTTTC-3′].

Results

To evaluate the potential for viral resistance to BI-RG-587, H9 target cells (1×10^7) were infected with HIV-1_{IIIB} (1×10^6 $TCID_{50}$) in the presence of 1.0 μM BI-RG-587 (≃25 times the IC_{50}) (1). The cells were washed 2 hr after infection and maintained in medium containing 1.0 μM BI-RG-587. Cell-free viral progeny were harvested 4 days later and used to initiate a new cycle of infection. After each cycle of infection, breakthrough virus was screened for drug sensitivity by quantitation of viral infectivity in the presence and absence of 1.0 μM BI-RG-587. Table 1 shows that the drug sensitivity of breakthrough virus had shifted dramatically after only one cycle of infection in the presence of drug. The drug sensitivity of parental virus (HIV-1_{IIIB}) passaged in parallel in the absence of drug did not change (data not shown). More detailed studies of drug susceptibility in MT-2 cells and HeLa-CD4 cells demonstrated that the IC_{50} of virus passaged twice in the presence of drug had increased >100-fold, compared with parental HIV-1_{IIIB} (Fig. 2). BI-RG-587-resistant virus replicated efficiently to high titer (>10^6 $TCID_{50}$/ml) in the presence of 1 μM drug (Table 1) and induced syncytia in MT-2 and HeLa-CD4 cells with the same kinetics and morphology as parental virus. Selection of resistant virus was repeated starting with parental HIV-1_{IIIB}; resistant virus again emerged after one cycle of infection in the presence of drug (data not shown).

TABLE 1

Altered drug sensitivity of HIV-1 passaged serially in the presence of BI-RG-587

Virus passage[a]	Infectivity[b]	Reduction in infectivity by 1 μM BI-RG-587[c]
	log_{10} $TCID_{50}$/ml	log_{10}
0	5.7	2.4
1	2.2	0
2	2.3	0
3	2.6	0
2 (stock)	6.1	0

[a] Number of viral passages in the presence of 1.0 μM BI-RG-587.

[b] Virus was harvested 4 days after infection, except for passage 2 stock virus, which was harvested on day 7. Infectivity was determined by triplicate end-point dilution in MT-2 cells (12).

[c] Calculated by subtracting the infectivity titer determined in the presence of 1 μM BI-RG-587 from the titer determined in the absence of drug.

The susceptibility of virion-associated RT to inhibition by BI-RG-587 was studied in detergent-disrupted preparations of parental and resistant virus, with poly(rA)-oligo$(dT)_{10}$ as the template-primer. RT activity from parental virus was inhibited by BI-RG-587 in a concentration-dependent manner, with an IC_{50} value of ≃3.3 μM (Fig. 3). In contrast, RT activity from resistant virus was not inhibited by BI-RG-587 at concentrations up to 10 μM.

The activity of other antiretroviral compounds against BI-RG-587-resistant virus was examined. Table 2 shows that resistant virus was cross-resistant to TIBO R82150 (≃50-fold shift in IC_{50}) and to HEPT (≥5-fold shift in IC_{50}). BI-RG-587-resistant virus remained susceptible to the nucleoside analogues AZT, D4T, DDI, DDC, sDDC (20), and PMEA (21) and the pyrophosphate analogue PFA.

To investigate the genetic basis of BI-RG-587 resistance, the full-length 1.7-kilobase coding sequence of HIV-1 RT was PCR

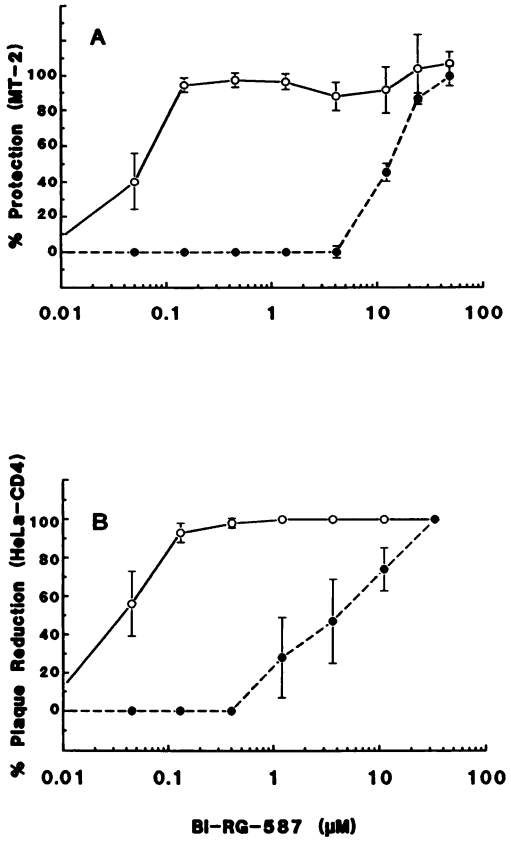

Fig. 2. Susceptibilities of parental (○) and resistant (●) HIV-1_{IIIB} to BI-RG-587, assayed in MT-2 cells (A) and in HeLa-CD4 cells (B). Mean values ± 1 SD are shown. Assays were performed as described in Materials and Methods.

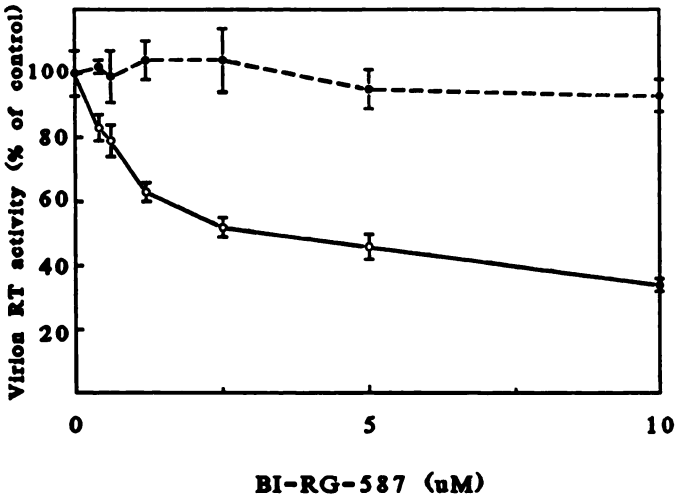

Fig. 3. Susceptibility of virion-associated RT from parental (○) and resistant (●) HIV-1_{MB} to inhibition by BI-RG-587. RT assays were performed as described in Materials and Methods.

TABLE 2

Sensitivity of BI-RG-587-resistant HIV-1 to other antiretroviral compounds

Drug susceptibilities were determined in MT-2 cells, as described in Materials and Methods. IC_{50} values were calculated from linear-log_{10} plots of percentage protection versus inhibitor concentration.

Compound	IC_{50}	
	Parental HIV-1_{MB}	BI-RG-587-resistant HIV
BI-RG-587	0.12	>11.1
TIBO R82150	0.13	6.3
HEPT	20.1	>100
AZT	0.11	0.13
D4T	2.4	3.5
DDC	0.94	0.84
sDDC	3.9	3.6
DDI	17.1	20.8
PMEA	10.8	17.6
PFA	229	281

amplified from viral RNA after cDNA synthesis (Fig. 1). The PCR product was subcloned into the pKK 233-2 expression vector and used to transform *E. coli* JM 109 (Fig. 1). About 40% of ampicillin-resistant bacterial colonies derived from parental or drug-resistant viral RNA expressed high level RT activity after ITPG induction. Recombinant RTs from >10 parental and resistant clones were examined for sensitivity to BI-RG-587, using poly(rC)-oligo$(dG)_{12-18}$ as the template-primer (Fig. 4). All RTs (13 of 13) cloned from resistant viral RNA showed reduced sensitivity to BI-RG-587, with the majority (8 of 13) exhibiting >90% of control activity in the presence of 600 nM BI-RG-587. In contrast, RTs (12 of 12) cloned from parental viral RNA had <50% of control activity in the presence of 600 nM BI-RG-587. Similar results were obtained with poly(rA)-oligo$(dT)_{10}$ as the template-primer (data not shown). Cloned RTs (13 of 13) from resistant virus were cross-resistant to TIBO R82150 but as sensitive as parentally derived RTs to PFA and AZTTP (Table 3).

DNA sequencing of six parental and nine BI-RG-587-resistant RT clones identified a single base pair change common to all resistant clones. This change alters the predicted amino acid at position 181 from tyrosine to cysteine (TAT → TGT). A total of seven additional amino acid changes were identified in

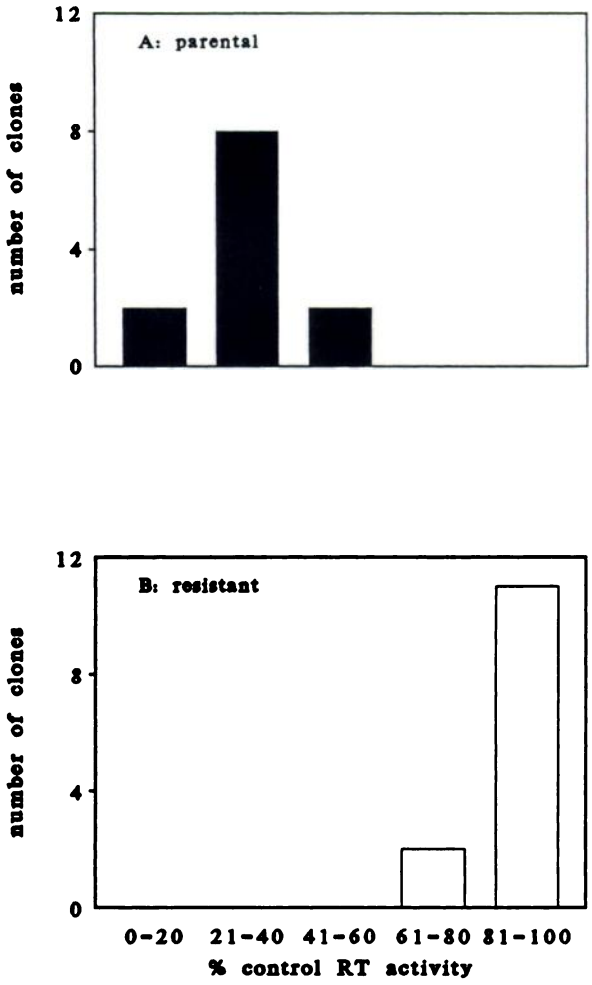

Fig. 4. Sensitivities of cloned recombinant RTs from parental (A) and resistant (B) HIV-1_{MB} to BI-RG-587 (600 nM). RT assays were performed as described in Materials and Methods.

TABLE 3

Sensitivities of recombinant HIV-1 RTs to PFA and AZTTP

Drug (concentration)	Type of RT clone[a]	% Control RT activity[b]				
		0–20%	21–40%	41–60%	61–80%	81–100%
		Number of RT clones				
PFA (6 μM)	Parental	10	1	1	0	0
	Resistant	12	1	0	0	0
AZTTP (20 nM)	Parental	1	6	2	2	1
	Resistant	0	11	2	0	0

[a] RT clones were derived from parental HIV-1_{MB} or BI-RG-587-resistant HIV-1.
[b] Reaction conditions were as described in Fig. 4, except that poly(rA)-oligo$(dT)_{10}$ was the template-primer.

the nine resistant RT clones, but none of these changes were common to more than one clone. The tyrosine-181 residue lies within the predicted polymerase domain of RT (22) and is absolutely conserved in all previously reported HIV-1 strains (23), strongly implicating its substitution in BI-RG-587 resistance. In addition, analysis of the predicted amino acid sequences of HIV-2, simian and murine retroviral RTs reveals the absence of tyrosine at the analogous position (22). This may explain the lack of activity of BI-RG-587 against these retroviral RTs.

Discussion

These studies demonstrate that HIV-1 with reduced susceptibility to BI-RG-587 can be readily selected *in vitro*. Resistance to BI-RG-587 was associated with a single amino acid change ($Tyr^{181} \rightarrow Cys$) in the conserved polymerase domain of HIV-1 RT. The rapidity with which resistant virus emerged in culture (4 days) suggests that the drug-resistant viral phenotype was pre-existent within the initial viral inoculum (1×10^6 $TCID_{50}$). Using a similar procedure, we have also readily selected HIV-1 that is resistant (>100-fold) to TIBO R82150.[1] Thus, the rapid development of viral resistance is not unique to BI-RG-587 and may be a common property of narrow spectrum RT inhibitors.

Recently, Nunberg *et al.* (24) reported the successful *in vitro* isolation of HIV-1 variants that are >1,000-fold resistant to pyridinone derivatives. Compounds of this class are also specific inhibitors of HIV-1 RT but not other retroviral RTs. Pyridinone-resistant virus was cross-resistant to BI-RG-587 and TIBO R82150. These investigators identified two amino acid changes in HIV-1 RT that were responsible for resistance, $Lys^{103} \rightarrow Arg$ and the $Tyr^{181} \rightarrow Cys$ substitution we have detected. Of the two mutations, the $Tyr^{181} \rightarrow Cys$ mutation was quantitatively more important (~800-fold pyridinone resistance) than the $Lys^{103} \rightarrow Arg$ change (~20-fold resistance). We did not detect the $Lys^{103} \rightarrow Arg$ substitution in any of the nine BI-RG-587-resistant clones examined. This mutation may be either specific for pyridinone resistance or observed only at higher degrees of BI-RG-587 or TIBO R82150 resistance.

Although these studies were performed in a cell culture system with laboratory strains of HIV-1, they raise the possibility that resistance to non-nucleoside RT inhibitors will develop *in vivo*. This possibility should, therefore, be carefully monitored in clinical trials of these compounds. Factors such as disease stage, viral burden, and duration of drug therapy will undoubtedly contribute to the frequency with which resistance develops *in vivo* (10). The finding that BI-RG-587-resistant virus is cross-resistant to both TIBO R82150 and HEPT suggests that these compounds have a similar site of action and would not be useful in combination. The lack of cross-resistance to 2′,3′-dideoxynucleosides is indicative of a distinct mechanism of action for these analogues. Combination therapy with a non-nucleoside derivative and a dideoxynucleoside may, therefore, provide synergistic antiviral activity (25) and forestall the development of viral resistance.

In contrast to the rapid emergence of BI-RG-587-resistant virus in the present studies, selection of AZT-resistant strains of HIV-1 *in vitro* has proved difficult (6, 13), although recent success has been reported after multiple passages (26). The reason for this difference is not clear but may relate to 1) the number of mutations in RT required for drug resistance and 2) the requirement for cellular activation of AZT but not BI-RG-587 (1). A combination of three or four nonconservative amino acid substitutions is required for high level (>100-fold) AZT resistance (16). One of these changes requires the rare occurrence of a two-nucleotide change within the same codon. In comparison, a single base change appears to be sufficient for BI-RG-587 resistance. Thus, the frequency of drug-resistant variants within an unselected virus population is probably much lower for AZT than for BI-RG-587. In addition, to amplify rare drug-resistant variants to detectable levels, antiviral selective pressure must be consistently applied to the virus population. Selective pressure with AZT may be difficult to maintain *in vitro*, because cell lines cultured in AZT may undergo adaptive changes in kinase function (e.g., loss of thymidine kinase activity) that reduce the anticellular as well as antiviral activity of AZT. This could allow breakthrough replication of nonresistant viral strains. Support for this concept is derived from the report by Pagano and co-workers (27) that breakthrough replication of HIV-1 in H9 cells in the presence of high AZT concentrations is due to drug-sensitive and not drug-resistant virus. Because BI-RG-587 does not appear to require cellular metabolism to inhibit HIV-1 RT (1), antiviral selective pressure is more likely to be maintained, resulting in specific amplification of drug-resistant viral variants.

A paradox has been noted with regard to AZT-resistant HIV-1. Virion-associated and cloned recombinant RT from AZT-resistant virus shows no apparent reduction in sensitivity to AZTTP in standard assays of enzyme activity (9, 16). Our studies with native and recombinant RT demonstrate a clear correlation between viral and RT sensitivity to BI-RG-587. This correlation provides convincing evidence that viral RT is the major site of action of this compound. The reasons for the dissociation between viral and enzyme sensitivity to AZT remain to be elucidated but may be related to a more complex mechanism of action of AZTTP that is not adequately represented in standard *in vitro* enzyme inhibition assays.

Note Added in Proof. After acceptance of this manuscript, Richman *et al.* (28) published a similar report of the emergence of HIV-1 mutants resistant to B1-R6-587 in tissue on culture. Resistant mutants were cross-insistant to T1130 derivatives, and encoded line $Try^{181} \rightarrow Cys$ change. This substitutor dramatically reduced the binding of radiolabeled B1-R6-S87 to RT.

Acknowledgments

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