JOURNAL OF VIROLOGY, June 1996, p. 3698–3705 Vol. 70, No. 6
0022-538X/96/$04.00+0

(Alkylamino)Piperidine Bis(Heteroaryl)Piperizine Analogs Are Potent, Broad-Spectrum Nonnucleoside Reverse Transcriptase Inhibitors of Drug-Resistant Isolates of Human Immunodeficiency Virus Type 1 (HIV-1) and Select for Drug-Resistant Variants of HIV-1_{IIIB} with Reduced Replication Phenotypes

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Received 22 December 1995/Accepted 11 March 1996

The (alkylamino)piperidine bis(heteroaryl)piperizines (AAP-BHAPs) are a new class of human immunodeficiency virus type 1 (HIV-1)-specific inhibitors which were identified by targeted screening of recombinant reverse transcriptase (RT) enzymes carrying key nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance-conferring mutations and NNRTI-resistant variants of HIV-1. Phenotypic profiling of the two most potent AAP-BHAPs, U-95133 and U-104489, against in vitro-selected drug-resistant HIV-1 variants carrying the NNRTI resistance-conferring mutation (Tyr→Cys) at position 181 of the HIV-1 RT revealed submicromolar 90% inhibitory concentration estimates for these compounds. Moreover, U-104489 demonstrated potent activity against BHAP-resistant HIV-1_{MF} harboring the Pro-236→Leu RT substitution and significantly suppressed the replication of clinical isolates of HIV-1 resistant to both delavirdine (BHAP U-90152T) and zidovudine. Biochemical and phenotypic characterization of AAP-BHAP-resistant HIV-1_{IIIB} variants revealed that high-level resistance to the AAP-BHAPs was mediated by a Gly-190→Glu substitution in RT, which had a deleterious effect on the integrity and enzymatic activity of virion-associated RT heterodimers, as well as the replication capacity of these resistant viruses.

The emergence of drug-resistant variants presents a major concern for the development and clinical utility of agents against human immunodeficiency virus type 1 (HIV-1). The rapidity with which these variants of HIV-1 are selected in vivo by nucleoside (for a review, see reference 26) and nonnucleoside (26, 27, 31, 35) inhibitors of reverse transcriptase (RT), as well as viral protease inhibitors (6), can be explained by recent studies of the dynamics of HIV-1 replication in infected individuals which revealed extremely high rates of virus replication and turnover (14, 35). High levels of replication combined with the infidelity of the RT DNA polymerase inevitably lead to the generation of the viral quasispecies from which drug-resistant variants are selected during drug therapy.

Considerable effort has been focused on the development of the HIV-1-specific nonnucleoside RT inhibitors (NNRTIs) as therapeutic treatments for HIV-1 infection and AIDS because of their desirable safety, selectivity, and antiviral activity profiles. However, the rate of resistance development in vitro (2, 19, 22, 28) and in vivo (27, 31, 35) has tempered enthusiasm for the potential clinical utility of this structurally diverse group of potent noncompetitive inhibitors, which includes the bis(heteroaryl)piperizines (BHAPs) (7, 29), pyridinones (12), nevirapine (21), α-anilinophenylacetamide (α-APA) (24), and tetrahydroimidizo[4,5,1-*jk*][1,4]-benzodiazepin-2(1H)- thione (TIBO) (25). When early studies showed that NNRTI-resistant HIV-1 variants selected with one drug displayed considerable cross-resistance to other NNRTIs (18, 22, 28, 32), the view that development of resistance to one inhibitor would preclude the utility of other NNRTIs for follow-up therapy emerged. For example, the Tyr-181→Cys substitution in RT confers broad cross-resistance to most NNRTIs currently in development (22, 28). However, this and other RT substitutions that confer NNRTI resistance (20) do not have equivalent effects on all NNRTIs (1, 8). For example, a Pro-236→Leu substitution in RT confers resistance to BHAP U-90152T but increases sensitivity to other NNRTIs (e.g., pyridinone L-697,661 and TIBO R82913) (8, 10). The Pro-236→Leu substitution also diminished the resistance conferred to L-697,661 by the Tyr-181→Cys substitution in recombinant RT assays. These studies suggested that treatment with one NNRTI could be followed effectively with other NNRTIs still able to inhibit virus resistant to the initial drug.

On the basis of these results, we set out to identify compounds with broad activity against several NNRTI-resistant variants of HIV-1. This report describes the identification of (alkylamino)piperidine-BHAP (AAP-BHAP) derivatives that have marked activity in vitro against RTs carrying key NNRTI resistance-conferring mutations (e.g., Pro-236→Leu, Tyr-181→Cys, Lys-103→Asn, and Leu-100→Ile) as well as wild-type (WT) RT. The AAP-BHAP U-104489 demonstrated potent activity against a panel of NNRTI-resistant HIV-1 variants and significantly suppressed the replication of clinical isolates of HIV-1 resistant to both delavirdine (DLV) and zidovudine (AZT). In vitro drug resistance selection studies revealed that

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resistance to the AAP-BHAPs was mediated by a Gly-190→Glu substitution in RT. Biochemical and phenotypic characterization of these AAP-BHAP-resistant HIV-1 variants linked the Gly-190→Glu substitution to decreased integrity and activity of virion-associated RT heterodimers and significantly diminished the replication capacity of these resistant viruses.

MATERIALS AND METHODS

Compounds. U-90152T, L-697,661 (pyridinone), and α-APA (R88703) were synthesized by previously published methods (12, 25, 29). The methods for the AAP-BHAP syntheses (U-92884, U-95133, and U-104489) will be described elsewhere (30). AZT was purchased from Sigma. Compound stocks (10 and 30 mM) were prepared in dimethyl sulfoxide and kept frozen at −20°C.

Cells and viruses. The T-lymphoid cell line MT4 and cell-free virus HTLV-IIIB/H9 (HIV-1_{IIIB}) were provided by Douglas Richman and Robert Gallo, respectively, and obtained through the AIDS Research and Reference Reagent Program. Human peripheral blood mononuclear cells (PBMCs) were prepared and maintained as previously described (4). The HIV-1_{MF} strain was provided by Mario Stevenson (34). Stock viruses were prepared in MT4 cells maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (RPMI/FBS). Infectious titers (50% tissue culture infectious dose [$TCID_{50}$] per milliliter) for individual cell-free virus stocks were determined in MT4 cells by p24 enzyme-linked immunosorbent assays (ELISAs) (Coulter).

Antiviral assay. Assessment of the activities of the NNRTIs versus the panel of viruses used in these studies was carried out mainly in MT4 cells. Cells were batch infected with the appropriate virus stock at a multiplicity of infection of 0.001 to 0.005 $TCID_{50}$ per cell for 2 h at 37°C. The cells were washed, resuspended in RPMI/FBS, and plated in 24-well dishes at a final concentration of 1.5×10^5 cells per ml to which were added 2×-concentrated drug treatments prepared in RPMI/FBS. All treatment concentrations were tested in duplicate. The final dimethyl sulfoxide concentration for all treatments or vehicle control cultures was 0.1%. At 4 days postinfection, culture fluid samples were collected for HIV-1 p24 core antigen quantitation to determine antiviral effects. Antiviral assays utilizing PBMC cultures were performed where indicated in the text and as described previously (4). Linear regression analysis was used to calculate the drug concentration necessary to inhibit 90% of non-drug-treated p24 antigen production (90% inhibitory concentration [IC_{90}]).

Selection of drug-resistant variants. MT4 cells were infected with HIV-1_{IIIB} or HIV-1_{MF} as described above for the antiviral assay and initially treated with a concentration of NNRTI approximating its IC_{50}. Infected cells were serially passaged (1:4), typically every 3 to 4 days. Each passage was initiated by pelleting one-quarter of the cells, resuspending in fresh medium with drug, and supplementing with uninfected cells to yield a cell density of $\sim 3 \times 10^5$ to 5×10^5/ml. The infections were monitored by the progression of the cytopathic effect (i.e., syncytium formation) and p24 production. Inhibitor concentrations were increased by 0.5 $\log_{10}$ increments during the passage series when p24 antigen levels approached 10^6 pg/ml. Resistant virus stocks were harvested once growth was sustained in the respective NNRTI (10 μM).

Genotypic analyses of NNRTI-resistant HIV-1_{IIIB} variants. Purification of virion RNAs from NNRTI-resistant variant virus-infected cell culture supernatants (0.1×10^6 to 1×10^6 pg of p24 per ml) was performed by extracting 250 μl of supernatant with Tri Reagent LS (Molecular Research Center, Inc.), extracting with chloroform, and precipitating with isopropanol. Glycogen served as the carrier during the precipitation. The pelleted RNA was resuspended in 50 μl of diethylpyrocarbonate-treated water. For RT-PCR, 1.0 μl of resuspended RNA was used for cDNA synthesis primed by random hexamers, followed by PCR amplification with a biotinylated upstream primer and nonbiotinylated downstream primer (8). PCR products were immobilized on streptavidin-coated magnetic beads, denatured with NaOH to yield single-stranded DNA, and sequenced by using Sequenase version 2.0. PCR fragment containing RT codons 1 through 255 was cloned into pCR II plasmid (Invitrogen, San Diego, Calif.). Plasmid RT clones were sequenced by using minipreparation (Instaprep [5 Prime→3 Prime, Boulder, Col.) DNA as the template for Sequenase version 2.0.

Generation, expression, and purification of mutant RTs. The *Eco*RI-*Hin*dIII fragment of DE 5.2 (33), which contains the entire RT coding region downstream from six histidine codons, was cloned into the *Eco*RI and *Hin*dIII sites of M13mp18. Site-directed mutagenesis was performed by using the Sculptor in vitro mutagenesis system (Amersham Life Science) according to kit instructions. M13 replicative-form DNAs containing the desired mutations were digested with *Eco*RI and *Hin*dIII to yield the 1.7-kb RT coding fragments which were gel purified and ligated into the vector pKK223-3. The quadruple mutant Thr-139→Ile/Gly-190→Glu/Thr-200→Ala/Leu-214→Phe was generated by replacing the *Eco*RV-*Hin*dIII fragment (codon 144 through the carboxy-terminal codon) from the Thr-139→Ile mutant in pKK223-3 with the same fragment from the triple mutant Gly-190→Glu/Thr-200→Ala/Leu-214→Phe. All plasmids were propagated in *Escherichia coli* DH5α.

Recombinant HIV-1 RT assays. Drug inhibition assays using purified RT preparations were performed as described previously (8, 33). Briefly, either p66 homodimer or HIV-1 protease-processed p66-p51 heterodimer was used at approximately 40 μM to incorporate [^{3}H]thymidine via reverse transcription of a poly(rA) · oligo(dT) template primer in the presence of various concentrations of inhibitor.

Virus replication studies in MT4 cells and PBMCs. Two replication experiments were performed in MT4 cells. Duplicate infections with each virus were done as follows: titered virus stocks were diluted in cell culture medium to an estimated infectious titer concentration of 1,000 $TCID_{50}$s/ml and incubated (100 μl) with 4×10^5 MT4 cells for 2 h at 37°C. Following incubation, extracellular virus was removed by two washes with phosphate-buffered saline (PBS) (5 ml), and the washed cell pellets were resuspended in 2 ml of RPMI/FBS. Each cell suspension was distributed to two wells (1 ml per well) in 24-well dishes and incubated at 37°C in 5% CO_2. At day 4 postinfection, each culture was split 1:3 by removal of two-thirds of each culture volume after mixing and replacing with fresh RPMI/FBS. Culture fluids were sampled for p24 core antigen quantitation at several time points out to 7 days postinfection. To confirm the amount of infectious virus used to initiate the infections, $TCID_{50}$ estimations for the virus inocula were performed in parallel in MT4 cells during the course of each experiment. For replication comparison purposes, the inoculum titrations provided estimations for the normalization and expression of p24 production as picograms of p24 per $TCID_{50}$.

To generate virus stocks for comparison of the replication phenotypes of mutant HIV-1_{IIIB}-104489^r and WT (HIV-1_{IIIB}) virus in PBMCs, the MT4 cell-derived U-104489-resistant and WT viruses were passaged once in PBMCs in the presence and absence of 10 μM U-104489, respectively. For this experiment, the inocula used to initiate the infections were prepared by dilution of the virus stocks to identical p24 concentrations, as an estimate of equivalent amounts of virion particles. Briefly, 10^6 phytohemagglutinin-stimulated PBMCs were incubated with 100 μl of virus stock, diluted to 5×10^5 pg of p24 per ml, for 2 h at 37°C. Following incubation, the cells were washed until they were free of extracellular virus and plated at 0.5×10^6 cells per ml in RPMI/FBS plus human recombinant interleukin-2 (80 U/ml). The HIV-1_{IIIB}-104489^r infection was carried out in the absence of U-104489. Culture fluids were sampled for p24 at several time points postinfection. One-half volume medium (only) changes were performed on days 3, 7, and 11 postinfection.

Virion-associated RT assay. The virion-associated RT activities of several NNRTI-resistant variants derived for this study were assessed by an enzyme-linked oligonucleotide hybridization technique as described previously (9) (RT-DETECT Reverse Transcriptase Assay; Dupont Medical Products, Billerica, Mass.). For preparation of the virion lysates, virus particles were pelleted from culture supernatants (0.5 to 1.0 ml; $\sim 10^6$ pg of p24 per ml) in an Eppendorf microcentrifuge (model 5415c; 60 min, 13,000 rpm), lysed in 45 μl of detergent-containing buffer from the kit and stored at −20°C until assayed. An aliquot of each lysate was assayed by p24 ELISA prior to the RT assay.

Western blot (immunoblot) analysis of virion-associated RT heterodimers. Cell-free supernatants from infected MT-4 cell cultures were spun in a microcentrifuge for 45 min at room temperature to pellet virions. Virus pellets were resuspended in PBS, and aliquots of each pellet were assayed to determine p24 concentrations. Equivalent amounts of each virus preparation, as measured by p24 content, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for Western blot analysis. Binding of rabbit polyclonal HIV-1 RT antibody (Intracell) to virion-associated RT subunits was detected by sequential incubation with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium; Kirkegaard & Perry Laboratories, Inc.).

RESULTS

Identification of the AAP-BHAPs as potent inhibitors of NNRTI-resistant RTs. The strategy implemented to search for broad-spectrum inhibitors involved the in vitro screening of a diverse series of BHAP analogs against recombinant RTs carrying either the Pro-236→Leu or Tyr-181→Cys substitution; these substitutions represent RT genotypes known to confer high-level BHAP resistance and broad NNRTI cross-resistance, respectively. Lead templates active in the primary screening were then assayed against a select panel of mutant RTs carrying single amino acid substitutions (i.e., Leu-100→Ile, Lys-101→Glu, Lys-103→Asn, Val-106→Ala, Tyr-188→Cys, Tyr-188→His, Glu-233→Val, and Lys-238→Thr) important in conferring resistance to the NNRTI classes of compounds (20). From the screening effort, two lead AAP-BHAP compounds, U-92884 and U-95133 (Fig. 1), were identified as potent inhibitors of both panels of mutant RTs (data not shown). Structure-activity relationship studies yielded the most potent AAP-BHAP analog, U-104489, which is the *N*-ethyl homolog of U-95133 (Fig.

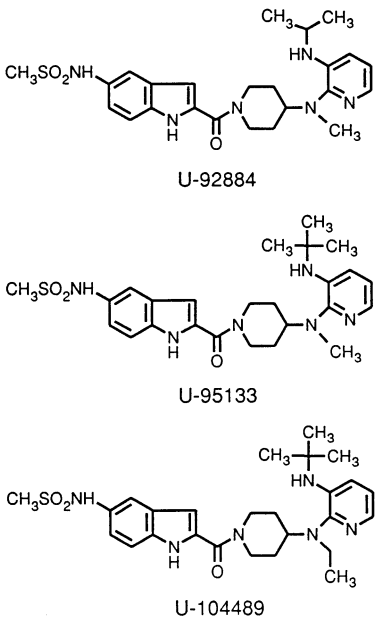

FIG. 1. Chemical structures of the U-92884, U-95133, and U-104489 AAP-BHAPs.

1). A detailed characterization of the AAP-BHAPs with respect to their antiviral activities and selection of virus resistance is described below.

AAP-BHAP RTIs potently block the replication of several drug-resistant HIV-1. In Table 1, the antiviral activities (IC_{90}s) of the AAP-BHAPs U-95133 and U-104489 are compared with the activities of the BHAP U-90152T (DLV), L-697,661 (pyridinone), and α-APA (R88703) against four cell-culture-selected NNRTI-resistant HIV-1 variants. The DLV-resistant HIV-1_{MF} variant, HIV-1_{MF}-BHAP^r, carrying the Pro-236→Leu substitution, showed considerable cross-resistance to the early AAP-BHAP derivatives U-92884 (data not shown) and U-95133, whereas the later generation derivative U-104489 displayed potent inhibitory activity against this variant. The two variants resistant to pyridinone and α-APA, both of which have the broad NNRTI cross-resistance-conferring mutation Tyr-181→Cys RT, and a second DLV-resistant virus having substitutions at amino acids 100 and 230 demonstrated significant cross-resistance to the antiviral effects of L-697,661, α-APA, and DLV. In contrast, these three variants remained susceptible in vitro to submicromolar concentrations of both AAP-BHAPs, with estimated IC_{90}s for U-104489 from 30 to 80 nM.

TABLE 2. Antiviral activity of U-104489 against clinical isolates of HIV-1 resistant to both DLV and AZT

RTI	IC_{90} (μM)[a]			
	HIV-1/#410	HIV-1/#472	HIV-1/#458	HIV-1/#461
U-104489	1.1	0.18	0.03	0.03
DLV	>10	>10	7.7	10.0
AZT	6.6	1.4	0.3	0.3

[a] IC_{90}, concentration of drug that inhibited p24 production by 90% in infected PBMC cultures.

A more critical assessment of the antiviral effectiveness of U-104489 is provided in Table 2. Shown are the antiviral activities of AAP-BHAP U-104489, DLV, and AZT against clinical isolates obtained from patients who had received 24 to 36 weeks of combination therapy with DLV and AZT. Although these isolates were highly resistant to both DLV and AZT, U-104489 demonstrated potent submicromolar activity against three of the four isolates in infected PBMCs. Against the most resistant isolate, HIV-1/#410, U-104489 displayed good activity with an IC_{90} estimate of 1.1 μM. These analyses demonstrated that these two AAP-BHAPs have excellent broad-spectrum activities against the NNRTI-resistant viruses tested, with U-104489, the *N*-ethyl homolog of U-95133, demonstrating more potent activity.

Selection of resistance to the AAP-BHAPs. The development of high-level resistance to the lead AAP-BHAPs was assessed by culturing HIV-1_{IIIB}-infected MT4 cells in escalating concentrations, up to 10 μM, of U-95133 or U-104489. In addition, for the antiviral studies (Table 1), cell-culture-derived variants of HIV-1_{IIIB} resistant to DLV, pyridinone, and α-APA were also selected. In general, the selection of variants capable of growing in 10 μM DLV, pyridinone, or α-APA was accomplished within 6 to 8 passages of 3 to 4 days per passage. In contrast, selection of variants with U-95133 and U-104489 was qualitatively more difficult, taking approximately 15 passages which was slightly longer (5 to 7 days) than for the other NNRTI-resistant variants (data not shown). Moreover, once obtained, the U-95133- and U-104489-resistant HIV-1_{IIIB} variants grew more slowly and to lower titers than HIV-1_{IIIB}-WT. The genotypic and phenotypic characterizations of these variants are described below.

Resistance to AAP-BHAPs is associated with specific genotypic changes in RT. The genotypic basis for resistance to the AAP-BHAPs was investigated by DNA sequence analysis of the RT gene after RT-PCR of virion RNAs from various AAP-BHAP-resistant HIV-1 stocks (Table 3). Compared with the parental WT drug-sensitive HIV-1_{IIIB}, U-95133-resistant HIV-1_{IIIB} contained five mutations in the RT gene that encoded the

TABLE 1. Antiviral activities of the AAP-BHAP RTIs against in vitro-selected NNRTI-resistant HIV-1 variants

RTI	IC_{90} (μM)[a]				
	HIV-1_{IIIB} (WT)	HIV-1_{MF}-BHAP^{r}[b] (Leu-236)	HIV-1_{IIIB}-BHAP^{r}[b] (Ile-100, Leu-230)	HIV-1_{IIIB}-Pyr^r (Cys-181)	HIV-1_{IIIB}-α-APA^r (Cys-181)
U-95133 (AAP-BHAP)	0.02	5.3	0.83	0.22	0.12
U-104489 (AAP-BHAP)	0.004	0.19	0.08	0.03	0.05
DLV (BHAP)	0.05	>10	>10	5.2	1.0
L-697,661 (Pyr[c])	0.11	0.43	4.0	>10	7.7
α-APA (R88703)	0.15	0.08	6.5	>10	>10

[a] IC_{90}, concentration of drug that inhibited p24 production by 90% in infected MT4 cells.
[b] DLV (U-90152T) was used for selection of the BHAP-resistant HIV-1_{MF} and HIV-1_{IIIB} variants.
[c] Pyr, pyridinone.

TABLE 3. Sequence analysis of the RT coding region of U-95133-resistant HIV-1_{IIIB}

Virus	No. of clones	RT amino acid[a]						
		Lys-103	Thr-139	Gly-190	Thr-200	Leu-214	Leu-234	Pro-236
HIV-1_{IIIB}-95133^r (parent stock)[b]		–	Thr + Ile	Gly + Glu	Ala	Leu + Phe	–	Pro + Leu
Clones[c]	1	Glu	Ile	Glu	Ala	Phe	–	–
	3	–	Ile	Glu	Ala	Phe	–	–
	2	–	Ile	–	–	–	–	Leu
	3	–	–	Glu	Ala	Phe	–	–
	1	–	–	Glu	Ala	Phe	Pro	–
HIV-1_{IIIB}-95133rd		–	Ile	Glu	Ala	Phe	–	–

[a] The sequence used for the genotypic comparison is the RT of HIV-1_{HXB2}. Dashes indicate identity with WT at that position. Genotypic mixtures are indicated where observed.
[b] Mutations detected from the direct DNA sequence analysis of the PCR-amplified RT coding region of the HIV-1_{IIIB}-95133^r parent stock.
[c] Molecular clones were derived from the PCR-amplified DNA from the parent stock.
[d] HIV-1_{IIIB}-95133^r was derived in MT4 cells by end-point titration of the parent stock in the presence of 10 μM U-95133.

following amino acid substitutions: Thr-139→Ile, Gly-190→Glu, Thr-200→Ala, Leu-214→Phe, and Pro-236→Leu. All of these codons, with the exception of codon 190, contained mixtures of WT and mutant bases which implied that not all of these nucleotide substitutions were present in the same viral genomic RNA molecule. To determine which of these mutations were linked on the same genomic RNA, 10 molecular clones of PCR fragment were prepared, and a portion of the RT coding region nucleotide sequence was determined. This analysis revealed not only that each RT gene had more than one mutation, with 8 of 10 clones containing at least three coding changes but also that the Pro-236→Leu substitution appeared independent of the clones containing the Gly-190→Glu substitution. Also, this analysis revealed the presence of two substitutions, Lys-103→Glu and Leu-234→Pro, which were present at a low frequency and thus not detected in the PCR fragment sequencing. The RT genotype of an additional stock of U-95133-resistant HIV-1_{IIIB} which was derived by end-point dilution in MT-4 cells in the presence of 10 μM U-95133 was determined by direct sequencing of PCR product. This analysis revealed the presence of the four most abundant amino acid substitutions described above (Thr-139→Ile, Gly-190→Glu, Thr-200→Ala, and Leu-214→Phe); no mixtures with WT sequence were detected at any of the mutant codons. Finally, we also determined the RT genotype of U-104489-resistant HIV-1_{IIIB}. In this case, direct sequencing of RT PCR fragments of the RT coding region demonstrated the presence of mutations encoding only the Gly-190→Glu and Leu-214→Phe substitutions, with no detectable WT sequence at these two codons.

The Gly-190→Glu amino acid substitution is a key determinant for HIV-1_{IIIB} resistance to the AAP-BHAPs. Selected RT mutations identified from the DNA sequence analysis described above were created by oligonucleotide-directed mutagenesis in an RT expression vector containing the HIV-1_{IIIB} RT coding region. The mutant RTs, expressed in bacteria, were purified as p66 homodimers to apparent homogeneity by immobilized metal affinity chromatography (IMAC) (the specific RT activity of the p66 homodimer is comparable to the p66-p51 heterodimeric RT [3]). Full-length Gly-190→Glu RT polypeptide, eluted from the IMAC column, was further purified over single-stranded DNA cellulose to minimize the presence of aberrantly folded RT polypeptide. The specific activities of most of the mutant polymerases were indistinguishable from that of the WT enzyme (data not shown), but the specific activities of RT enzymes carrying the Gly-190→Glu substitution, either alone or in combination with the Thr-139→Ile, Thr-200→Ala, and Leu-214→Phe substitutions, were significantly diminished (i.e., 75 and 40% of WT RT, respectively). Previous data by Kleim et al. (15, 16) showed that the Gly-190→Glu substitution conferred high-level resistance to the quinoxaline NNRTIs and also caused significant reductions in polymerase specific activity in RTs expressed as maltose-binding protein fusions. Despite the low specific activity of our Gly-190→Glu-containing enzymes, all mutant RTs exhibited sufficient activity to allow determination of their in vitro sensitivities to the AAP-BHAPs.

Table 4 shows the inhibition of various mutant HIV-1 RTs by U-95133 and U-104489. The Pro-236→Leu substitution, either alone or in combination with the Thr-139→Ile substitution, was inhibited by U-95133 with IC_{50}s of 2.4 and 1.4 μM, respectively, indicating that these substitutions have little effect on AAP-BHAP inhibition of RT. Similarly, U-95133 inhibited the mutant RTs containing Thr-139→Ile and Thr-200→Ala, alone and in combination, with IC_{50}s comparable to that of the WT enzyme, suggesting that these substitutions have little to no effect on AAP-BHAP inhibition of RT. However, when the Gly-190→Glu and Leu-214→Phe substitutions were combined with the Thr-139→Ile and Thr-200→Ala substitutions to make the quadruple mutant, the resulting RT was highly resistant to the AAP-BHAPs, with only 17 and 13% of the activity inhibited at 100 μM U-95133 and U-104489, respectively. Analysis of the RT containing only the Gly-190→Glu substitution revealed that the Gly-190→Glu substitution alone was able to confer high-level resistance to AAP-BHAPs, with inhibition

TABLE 4. Sensitivities of mutant RTs to the AAP-BHAPs U-95133 and U-104489

RT	IC_{50} (μM)[a]	
	U-95133	U-104489
WT	0.9 ± 0.1	0.47 ± 0.04
Thr-139→Ile	1.2 ± 0.2	0.95 ± 0.07
Thr-200→Ala	1.4 ± 0.3	ND
Thr-139→Ile/Thr-200→Ala	1.6 ± 0.4	ND
Pro-236→Leu	2.35 ± 0.1	ND
Pro-236→Leu/Thr-139→Ile	1.41 ± 0.08	ND
Gly-190→Glu	>100 (13)	>100 (12)
Thr-139→Ile/Gly-190→Glu Thr-200→Ala/Leu-214→Phe	>100 (17)	>100 (13)

[a] Values in parentheses are the percent inhibitions at 100 μM drug concentration. ND, not done.

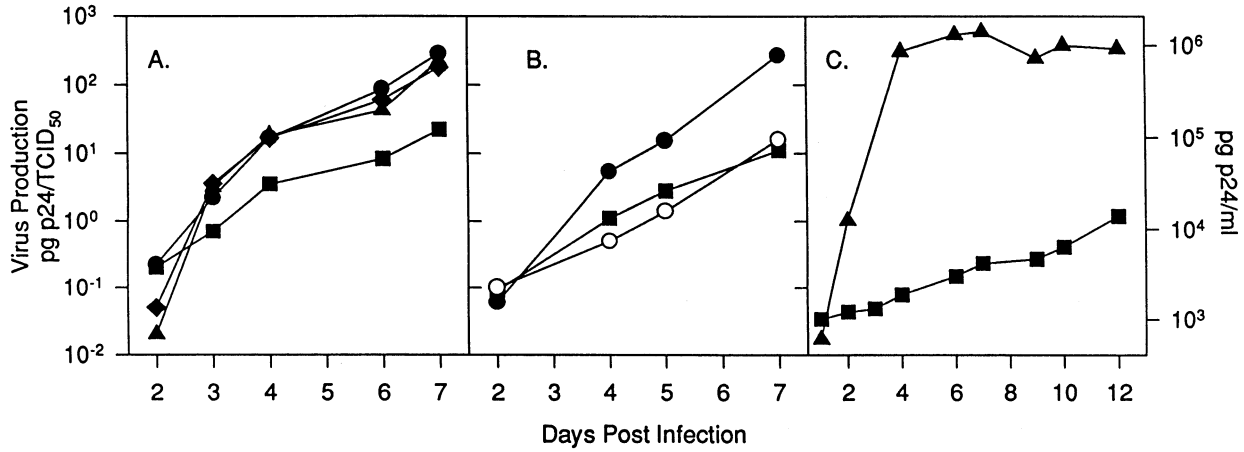

FIG. 2. Replication of the U-95133- and U-104489-resistant HIV-1_{IIIB} variants in MT4 cells and PBMCs. The results of three different experiments are shown in panels A, B, and C. (A and B) Results from MT4 cells infected with HIV-1_{IIIB}-104489^r (■), HIV-1_{IIIB}-95133^r (○), HIV-1_{IIIB}-92884^r (●), HIV-1_{IIIB}-DLV^r (◆), or HIV-1_{IIIB} (▲). Virus production at the indicated day postinfection is shown on the left *y* axis and expressed as the measured concentration of p24 (picogram per milliliter) divided by the inoculum infectious titer ($TCID_{50}$ per milliliter) to yield picograms of p24 per $TCID_{50}$. The infectious titer estimations ($TCID_{50}$ per milliliter) determined for the inocula used in the experiments shown in panels A and B were as follows: HIV-1_{IIIB}-104489^r, 2.2×10^3 (A) and 3.2×10^3 (B); HIV-1_{IIIB}-95133^r, 2.5×10^3 (B); HIV-1_{IIIB}-92884^r, 7.1×10^2 (A) and 2.4×10^3 (B); HIV-1_{IIIB}-DLV^r, 2.8×10^2 (A); and HIV-1_{IIIB}, 2.8×10^2 (A). (C) Replication of HIV-1_{IIIB}-104489^r (■) and HIV-1_{IIIB} (▲) in PBMCs. The inocula used for the infections were estimated to contain an equivalent number of virus particles based on p24 concentration determinations (5×10^5 pg of p24 per ml). Virus production (in picograms of p24 per milliliter) is shown on the right *y* axis.

values by U-95133 and U-104489 very similar to those obtained with the quadruple mutant. This result is consistent with the observations that the Gly-190→Glu substitution was the most common substitution found in all of the resistant viruses genotyped. Because the residue at position 214 is variable, with either leucine or phenylalanine, and isolates which contain a phenylalanine at position 214 (e.g., HIV-$1_{JR\text{-}CSF}$ [17] [GenBank accession no. M34829]) are sensitive to AAP-BHAPs (23), it is unlikely that this amino acid change contributes to AAP-BHAP resistance and was therefore not studied alone. Taken together, the above results suggest that a key determinant for the observed HIV-1_{IIIB} resistance to U-95133 and U-104489 is the Gly-190→Glu RT substitution.

Effect of the Gly-190→Glu RT substitution on AAP-BHAP-resistant HIV-1_{IIIB} replication. As mentioned above, selection of the HIV-1_{IIIB} variants resistant to the AAP-BHAPs U-95133 and U-104489 was longer than for other NNRTI-resistant viruses. Once virus stocks were derived, they were found to be of lower infectious titer and to grow more slowly than WT virus in cell culture. To examine the effect of the Gly-190→Glu RT substitution on virus replication, we conducted a more detailed characterization of the replication phenotypes of the two AAP-BHAP-resistant viruses (Gly-190→Glu RT genotype) in parallel with HIV-1_{IIIB} and the drug-resistant variants HIV-1_{IIIB}-DLV^r and HIV-1_{IIIB}-92884^r, all three of which harbor the WT codon for amino acid 190 of RT.

Three cell-culture virus replication experiments are shown in Fig. 2. Figure 2A and B show experiments in MT4 cells which were infected in duplicate with virus inocula estimated to contain equivalent amounts of infectious virus on the basis of previously determined $TCID_{50}$ estimations. In both experiments the variants HIV-1_{IIIB}-104489^r (Fig. 2A and B) and HIV-1_{IIIB}-95133^r (Fig. 2B), which contain the mutant residue at position 190, displayed markedly reduced replication, as measured by p24 production per infectious unit, at each time point than did HIV-1_{IIIB}-92884^r, HIV-1-DLV^r, and HIV-1_{IIIB}-WT, each of which has the WT RT residue at position 190 (i.e., Gly). Specifically, at day 7 postinfection, the normalized levels of p24 production were reduced as much as 24-fold (Fig. 2B) for HIV-1_{IIIB}-104489^r and 17-fold (Fig. 2B) for the U-95133-resistant variant from the levels of the Gly-190 RT viruses.

To determine if the retarded growth phenotype of the Gly-190→Glu RT-containing viruses was cell type specific, we compared the replication of HIV-1_{IIIB}-104489^r to HIV-1_{IIIB} in primary PBMC cultures. In contrast to the replication studies described above, the viral inocula used to initiate the PBMC infections were prepared to contain equivalent p24 concentrations as an estimate of the presence of an equivalent number of virion particles in each inoculum (see Materials and Methods). Figure 2C shows that, as with the MT4-derived virus assayed in MT4 cells, the PBMC-derived U-104489-resistant virus grows slower than WT virus in PBMCs. By day 4 postinfection, the HIV-1_{IIIB}-WT p24 levels increased by nearly 3 orders of magnitude, whereas over the 12-day growth period, the HIV-1_{IIIB}-104489^r p24 levels increased only 10-fold. The results from this set of three experiments strongly suggest that the Gly-190→Glu RT mutation compromises the replicative capacity of HIV-1_{IIIB} in cell culture.

AAP-BHAP-resistant variants display significantly reduced virion-associated RT activities. We knew from the in vitro data that the Gly-190→Glu RT had low specific activity and that the presence of this substitution in AAP-BHAP-resistant variants reduced their replicative capacity. Taken together, these observations lead us to examine the activity of RT packaged in the virus particles of the AAP-BHAP-resistant virus stocks. In this assay, DNA product synthesis by virion-associated RT from an exogenous RNA template was measured by an enzyme-linked oligonucleotide hybridization technique (Table 5). Not unexpectedly, the U-95133- and U-104489-resistant viruses that have the Gly-190→Glu RT genotype demonstrated minimal activity in this assay. Normalization of the DNA product concentration to input p24 indicated that the product yields per p24 equivalent for HIV-1_{IIIB}-104489^r and HIV-1_{IIIB}-95133^r were only 0.3 to 0.5% and 2%, respectively, of those measured for HIV-1_{IIIB}-WT. Interestingly, the DLV-resistant HIV-1_{IIIB}, which harbors the RT substitutions Leu-100→Ile, Arg-172→Lys, His-198→Asn, Leu-214→Phe, and Met-230→Leu displayed only 16 to 22% of the activity of

TABLE 5. Virion-associated RT activities of in vitro-selected HIV-1 variants resistant to NNRTIs

Virus	Virion lysate (ng of p24/reaction)	DNA product[a] (fmol/ml/ng of p24)	% of WT
HIV-1_{IIIB}	2.2	14.9 ± 0.8	100
HIV-1_{IIIB}-104489^r	18.3	0.06 ± 0.01	0.5
	61.3	0.05 ± 0.0	0.3
HIV-1_{IIIB}-95133^r	14	0.24 ± 0.01	2
	28	0.25 ± 0.01	2
HIV-1_{IIIB}-DLV^r	2.4	3.3 ± 0.8	22
	7.2	2.4 ± 0.4	16
HIV-1_{IIIB}-Pyr^r	1.7	6.7 ± 0.2	45
	5.5	7.5 ± 0.2	50
HIV-1_{IIIB}-α-APA^r	2.0	8.1 ± 0.5	54
	6.0	7.9 ± 0.0	53
HIV-1_{IIIB}-92884^r	4.0	14.9 ± 0.7	100

[a] For comparison, the DNA product concentrations (femtomoles of DNA per milliliter) for the RT reactions were normalized by dividing by the amount (nanograms of p24) of virion lysate used. Each value shown is the mean ± standard deviation.

HIV-1_{IIIB}-WT. However, as shown above (Fig. 2), this variant appeared to replicate as efficiently as HIV-1_{IIIB}-92884^r (Val-106→Ile and Tyr-181→Cys) and HIV-1_{IIIB}-WT. The RT activities for the pyridinone- and α-APA-resistant variants which carry the Tyr-181→Cys and Leu-214→Phe substitutions were reduced only approximately twofold from the WT RT activity.

Western blot analysis of virion-associated RT. The significantly reduced endogenous RT activity observed for the AAP-BHAP-resistant variants next lead us to directly examine and compare the amounts and integrities of the virion-associated heterodimeric RTs from HIV-1_{IIIB}-104489^r and HIV-1_{IIIB}. Equivalent amounts of concentrated virus preparations, as measured by p24 ELISA, from infected MT4 cells were analyzed by Western blotting with a monospecific polyclonal antibody to HIV-1 RT which recognized with equal efficiency by immunoblotting WT and Gly-190→Glu recombinant p66 homodimer and p66-p51 heterodimer preparations (data not shown). Compared with the normally processed p66 and p51 subunits of the HIV-1_{IIIB}-WT RT heterodimer (Fig. 3, lane A), only faint detection of the p66 RT subunit of HIV-1_{IIIB}-104489^r was observed (lane B) and the p51 subunit was not detected. Thus, the amount of full-length heterodimeric RT detected from U-104489-resistant virions was significantly less than from WT virus. The immunoreactivity pattern of the lower-molecular-weight peptides (lane B) suggested that the RT heterodimer for the U-104489-resistant HIV-1_{IIIB}, which has the Gly-190→Glu substitution in RT, was proteolytically degraded during either particle assembly or maturation. This apparent degradation is not cell specific, as an identical banding pattern was observed for U-104489-resistant HIV-1_{IIIB} prepared in both MT4 cells and PBMCs (data not shown). The pattern of staining for the U-92884-resistant virus, which was derived in the same manner by passage in MT4 cells but encodes the Val-106→Ile and Tyr-181→Cys RT substitutions, was similar to the pattern observed for WT virus, which suggested that the aberrant processing detected in U-104489-resistant virions was RT genotype specific and not due to serial passage in cell culture (data not shown).

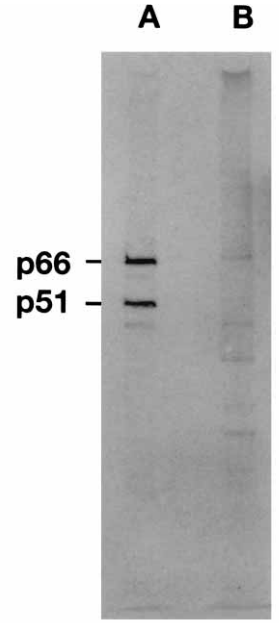

FIG. 3. Western blot analysis of virion-associated heterodimeric RT from HIV-1_{IIIB}-104489^r and HIV-1_{IIIB}. Equivalent amounts of virion lysates of HIV-1_{IIIB} (lane A) and HIV-1_{IIIB}-104489^r (lane B) were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with monospecific polyclonal antibody to HIV-1 RT. The p66 and p55 RT subunits of HIV-1 heterodimeric RT are indicated.

DISCUSSION

In the experiments described here, we have identified the AAP-BHAPs as a class of potent HIV-1-specific inhibitors with broad-spectrum activity against several NNRTI-resistant HIV-1 variants and recombinant RT enzymes. Specifically, the most potent AAP-BHAP U-104489 has excellent activity against the cell-culture-selected panel of HIV-1 variants resistant to BHAP (Pro-236→Leu or Leu-100→Ile), pyridinone (Tyr-181→Cys), and α-APA (Tyr-181→Cys). U-104489 was also inhibitory at submicromolar concentrations to three of four clinical isolates tested which were resistant to both DLV and AZT (Tables 1 and 2). Thus, U-104489 has the requisite broad-spectrum activity we set out to identify in our discovery efforts.

An obstacle for the successful development of HIV-1 antiviral drugs is the emergence of resistant variants, which may compromise their therapeutic utility for the prolonged treatment of HIV-1 infection and AIDS. Therefore, a desired property of an effective anti-HIV-1 agent would be the protracted emergence of resistant variants during treatment. The in vitro selection for high-level resistance to either of the AAP-BHAPs, U-95133 or U-104489, in HIV-1_{IIIB}-infected MT4 cells appeared to be longer than the resistance development to DLV, L-697,661, α-APA, and the early generation AAP-BHAP derivative U-92884. The genotypic analyses of the two HIV-1_{IIIB}-95133^r stocks indicated that simultaneous multiple amino acid substitutions in RT, including Gly-190→Glu, appear to be required for expression of high-level resistance to the AAP-BHAP U-95133 (Table 3). Assuming that the breadth of genotypic diversity of the virus population present at the beginning of the selection influences the rate of emergence of drug-resistant variants, greater diversity or prolonged selection would be required if multiple amino acid substitutions were needed to confer significant resistance to an inhibitor. Although the in vitro recombinant RT experiments revealed that the Gly-

190→Glu substitution was sufficient to cause high-level resistance to the AAP-BHAPs, it is possible that the other substitutions are necessary for maintaining enzymatic function or integrity of the RT (Table 4). While the genotypic analysis of the U-104489-resistant HIV-1_{IIIB} revealed only one RT substitution, Leu-214→Phe, in addition to Gly-190→Glu, we cannot rule out the possibility that the other substitutions (Thr-139→Ile and Thr-200→Ala) were present earlier in the passage series at lower U-104489 concentrations or were not present in sufficient proportions for detection by direct PCR fragment sequencing from the quasispecies. The ordered appearance of multiple drug-resistant RT mutations during passage of virus in escalating concentrations of the NNRTI quinoxaline S-2720, leading to the selection of Gly-190→Glu or Tyr-181→Cys, has been reported elsewhere (1). Further evidence for a selective benefit of the Thr-200→Ala substitution in a Gly-190→Glu-containing RT (e.g., HIV-1_{IIIB}-95133^r) comes from the observations that HIV-1_{MN}-WT (13) (which has alanine at position 200; GenBank accession no. M17449), passaged in the presence of quinoxaline S-2720, acquired the Gly-190→Glu substitution while retaining the alanine at RT residue 200, whereas the threonine-to-alanine change was acquired by HIV-1_{IIIB} grown in the presence of U-95133. In addition, the Leu-214→Phe RT substitution accumulated in both of the Gly-190→Glu RT-containing viruses (i.e., HIV-1_{IIIB}-104489^r and HIV-1_{IIIB}-95133^r) but is not present in the RT of virus resistant to the early generation AAP-BHAP analog U-92884, which retains glycine at residue 190, suggesting that the presence of the Leu-214→Phe substitution is in some way beneficial to the RT in combination with Gly-190→Glu. Nonetheless, the Gly-190→Glu RT substitution, either in the presence or absence of other RT mutations, appears to be required for HIV-1_{IIIB} replication in the presence of high concentrations (10 μM) of U-95133 or U-104489. Drug sensitivity phenotyping of HIV-1_{IIIB}-104489^r demonstrated that the presence of the Gly-190→Glu RT substitution confers significant cross-resistance to pyridinone, α-APA, nevirapine (IC_{90}s, >10 μM), and DLV (IC_{90}, 4.2 μM). These data are in good agreement with previously reported drug sensitivities of quinoxaline S-2720-resistant HIV-1 variants carrying the Gly-190→Glu RT mutation to various NNRTIs (1).

Of equal importance, phenotypic characterization of the U-104489- and U-95133-resistant HIV-1_{IIIB} variants showed that these variants grew to lower infectious titers and displayed a marked reduction in replication capacity in tissue culture than viruses with the WT Gly-190 RT genotype (i.e., HIV-1_{IIIB}-WT, HIV-1_{IIIB}-92884^r, and HIV-1_{IIIB}-DLV^r). The replication deficiency observed for the Gly-190→Glu RT-containing viruses was more pronounced in PBMCs than in MT4 cells, since an exponential increase in virus production was not observed in the HIV-1_{IIIB}-104489^r-infected PBMC cultures (Fig. 2C). Although it was not quantitated, a lower infectious inoculum titer for the U-104489-resistant virus than for the WT virus could explain the low p24 levels soon after PBMC infection. Even so, after several rounds of replication (i.e., 3 to 5 days), one could predict that the initial bursts of virus production would be sufficient to lead to a logarithmic increase in p24 levels by a replication-competent virus population. This was not observed for the U-104489-resistant variant. We also attempted in several experiments, albeit unsuccessfully, to generate molecularly defined virus stocks to directly assess the phenotypic effect of the Gly-190→Glu substitution either alone or in combination with the Thr-139→Ile, Thr-200→Ala, and Leu-214→Phe substitutions. MT4 cells transfected with plasmid containing HIV-1_{HXB2}-WT proviral DNA consistently produced high-titered virus stocks ($>10^6$ pg of p24 per ml) within 4 to 6 days posttransfection, whereas cultures transfected with the mutant RT proviral DNAs yielded peak p24 levels of only 300 to 400 pg of p24 per ml at 7 to 9 days posttransfection followed by a decrease to the baseline level (data not shown). The results of analyses reported here and elsewhere (11, 15, 16) implicate the Gly-190→Glu RT substitution encoded by these variants as being largely responsible for the retarded growth characteristic.

The significantly reduced specific activities of the recombinant RTs carrying the Gly-190→Glu mutation lead us to examine the virion-associated RT activities of the various drug-resistant variants and WT HIV-1 used in these studies. The variants expressing the Gly-190→Glu RT substitution displayed virion-associated RT activities that were 2% or less of the HIV-1_{IIIB} endogenous RT activity (Table 5). The significantly diminished endogenous RT activity levels suggested that the packaged RT molecules of the U-95133- and U-104489-resistant HIV-1_{IIIB} variants are functionally impaired. Partial explanation for the impaired activities comes from the immunoblot analyses performed on virion-associated RT which indicated aberrant heterodimer processing or proteolytic degradation in variants with the Gly-190→Glu RT genotype (Fig. 3). The aberrant processing of RT in these preparations could well explain the significantly reduced endogenous RT activities and viral replication observed. If, as suggested by Coffin (5), selection of a virus population with a replication disadvantage would likely lead to a decreased steady-state level of virus replication and potential clinical benefit, compounds with in vitro properties like those of the AAP-BHAPs have a theoretical basis for in vivo efficacy.

ACKNOWLEDGMENTS

We thank H. W. Smith and J. Morris for kindly supplying L-697,661 and R-88703 and the HIV-1 RT Program Team for their support. We also thank A. Kiewiet and M. Meissner for assistance in manuscript preparation.

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