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A mutation in reverse transcriptase of bis(heteroaryl)piperazine-resistant human immunodeficiency virus type 1 that confers increased sensitivity to other nonnucleoside inhibitors

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ABSTRACT **Several nonnucleoside inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) have been described, including Nevirapine, thiobenzimidazolone (TIBO) derivatives, pyridinone derivatives such as L-697,661, and the bis(heteroaryl)piperazines (BHAPs). HIV-1 resistant to L-697,661 or Nevirapine emerges rapidly in infected patients treated with these drugs, and the resistance is caused primarily by substitutions at amino acids 181 and 103 of RT that also confer cross resistance to the other nonnucleoside inhibitors. We describe derivation and characterization of two BHAP-resistant HIV-1 variants that differ from this pattern of cross resistance. With both variants, HIV-1 resistance to BHAP RT inhibitors was caused by a RT mutation that results in a proline-to-leucine substitution at amino acid 236 (P236L). Rather than conferring cross resistance to other RT inhibitors, this substitution *sensitized* RT 7- to 10-fold to Nevirapine, TIBO R82913, and L-697,661 without influencing sensitivity to nucleoside analogue RT inhibitors. This sensitization caused by P236L was also observed in cell culture with BHAP-resistant HIV-1. The effects of the P236L RT substitution suggest that emergence of BHAP-resistant virus *in vivo* could produce a viral population sensitized to inhibition by these other nonnucleoside RT inhibitors.**

Treatment of human immunodeficiency virus type 1 (HIV-1) infection with the nucleoside analogues 3′-azido-3′-deoxythymidine (AZT) or 2′,3′-dideoxyinosine (ddI) results in emergence of resistant HIV-1 variants with specific mutations in the reverse transcriptase (RT) gene (1–3). Drug-resistant HIV-1 also emerges rapidly in patients treated with nonnucleoside RT inhibitors such as the pyridinone L-697,661 (4, 5) and Nevirapine (6–8). Earlier studies have shown that these compounds, as well as the bis(heteroaryl)piperazines (BHAPs) (9–11) and thiobenzimidazolone (TIBO) derivatives (12, 13), interact with RT at a common site (5, 8, 14, 15). Therefore, the observation of cross resistance conferred against all the nonnucleosides by substitution at amino acids 103, 181, or 188 of RT led to the view that emergence of HIV-1 resistant to one inhibitor would obviate usefulness of the others (16).

This report describes serial HIV-1 passage *in vitro* in increasing concentrations of the BHAP class of nonnucleoside RT inhibitors to establish highly resistant HIV-1 variants. Development of HIV-1 resistance to the BHAPs differed from that observed with other nonnucleoside RT inhibitors because BHAP resistance was caused by a proline-to-leucine substitution at amino acid 236 of RT (P236L). The effects of the P236L substitution were quantitatively assessed in both recombinant RT and HIV-1_{HXB2} chimeric for BHAP-resistant RT. While this substitution conferred resistance to the BHAPs as expected, it had the intriguing property of sensitizing RT to inhibition by Nevirapine, TIBO R82913, and L-697,661.

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MATERIALS AND METHODS

Cells, Virus, and Compounds. HIV-1_{MF} is cloned from a primary virus isolate and is highly cytopathic for both peripheral blood mononuclear cell (PBMC) populations and $CD4^+$ cell lines (17). HIV-1_{MF} was propagated in MT-4 cells (18). HIV-1_{JR-CSF} is a monocytotropic variant that replicates in PBMCs but not in $CD4^+$ cell lines (19) and was propagated in PBMCs. U-87201E, U-90152S, L-697,661, and Nevirapine were synthesized by Upjohn according to published methods (4, 11, 20, 21). TIBO R82913 was purchased from PharmaTech International (West Orange, NJ). $(dG)_{12-18}$, $(dT)_{10}$, $(rC)_{\sim 430}$, and $(rA)_{\sim 550}$ were obtained from LKB Pharmacia. HPLC-grade deoxynucleotides and 3′-deoxythymidine triphosphate (ddTTP) were obtained from United States Biochemical. Phosphonoformate was obtained from Sigma.

Establishment of BHAP-Resistant HIV-1 Variants. High-level resistance to BHAP inhibitors was generated by serial passage of HIV-1-infected MT-4 cells or PBMCs in increasing concentrations of inhibitor. For selection of HIV-1_{MF} resistant to the BHAP U-87201E (11), virus was maintained for 10 passages at 1 μM U-87201E, 5 passages at 5 μM, 6 passages at 15 μM, and 4 passages at 30 μM. Cells were passed every 2–3 days by 1:4 dilution into fresh medium with drug to maintain a cell density of $\approx 7 \times 10^5$ per ml. Propagation of HIV-1 in the presence of U-87201E at concentrations exceeding 30 μM was not performed, to avoid potential cytotoxic effects of the drug. Selection of high-level HIV-1 resistance to the BHAP U-90152S in PBMCs was obtained by serial passage of virus with drug concentrations of 0.3–3 μM. Each passage was initiated by infection of 5×10^5 PBMCs with an inoculum of HIV-1_{JR-CSF} corresponding to 30 ng of p24 antigen and consisted of sustained growth for 7–10 days.

Cloning of BHAP-Resistant HIV-1 RT. Total cellular DNA from parental HIV-1_{MF}-infected MT-4 cells and BHAP-resistant HIV-1_{MF}-infected MT-4 cells was isolated by guanidinium isothiocyanate lysis (17). A 1.93-kb fragment including the RT coding region was generated by 30 cycles of PCR using primers corresponding to nucleotides 2518–2538 (5′-GTTGACTCAGATTGGTTGCAC-3′) and 4636–4654 (5′-GGGAATTCCAAATTCCTGC-3′) of the HIV-1_{HXB2} se-

Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; BHAP, bis(heteroaryl)piperazine; TIBO, thiobenzimidazolone; AZT, 3′-azido-3′-deoxythymidine; PBMC, peripheral blood mononuclear cell.

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quence (GenBank accession no. M38432). Primer binding sites were chosen to allow use of the *Bal* I restriction sites at nucleotides 2620 and 4550 of the HIV-1_{MF} *pol* gene (17). PCR products were cloned directly into the vector PCR 2000 (Invitrogen, San Diego), and full-length HIV-1 RT clones were identified by restriction mapping.

Construction of Mutant Proviral HIV-1 Clones. To assess the phenotype of individual RT clones from BHAP-resistant HIV-1_{MF} in cell culture, 1.93-kb *Bal* I fragments containing the entire RT gene were excised from several PCR 2000 clones and ligated individually into *Bal* I-digested pHXB2gpt, an infectious DNA molecular clone of HIV-1 (22). The integrity and orientation of HIV-1_{HXB2} clones chimeric for HIV-1_{MF} RT were confirmed by restriction mapping. Chimeric virus stocks were rescued after transfection of HeLa cell cultures as described (17). The RT coding regions of HIV-1_{HXB2}/HIV-1_{MF} RT chimeric viral clones with high BHAP resistance were characterized by nucleotide sequencing of the corresponding plasmid DNA. For one viral clone, MFE 30.3, the entire RT polymerase domain encoded by the *Bal* I fragment transferred from resistant HIV-1_{MF} was sequenced, and this mutant viral stock was used to determine the IC_{50} and IC_{90} values of the BHAPs and other RT inhibitors in cell culture (see below).

Assay of Virus Replication and Drug Sensitivity. HIV-1 viral replication in MT-4 cultures was monitored by p24 antigen production (p24 antigen-capture assay, Coulter) and/or by number of HIV-1 antigen-expressing cells as determined by immunofluorescence assay utilizing HIV-1-seropositive patient serum (17). For drug-sensitivity analysis of HIV-1, MT-4 cells were pretreated with the respective RT inhibitors for 18 hr at a cell density of 5×10^5 per ml. Virus inoculum (1000 50% tissue culture infectious doses per 10^6 cells) was added and cultures were incubated for 1 hr at 37°C. Cells were washed and resuspended at $0.3–0.5 \times 10^5$ per ml in 5–6 ml of fresh culture medium. Virus replication was monitored over 5–6 days postinfection at 24-hr intervals by p24 antigen assay or immunofluorescence. In control (no drug) cultures, these conditions yielded 10–20% HIV-1 antigen-positive cells by 3 days postinfection.

Identification of Mutations in BHAP-Resistant HIV-1_{JR-CSF}. Approximately 10^7 PBMCs from a culture infected with U-90152S-resistant HIV-1_{JR-CSF} (>100-fold more resistant than the parent virus) and grown for 7 days were pelleted and frozen. Mutations thought to confer BHAP resistance were identified by nucleotide sequencing of the RT coding region after PCR amplification of viral RNA. Total cellular RNA was extracted from the infected PBMCs with RNAzol B (Biotecx Laboratories, Houston) as described (23). To control for potential amplification errors, six replicate RNA samples (1 μg) were used for cDNA synthesis and amplification using the RT RNA PCR kit (Perkin–Elmer/Cetus) according to the manufacturer's recommendations. The RT coding region was amplified as two overlapping fragments by using primer pairs based on the HIV-1_{JR-CSF} sequence (GenBank accession no. M38429): for the 500-bp 5′ fragment, nucleotides 2531–2551 (5′-GTTGACTCAGATTGGTTGCAC-3′) and 3786–3806 (5′-CTCCCACTCAGGAATCCAGGT-3′); for the 772-bp 3′ portion, nucleotides 2927–2947 (5′-AGACTTCAGGAAGTATACTGC-3′) and 4035–4055 (5′-GTCTGTTACTATGTTTACTTC-3′). Template for chain-termination sequencing (Sequenase, United States Biochemical) was generated by a second round of PCR using nested primers corresponding to nucleotides 2562–2583 (5′-CCCATTAGTCCTATTGAAACTG-3′), 3062–3082 (5′-TGTTTTCTAAAAGGCTCTAAG-3′), 3014–3034 (5′-GGGATGGAAAGGATCACCAGC-3′), and 3786–3806 (5′-CTCCCACTCAGGAATCCAGGT-3′). One primer of each pair was linked to biotin to facilitate isolation of single-stranded template with streptavidin-coated magnetic beads (24) according to the manufacturer's recommendations (Dynal, Great Neck, NY).

***In Vitro* Characterization of RT Mutants.** Specific point mutations in the RT gene derived from HIV_{IIIb} were created by using an oligonucleotide-directed mutagenesis system (Amersham). Mutant RT genes were cloned into plasmid DE 5.2 (25), which expresses RT with an amino-terminal hexahistidine linker. Mutant RTs were purified by metal affinity chromatography (26) and used as p66 homodimer. RNA-dependent DNA polymerase activity of the mutant RTs was assayed with 40 nM enzyme. The reaction mixture contained 2.5 μM $(rA)_{\sim 550}\cdot(dT)_{10}$ (1:1), 50 μM dTTP with [^{3}H]dTTP at 20 μCi/ml, 50 mM Tris·HCl (pH 8.3), 10 mM dithiothreitol, 60 mM NaCl, 10 mM $MgCl_2$, and 0.05% Nonidet P-40. Nonnucleoside drugs were added from Me_2SO stocks. Phosphonoformate and ddTTP were added from aqueous stocks. In all cases the final Me_2SO concentration was 1% (vol/vol). Assays were initiated by addition of the [^{3}H]dTTP mixture and stopped by addition of EDTA to 150 μM. Three 10-μl portions of each reaction mixture were spotted on DE-81 paper (Schleicher & Schuell), allowed to dry, and washed three times for 5 min with 0.3 M NaCl/30 mM sodium citrate, pH 7.0, followed by three washes in 95% ethanol. Bound radioactivity was quantitated by scintillation counting.

RESULTS AND DISCUSSION

To assess development of resistance to the BHAPs, two HIV-1 variants with widely different phenotypic properties *in vitro* were cultured for extended periods in the presence of BHAP inhibitors. Sequential propagation of HIV-1_{JR-CSF} in PBMCs and of HIV-1_{MF} in MT-4 cells with increasing concentrations of the BHAP compounds U-90152S and U-87201E, respectively (Fig. 1), resulted in the generation of virus highly resistant to these drugs. The IC_{90} for U-90152S against parental HIV-1_{JR-CSF} was ≈0.1 μM compared with an IC_{90} >10 μM for BHAP-resistant HIV-1_{JR-CSF}. The IC_{90} against parental HIV-1_{MF} was 0.45 μM and 0.06 μM for U-87201E and U-90152S compounds, respectively, while the IC_{90} values for BHAP-resistant HIV-1_{MF} were >30 μM and 3.0 μM. In the absence of inhibitors, BHAP-resistant HIV-1_{MF} elicited a spreading infection in $CD4^+$ MT-4 cells with kinetics very similar to parental, drug-sensitive virus (data not shown), so the drug resistance observed did not significantly attenuate viral replication *in vitro*.

To confirm that mutation of the RT gene caused the BHAP-resistant phenotype, a HIV-1 *pol* fragment containing the entire RT coding region was amplified by PCR from MT-4

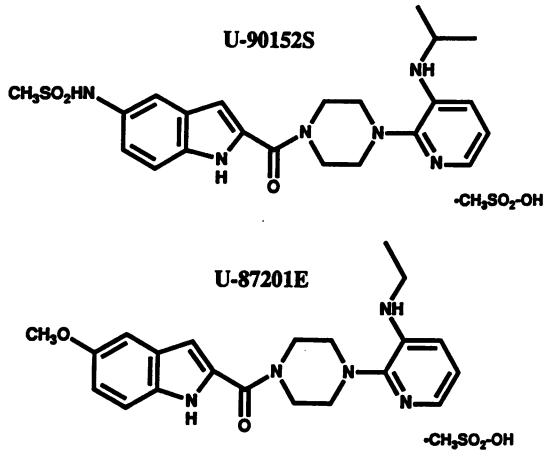

FIG. 1. Chemical structures of the BHAP compounds U-90152S and U-87201E.

cells infected with BHAP-resistant HIV-1_{MF}. This DNA was cloned into the genetic background of the prototypic HIV-1_{HXB2} variant and several chimeric viral clones (HIV-1_{HXB2}/HIV-1_{MF} RT) were obtained after transfection of human cells. Transfer of the RT coding region from BHAP-resistant HIV-1_{MF} conferred drug resistance on the viral clones; the U-90152S IC_{90} for parental HIV-1_{HXB2} was 0.06 μM, while for chimeric virus evaluated in parallel the IC_{90} was 1 μM. As observed with the BHAP-resistant HIV-1_{MF}, in the absence of drug the BHAP-resistant phenotype did not markedly attenuate virus replication in $CD4^+$ cells *in vitro* (data not shown).

The nucleotide sequence of the HIV-1_{MF} RT fragment (clone MFE30.3, encoding amino acids 1–483) that conferred BHAP-resistance as described above was determined and compared with the parental HIV-1_{MF} DNA sequence. This analysis indicated that two substitutions had occurred in RT: proline to leucine at amino acid 236 (P236L) and glycine to arginine at amino acid 273 (G273R). Partial sequencing of seven additional *pol*-region clones established from BHAP-resistant HIV-1_{MF} indicated that all possessed the P236L mutation, while only one (MFE30.3) also contained the G273R substitution. Thus, the P236L substitution was apparently sufficient to confer the BHAP-resistant phenotype on HIV-1_{MF}. Consistent with these data, the DNA nucleotide sequence encoding amino acids 38–323 of RT of BHAP-resistant HIV-1_{JR-CSF} was determined after RNA/DNA PCR and compared with the parental HIV-1_{JR-CSF} DNA sequence. The sequence changes observed encoded two RT substitutions: leucine to phenylalanine at amino acid 228 (L228F), and the P236L mutation also observed in the BHAP-resistant HIV-1_{MF}. Since the RT gene of both BHAP-resistant HIV-1_{MF} and HIV-1_{JR-CSF} carried the P236L substitution, these data strongly suggested that this single substitution conferred high-level drug resistance.

RT substitutions identified from the sequence analysis of BHAP-resistant virus were created in HIV-1_{IIIb} RT by oligonucleotide-directed mutagenesis. Similarly, enzymes carrying previously described substitutions known to confer resistance to Nevirapine, TIBO derivatives, or L-697,661 [tyrosine-181 to cysteine (Y181C) and lysine-103 to asparagine (K103N)] (5, 7, 8, 13) were also prepared. The sensitivity of purified mutant RTs to the BHAPs, L-697,661, TIBO R82913, and Nevirapine was determined *in vitro* by using poly(rA)·oligo(dT) template/primer (Table 1; Fig. 2). The Y181C and K103N substitutions conferred some resistance to all the nonnucleoside inhibitors tested, suggesting that these amino acid substitutions define areas of drug interaction with RT common to the BHAPs, Nevirapine, TIBO R82913, and L-697,661. Of these compounds, U-90152S was the most potent inhibitor of both the K103N- and the Y181C-substituted RTs, with IC_{50} values of $\approx$8 μM (Table 1). The mutant P236L RT was highly resistant to the BHAPs U-90152S and U-87201E (about 70-fold, Table 1), consistent with the presence of P236L in both the BHAP-resistant

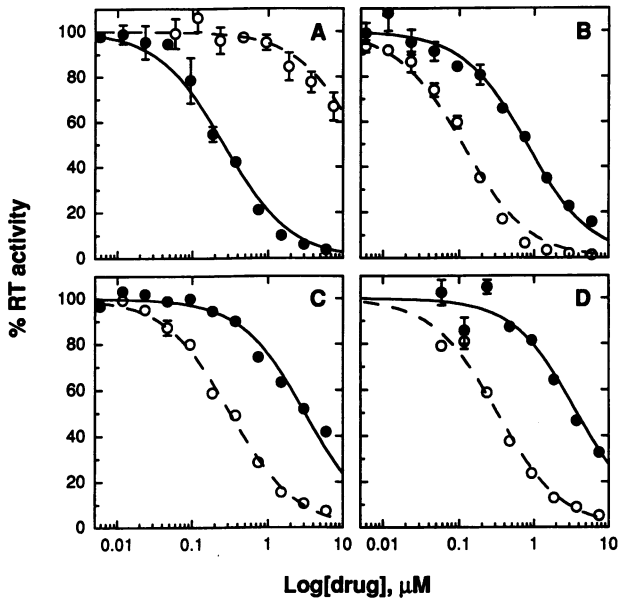

FIG. 2. Inhibition of recombinant wild-type (●) and P236L mutant (○) RT by U-90152S (*A*), L-697,661 (*B*), Nevirapine (*C*), or TIBO R82913 (*D*). Recombinant wild-type and mutant RT homodimers were made, purified, and assayed as described in *Materials and Methods*. Curve fits for theoretical competitive inhibition are shown.

HIV-1 isolates described above. However, RT with the P236L substitution was not cross resistant to L-697,661, TIBO R82913, or Nevirapine but, remarkably, was 7- to 10-fold more sensitive to inhibition than wild-type enzyme (Table 1; Fig. 2). The sensitizing effect was also evident when drug sensitivities were determined with poly(rC)·oligo(dG) template/primer (data not shown). Both the mutant P236L and the wild-type RT had identical sensitivity to ddTTP (IC_{50} = 0.09 μM) and phosphonoformate (IC_{50} = 0.39 μM), and displayed similar K_Ms of about 25 μM with respect to the dTTP substrate. Recombinant RT with the Y181C substitution in combination with P236L was much more resistant to U-90152S than the corresponding single mutants *in vitro* (Table 1). As might be expected, however, the presence of the P236L substitution in combination with Y181C decreased the resistance conferred by Y181C alone toward Nevirapine, TIBO R82913, and L-697,661 (Table 1). Recombinant RT carrying the L228F substitution identified in BHAP-resistant HIV-1_{JR-CSF} had drug sensitivities similar to wild-type RT. The role of L228F in combination with P236L was also negligible, since the L228F/P236L double mutant had drug sensitivities nearly identical to those of RT with the P236L mutation alone (data not shown). Finally, RT carrying the G273R substitution that was observed in two of the clones derived from BHAP-resistant HIV-1_{MF} also had drug sensi-

Table 1. *In vitro* inhibition of recombinant HIV_{IIIB} RT mutants

RT	IC_{50}, μM				
	U-90152S	U-87201E	Nevirapine	L-697,661	R82913
Wild type	0.26 ± 0.04	2.3 ± 0.2	3.1 ± 0.3	0.80 ± 0.08	3.8 ± 0.6
Y181C	8.32 ± 0.70	>60*	>60*	>60*	38 ± 7
K103N	7.7 ± 0.6	>60*	>60*	15 ± 4.1	>60*
P236L	18.0 ± 2.1	>60*	0.32 ± 0.02	0.11 ± 0.01	0.32 ± 0.05
Y181C/P236L	>60*	>60*	6 ± 1	10.0 ± 1.6	8.7 ± 1.1

RNA-dependent DNA polymerase activity of the mutant RTs was assayed as described in *Materials and Methods*. IC_{50} values were determined by nonlinear least-squares fit of data from triplicate points at 12 drug concentrations as shown in Fig. 2. Computed best-fit value for IC_{50} ± asymptotic standard deviation is shown.

*Highest.

tivities that were indistinguishable from those of the wild-type enzyme for all the inhibitors tested (data not shown).

To confirm results observed with the recombinant RTs, the drug sensitivities of BHAP-resistant HIV-1_{MF} (HIV-1_{MFE30}) and the chimeric clone which carried the resistant HIV-1_{MF} RT gene (HIV-1_{HXBE30}) were compared with those of wild-type HIV-1_{MF} and HIV-1_{HXB2} virus. While both HIV-1_{MFE30} and HIV-1_{HXBE30} were resistant to U-90152S (Fig. 3*A*), they were more sensitive to the pyridinone L-697,661 than the corresponding wild-type virus (Fig. 3*B*). L-697,661 IC_{90} values for wild-type HIV-1_{MF} and HIV-1_{HXB2} were 0.1 μM and 0.085 μM, respectively, while complete inhibition of viral replication required drug concentrations in excess of 0.2 μM. In contrast, 0.01 μM L-697,661 was sufficient for complete inhibition of BHAP-resistant HIV-1 replication (both HIV-1_{MFE30} and HIV-1_{HXBE30}). BHAP-resistant HIV-1 variants were also sensitized to Nevirapine (Fig. 3*C*), with IC_{90} values of 0.19 μM and 0.038 μM for HIV-1_{HXB2} and HIV-1_{HXBE30}, respectively. The BHAP-resistant phenotype did not affect sensitivity to the nucleoside analogue inhibitor AZT (data not shown).

These data demonstrate that the P236L substitution in RT is sufficient to confer resistance to the BHAPs. Because P236L arose in both HIV-1_{MF} and HIV-1_{JR-CSF}, this substitution may represent the primary means of BHAP-resistance development. Although we did not derive BHAP-resistant HIV-1 with RT substitutions such as Y181C and K103N, these changes do confer relative resistance to the BHAPs (Table 1; refs. 9 and 27). The frequency and character of mutations that occur during clinical evaluation of U-87201E and U-90152S in HIV-1-infected patients remain to be determined.

Like P236L, other amino acid substitutions in HIV-1 RT have been observed to cause resistance to one RT inhibitor while increasing sensitivity to a related inhibitor (3, 28). In these cases, the Y181C substitution or a leucine-74 to valine (L74V) substitution in RT suppressed AZT resistance despite the continued presence of other RT substitutions conferring AZT resistance. Because HIV-1 rapidly develops resistance to single RT inhibitors, knowledge of the specific means by which HIV-1 acquires resistance to the various RT inhibitors should be exploited in designing combination therapies for HIV-1-infected individuals. It appears that as HIV-1 becomes resistant to either the BHAPs or ddI, it increases its vulnerability to other inhibitors. If the selective pressure exerted by the BHAPs on HIV-1 predominantly causes the P236L substitution in RT, the initial use of BHAPs in a clinical setting may lead to derivation of a population of BHAP-resistant virus that is sensitized to inhibition by other nonnucleoside inhibitors.

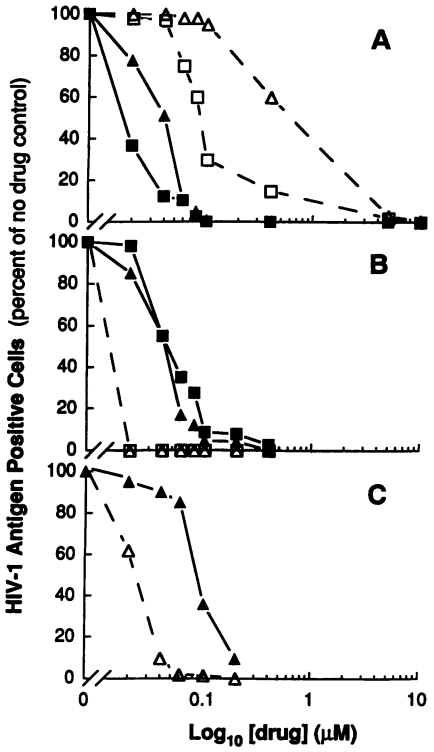

FIG. 3. Inhibition of wild-type HIV-1_{MF} (▲), wild-type HIV-1_{HXB2} (■), BHAP-resistant HIV-1_{MF} (HIV-1_{MFE30}, △), and BHAP-resistant HIV-1_{HXB2} (HIV-1_{HXBE30}, □) by U-90152S (*A*), L-697,661 (*B*), or Nevirapine (*C*). Percent of antigen-positive cells relative to untreated (no drug) control culture was determined by indirect immunofluorescence 5–6 days postinfection.

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