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In Vitro Selection and Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) Isolates with Reduced Sensitivity to Hydroxyethylamino Sulfonamide Inhibitors of HIV-1 Aspartyl Protease

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Human immunodeficiency virus type 1 (HIV-1) variants with reduced sensitivity to the hydroxyethylamino sulfonamide protease inhibitors VB-11,328 and VX-478 have been selected in vitro by two independent serial passage protocols with HIV-1 in CEM-SS and MT-4 cell lines. Virus populations with greater than 100-fold-increased resistance to both inhibitors compared with the parental virus have been obtained. DNA sequence analyses of the protease genes from VB-11,328- and VX-478-resistant variants reveal a sequential accumulation of point mutations, with similar resistance patterns occurring for the two inhibitors. The deduced amino acid substitutions in the resistant protease are Leu-10→Phe, Met-46→Ile, Ile-47→Val, and Ile-50→Val. This is the first observation in HIV protease resistance studies of an Ile-50→Val mutation, a mutation that appears to arise uniquely against the sulfonamide inhibitor class. When the substitutions observed were introduced as single mutations into an HIV-1 infectious clone (HXB2), only the Ile-50→Val mutant showed reduced sensitivity (two- to threefold) to VB-11,328 and VX-478. A triple protease mutant infectious clone carrying the mutations Met-46→Ile, Ile-47→Val, and Ile-50→Val, however, showed much greater reduction in sensitivity (14- to 20-fold) to VB-11,328 and VX-478. The same mutations were studied in recombinant HIV protease. The mutant protease Ile-50→Val displays a much lower affinity for the inhibitors than the parent enzyme (≤80-fold). The protease triply mutated at Met-46→Ile, Ile-47→Val, and Ile-50→Val shows an even greater decrease in inhibitor binding (≤270-fold). The sulfonamide-resistant HIV protease variants remain sensitive to inhibitors from other chemical classes (Ro 31-8959 and L-735,524), suggesting possibilities for clinical use of HIV protease inhibitors in combination or serially.

The human immunodeficiency virus (HIV) aspartyl protease is required for processing the Gag and Gag-Pol polypeptide precursors during virion maturation (5, 11, 18). Given the essential function of the virus-encoded protease, a considerable effort is being made to design compounds that potently inhibit the protease and suppress HIV replication. To date, a wide variety of HIV protease inhibitors have been designed. Many of these are potent inhibitors of HIV replication in vitro, and several have shown acceptable oral bioavailability and pharmacokinetic profiles in laboratory animals (35, 39, 40). Several inhibitors are undergoing clinical evaluation in patients with mid-stage AIDS (2, 14).

The appearance of HIV drug resistance is a concern for antiviral therapy with reverse transcriptase inhibitors. Drug resistance is a particularly difficult problem for anti-HIV agents because of the relatively low fidelity of the viral reverse transcriptase (30) and replication of the virus over extended periods in infected individuals. HIV drug resistance is exemplified by the emergence, both in cell culture experiments and in the clinic, of reverse transcriptase mutants resistant to nucleoside and nonnucleoside inhibitors (4, 32). Although the clinical significance of HIV drug resistance to the nucleoside and nonnucleoside inhibitors is still being assessed (22, 33), resistance must now be considered a key concern in the clinical development of all new HIV antiviral agents.

Recently, a number of investigators have succeeded in selecting HIV type 1 (HIV-1) variants in vitro with reduced sensitivity to several protease inhibitors (3, 6, 8, 13, 15, 23, 27, 28). Such work suggests that HIV-1 protease can sustain multiple mutations that interfere with inhibitor activity while maintaining protease function. Even after resistance-conferring mutations have been identified in vitro, the potential clinical significance of these data is difficult to evaluate. Clinical trials with HIV protease inhibitors are in early stages, and very few examples are available for correlation between in vitro and in vivo resistance results (2, 14).

We have performed an in-depth study of the development of HIV-1 resistance in cell culture against two compounds derived from a class of HIV-1 protease inhibitors composed of an N,N-disubstituted hydroxyethylamino sulfonamide scaffold. These inhibitors show high intracellular antiviral potency and good oral availability across several animal species (17, 39). VX-478 is currently undergoing clinical evaluation. The effects of the individual amino acid substitutions obtained in our in vitro passage of VB-11,328 and VX-478 were evaluated bio-

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chemically and by infectivity experiments with defined molecular clones.

MATERIALS AND METHODS

Cells and virus. Two human lymphoblastoid T-cell lines, CEM-SS (10, 26) and MT-4 (25), were used to propagate HIV-1_{IIIB} and HIV-1_{HXB2}, respectively. Cells were maintained in RPMI 1640 containing 10% (vol/vol) fetal bovine serum and antibiotics. The HIV strains used in this study were HIV-1_{IIIB}, originally obtained from Robert Gallo, National Cancer Institute, Bethesda, Md., and the infectious molecular clone HIV-1_{HXB2}, derived from HIV-1_{IIIB} (9). Virus stocks were stored as cell-free supernatants at −70°C.

Selection of HIV-1_{IIIB}-resistant variants. HIV-1_{IIIB} was serially passaged in CEM-SS cells in the presence of twofold-increasing concentrations of VB-11,328 and VX-478. CEM-SS cells were acutely infected at a density of 5.0×10^6/ml with HIV-1_{IIIB} at a multiplicity of infection of 0.001 (5×10^3 50% tissue culture infective doses [$TCID_{50}$] per ml). After adsorption for 2 h at 37°C, the cells were pelleted, washed, and resuspended in 10 ml of complete RPMI at a density of 2.0×10^5/ml. The concentrations of VB-11,328 and VX-478 used at passage 1 were 10 and 50 nM, respectively. When extensive cytopathic effect (syncytium formation) was observed, or when 50% of the cells were infected as detected by immunofluorescence analysis of methanol-fixed cells (43), the cells were pelleted, and 2.5 ml of cell-free viral supernatant was used for the next round of infection. After the initial cycle of infection, infections were performed at the original concentration and at a twofold-higher concentration, and these concentrations were increased twofold whenever cytopathic effect was equivalent at the higher concentration. Cell pellets were washed once in phosphate-buffered saline (Gibco) and stored at −70°C for subsequent DNA sequence analysis. Passage of HIV-1_{HXB2} in MT-4 cells was essentially as described previously (20, 37), using 50 nM VB-11,328 at the initiation of the passage experiment.

Virologic analysis of protease inhibitor sensitivity. (i) CEM-SS acute infection assay. The infectivity of virus stocks was first analyzed by a standard 7-day $TCID_{50}$ culture system. Fourfold dilutions of the stocks were prepared in 96-well plates, in sextuplicate. CEM-SS cells were added to the wells and incubated for 4 days, the cultures were split 1/4, and then on day 7, culture supernatants were collected and p24 assays were performed (DuPont HIV-1 enzyme-linked immunosorbent assay [ELISA]). Using a cutoff of p24 > 50 pg/ml, $TCID_{50}$ values were calculated for each stock by the Spearman-Karber method. The sensitivity of the virus stocks to inhibition by different protease inhibitors was determined by a modification of standard methods. Briefly, CEM-SS cells were bulk infected at a ratio of 100 $TCID_{50}$/4×10^4 cells, washed twice, and suspended to 4×10^5/ml, and 100 μl of this suspension added to wells of 96-well plates containing dilutions of inhibitors in triplicate. Inhibitor dilutions and control wells were prepared in medium containing dimethyl sulfoxide (DMSO) so that all wells contained a final concentration of 1% DMSO. The plates were incubated for 4 days and split 1/4 into medium containing the same concentration of inhibitors, and on day 7, the culture supernatants were harvested. Percent inhibition values were calculated by comparing the amounts of supernatant p24 produced by cultures in the presence or absence of inhibitors.

(ii) MT-4 acute infection assay. Determination of the infectivity ($TCID_{50}$) of the virus stocks and the virus sensitivity assays were performed by the dye uptake method as described previously (29). Briefly, for sensitivity assays, serial dilutions of the test compounds were made in 100% DMSO to 100 times the final desired concentrations. One-microliter volumes of the dilution series were added to each well of a 96-well microplate, using eight replicate wells per dilution. Control wells received 1 μl of DMSO. MT-4 cells, either mock infected or infected with HIV at a multiplicity of infection of 0.001 for 1 h at 37°C, were added to the plate at a concentration of 40,000 cells per well in 100 μl of RPMI medium containing 10% fetal calf serum, and the cultures were incubated at 37°C for 5 days. Cultures were assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MT-4/MTT assay), and the concentration of drug required to protect 50% of the cells from virus killing (IC_{50}) was determined from regression analysis of the plot of percent cell death against drug concentration.

Genetic analysis of protease gene by direct PCR sequencing. The complete protease gene was amplified from cellular DNA extracts by PCR using Amplitaq polymerase and standard external primers (F1 [CTGTTTGAAATGTGGAAAG GAAGGA; 2024 to 2052] and R1 [CTTTATGGCAAATACTGGAGTATTGT ATGGA; 2710 to 2740]). The PCR synthesis was performed for 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and subsequent synthesis at 72°C for 2 min. Single-stranded DNA was prepared by asymmetric PCR using a 100:1 or 1:100 ratio of the same primers and similar reaction conditions. The annealing condition was changed to 55°C, and 35 cycles were performed. Dideoxy sequencing reactions were performed with internal fluorescent primers, and the samples were analyzed, in both directions, by using the Fluorescent A.L.F. DNA Sequencer (Pharmacia) nonradioactive system (42).

Construction of infectious clones containing mutant protease. Approximately 6 kb of human sequences flanking the HIV-1_{HXB2} provirus in pIB120HIV were removed to increase the stability of the resulting proviral vector, pHSBΔFLK. The Gag-Pro-reverse transcriptase *Kpn*I-*Sna*I fragment was cloned into M13, site-directed mutagenesis was performed to introduce unique *Not*I and *Asp*I sites 39 bp 5′ and 27 bp 3′, respectively, of the protease open reading frame, and the entire fragment was cloned back into pHXBΔFLK, to give pHXB-NAPro (24). Mutant protease *Not*I-*Asp*I gene fragments were created by PCR mutagenesis, or the complete protease gene was amplified from cellular DNA extracts by PCR. The recombinant viruses were produced by electroporation of MT-4 cells following direct cloning into pHXB-NAPro as previously described (16). Clones were sequenced, using fluorescence-labeled sequencing primers on an Applied Biosystems (Foster City, Calif.) automated sequencer, to confirm the presence of mutations after PCR or to obtain the mutational linkage from the passaged virus. Recombinant viruses were used in MT-4 sensitivity assays as described above.

Site-directed mutagenesis of recombinant protease. All oligonucleotide-directed mutagenesis was performed on pT7-HIV-1, using uracil enrichment of single-stranded DNA by the modification of Kunkel et al. (19). Mutagenesis was performed with the reagents provided in the Bio-Rad Mutagene kit. Mutations were confirmed by PCR sequencing using a dideoxy terminator sequencing kit from Applied Biosystems, and samples were analyzed on an Applied Biosystems sequencer (model 373A). *Nde*I-*Eco*RI fragments coding for the HIV-1 protease open reading frame were then subcloned into a pSPC27 vector (S. P. Chambers, Vertex Pharmaceuticals Inc.) for expression, using an isopropylthiogalactopyranoside (IPTG)-inducible Tac promoter. The amino acid sequence of recombinant protease was identical to the amino acid sequence of the HIV-1_{IIIB} protease. *Escherichia coli* RB791 (from R. Brent, Harvard University) was used as the host.

Purification of mutant HIV proteases. A typical purification involved suspending 25 g of cell paste in buffer A (25 mM morpholineethanesulfonic acid [MES] buffer [pH 6.0] containing 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol) containing protease inhibitor cocktail (final concentrations, 1 μg of leupeptin per ml, 2 mM benzamidine, and 3 mM phenylmethylsulfonyl fluoride) and nonionic detergent (0.5% Nonidet P-40), and the cells were lysed either by sonic disruption or in a microfluidizer. The lysate was harvested by centrifugation at 16,000 rpm in a Beckman JA17 rotor for 30 min, batch extraction with DEAE-53, and passage through an S-Sepharose column. The column was eluted with 0 to 1.0 M NaCl gradient in buffer A, with the active fractions eluting at 0.3 M NaCl. The pooled fractions were adjusted to 40% ammonium sulfate, and the resulting precipitate was collected by centrifugation at 16,000 rpm in a Beckman JA17 rotor for 30 min. The pellet was dissolved in buffer B (25 mM MES buffer [pH 6.0] containing 1 mM dithiothreitol, 1 mM EDTA, 0.5 M NaCl, and 5% glycerol) and applied to a Sephadex G-75 gel permeation column. The active fractions were pooled, and the protein concentration was determined. The concentration of active protease was determined by active-site titration (12).

Determination of K_i values. The assays were carried out in the presence of 1 mM dithiothreitol, 1 mM EDTA, and 50 mM citrate buffer at pH 4.5. A stock solution was prepared for 10 assays containing all of the above-specified components and enzyme to yield a final concentration of approximately 1 nM. The stock solution was aliquoted into vials, a fixed volume of DMSO or inhibitor in DMSO (the final concentration of DMSO in the reaction mixture was 1.7%) was introduced, the components were mixed, and the mixture was incubated for 15 min at room temperature (12). The reaction was initiated with the *p*-nitrophenylalanine substrate His-Lys-Ala-Arg-Val-Leu/p(NO_2)Phe-Glu-Ala-Nle-Ser-NH_2 and incubated at 37°C. After 30 min, the reaction was quenched with 20% trifluoroacetic acid, and the product of the reaction was quantified by high-pressure liquid chromatography analysis (36). The data were fitted to a tight-binding competitive inhibition model, using the software KineTic (BioKin Ltd., Madison, Wis.) to evaluate the inhibitory constants (K_i).

RESULTS

Passage of HIV-1_{IIIB} in the presence of protease inhibitors VB-11,328 and VX-478. CEM-SS cells were infected with HIV-1_{IIIB}, and the virus produced was serially passaged in the presence of increasing concentrations of the inhibitors. Spread of infection was monitored by microscopic observation of syncytium formation, indirect immunofluorescence, or both. When the cells displayed extensive syncytium formation or when >50% were positive for viral antigen expression by immunofluorescence, cell-free viral supernatant was harvested and used to infect fresh CEM-SS cells (one passage) at the current concentration and at twofold-higher concentrations of inhibitor. When these two conditions resulted in similar rates of infection, the next passage was initiated by using another twofold-higher increase in inhibitor concentration. The amount of virus ($TCID_{50}$ per milliliter) recovered in culture supernatants was determined by endpoint dilution in CEM-SS cells, and samples of infected cell pellets were stored at −70°C for genetic analysis.

In the first study using VB-11,328, virus was passaged 20 times, beginning with a concentration slightly below the IC_{90}

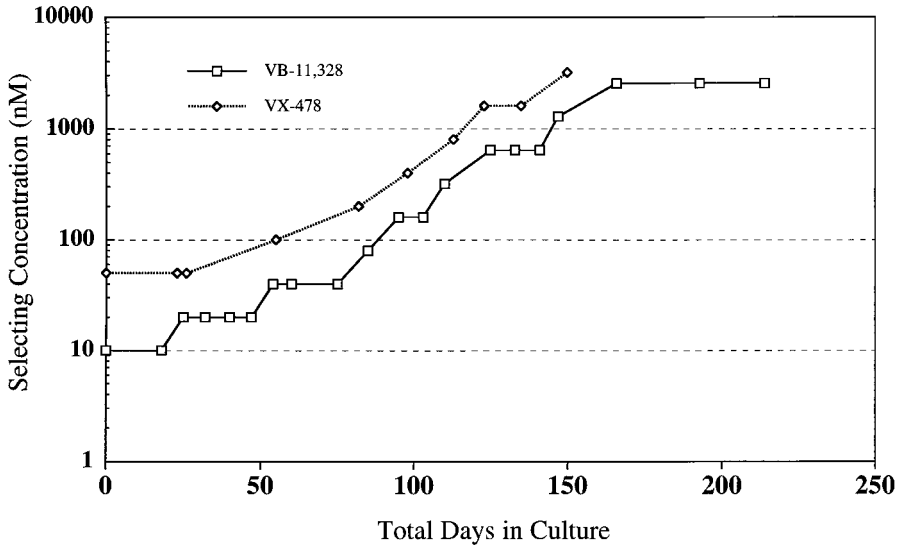

FIG. 1. Summary of selecting concentrations of VB-11,328 and VX-478 in cultures of HIV-1_{IIIB}-infected CEM-SS cells. Each point on the curve represents a single passage.

(10 nM) and ending at 2,560 nM (Fig. 1). Within the first 50 days of the experiment, we observed a delay in resistance progression at 20 nM. At this point, the concentration of VB-11,328 was doubled to 40 nM, and subsequent passages remained at the same selecting concentration for an average of two passages at each concentration; passages were made approximately every 10 days. Drug resistance at 2,560 nM was reached after a total of 225 days in culture.

Our method for selection of HIV-1 variants with reduced sensitivity to VX-478 was slightly modified on the basis of our experience with VB-11,328. In the case of VX-478, virus was passaged to fresh cells when the infection had spread to 90% of the cells. The virus was passaged 10 times beginning at an inhibitor concentration of 50 nM (slightly above the IC_{90}) and ending at 3,200 nM (Fig. 1). As with VB-11,328, we observed a delay in selection progression at the beginning concentration of 50 nM, and it required 36 days in culture until we could double the concentration to 100 nM. Subsequent passages with virus supernatants were made on average every 16 days, and each passage represented a doubling in selecting concentration.

Sensitivity of passaged virus to VB-11,328 and VX-478. To confirm that the passaged viruses were resistant to VB-11,328 and VX-478, cell-free virus supernatants collected at various passages were used in an acute infection assay to test for reduced sensitivity to the inhibitors (see Materials and Methods). Percent inhibition values were calculated by comparing the amounts of supernatant p24 produced by cultures in the presence or absence of inhibitors (Table 1). A gradual increase in VB-11,328 resistance as the selecting concentration of VB-11,328 increased was observed. The IC_{90} for the resistant virus increased from 29 nM at passage 10 to >2,000 nM at passage 20, representing an approximate 100-fold reduction in sensitivity in comparison with the level for the control virus passaged in the absence of drug. At each step in the selection procedure, the IC_{90} for the virus stock was approximately

TABLE 1. Antiviral activity of protease inhibitors[a]

Protease inhibitor	Passage no.	Selecting concn (nM)	Amino acid substitution(s)	VB-11,328			VX-478			Ro 31-8959			L-735,524		
				IC_{50}	IC_{90}	Ratio[b]	IC_{50}	IC_{90}	Ratio	IC_{50}	IC_{90}	Ratio	IC_{50}	IC_{90}	Ratio
VB-11,328	0	None	None	7	23	1	12	26	1	7	22	1	7	27	1
	10	80	L10F	ND[c]	29	1	ND	ND		ND	ND		ND	ND	
	12	160	L10F, I84V	ND	85	4	ND	ND		ND	ND		ND	ND	
	13	320	L10F, I84V	40	180	8	50	350	13	9	39	2	ND	ND	
	15	640	L10F, I50V	170	430	19	590	1,400	53	10	32	1	ND	ND	
	17	1,280	L10F, M46I, I50V	190	1,500	65	800	2,600	100	2	17	1	6	120	4
	20	2,560	L10F, M46I, I47V, I50V	550	2,500	100	1,694	6,243	240	19	50	2	25	100	4
VX-478	0	None	None	12	26	1	7	23	1	7	22	1	7	27	1
	7	800	L10F, I84V	100	210	8	500	880	38	12	20	1	27	54	2
	8	1,600	L10F, I50V	ND	ND		180	2,000	87	ND	ND		ND	ND	
	9	1,600	L10F, M46I, I47V, I50V	790	2,800	108	1,500	3,400	150	9	28	1	18	79	3
	10	3,200	L10F, M46I, I47V, I50V, D60V	450	1,200	46	1,100	4,000	170	8	28	1	27	160	6

[a] HIV-1_{IIIB} was passaged in CEM-SS cells in the presence of increasing concentrations of VB-11,328 or VX-478. The selecting concentration at each passage is indicated. Sensitivity testing was performed on virus stocks derived from infected CEM-SS cells at various passages. The IC_{50} and IC_{90} values were determined as described in Materials and Methods.

[b] Increase in IC_{90} with respect to that of the zero-passage virus.

[c] ND, assay not done.

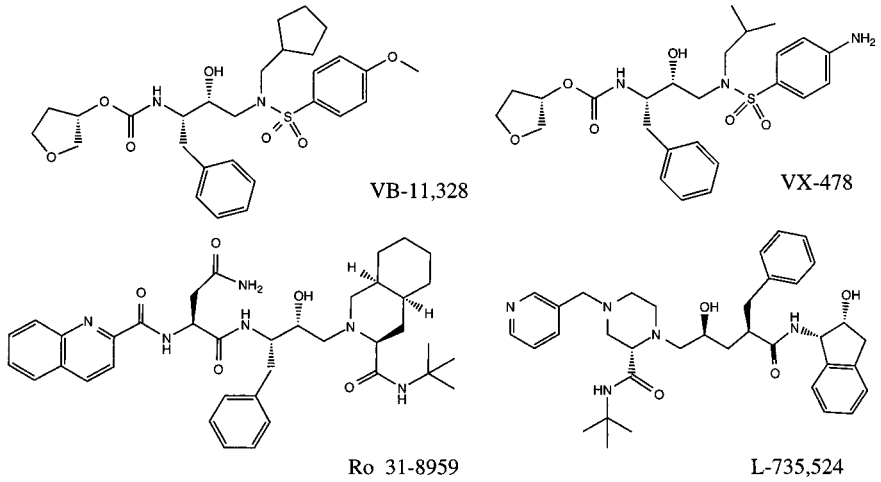

FIG. 2. Structures of protease inhibitors used in this study. VB-11,328 and VX-478 have been described by Tung et al. (39) and Kim et al. (17), Ro 31-8959 has been described by Roberts et al. (34), and L-735,524 has been described by Dorsey et al. (7).

equal to the selecting concentration in use at that time. We found a similar trend for the sensitivity of the VX-478-resistant virus when tested against VX-478 (Table 1). The IC_{90} for the VX-478-resistant virus increased from 880 nM at passage 7 to 4,000 nM at passage 10.

Sequence analysis of VB-11,328- and VX-478-resistant viruses. The results of the sensitivity assay shown in Table 1 suggested that genetic changes had occurred during the VB-11,328 and VX-478 passage experiments. Although genotypic changes outside protease region might conceivably contribute to resistance, we were interested in identifying those amino acid substitutions within the protease region that have a direct or indirect effect on the activities of VB-11,328 and VX-478. The complete protease gene was amplified from DNA extracts obtained from frozen cell pellets that we retained at each passage. Sequencing was performed with internal fluorescent primers and PCR. The samples were analyzed on a semiautomated DNA sequencer, with the resulting chromatograms representing a consensus sequence from the pooled viral DNA in the test sample. In preliminary mixing experiments using molecular clones, this sequencing method was able to detect minority populations of 25% or greater (results not shown). The sequence results of the proteases obtained from passages 0, 10, 12, 13, 15, 17, and 20 from the VB-11,328 selection experiment are shown in Table 1. At passage 10, we observed the Leu-10→Phe (L10F; CTC-TTC) mutation, which remained throughout the entire selection period. At passage 12, when we observed the first change in IC_{90}, both L10F and Ile-84→Val (I84V; ATA-GTA) were present. Analysis of the sequencing chromatogram suggested that approximately 50% of the virus population contained the I84V mutation. By passage 13, more than 90% of the viral proteases contained the I84V mutation. Interestingly, by passage 15, the I84V population was reduced to undetectable levels and a substitution of I50V (ATA-GTA) predominated. The I50V mutation persisted throughout the selection experiment, while Met-46→Ile (M46I; ATG-ATA) and, finally, I47V (ATA-GTA) arose at later passages. The drug-resistant virus population from passage 4 was further propagated on CEM-SS cells in the absence of drug for 11 days. Sequencing results from the protease region of this virus population were essentially identical to those of the passage 4 virus, suggesting that the genotypes were stable in the absence of selective pressure, at least for short time periods.

Since VB-11,328 and VX-478 are structurally related (Fig. 2), it was not surprising that the mutations found in the resistant viruses selected by these two agents were similar. All mutations selected by VB-11,328 were also selected by VX-478, with the exception of Asp-60→Val (D60V), which appeared at passage 10 of the VX-478 experiment (Table 1) together with L10F, M46I, I47V, and I50V.

Passage of HIV-1_{HXB2} in MT-4 cells in the presence of VB-11,328. In an independent selection protocol, MT-4 cells were acutely infected with an HIV-1_{HXB2} molecular clone, and virus was sequentially passed in increasing concentrations of VB-11,328. Sixty-five days later, at passage 6, the concentration of inhibitor reached 800 nM. Genetic analysis at each pass revealed at passage 3 an isoleucine-to-valine change at position 50 of protease, and by passage 6, M46I I47V I50V triple mutations were observed. MT-4/MTT assays revealed 8- and 30-fold increases in IC_{50} for the passage 4 and passage 6 viruses, respectively, compared with the HXB2 control. Our observation of the same amino acid substitutions at positions 46, 47, and 50 in CEM-SS and MT-4 cells confirmed that these residues are important in conferring resistance to VB-11,328 and VX-478. Sequencing of individual protease clones at passage 4 confirmed that the I50V substitution was present as a single mutation, and by passage 6, the M46I, I47V, and I50V mutations were linked in six of six clones (36a). The L10F and I84V mutations were not observed in our accelerated selection protocol using HIV-1_{HXB2} in MT-4 cells and VB-11,328, possibly because of the higher selection pressure used in this procedure. Alternatively, different patterns of resistance may emerge by the use of different starting genetic backgrounds or different host cell lines; i.e., HIV-1_{IIIB} is more heterogeneous than the infectious clone HIV-1_{HXB2}.

Characterization of mutant proteases in infectious molecular clones. PCR mutagenesis was used to construct the HXB2 single protease mutants L10F, M46I, I47V, I50V, and I84V, the double mutant M46I I47V, and the triple mutant. In every case, the genotype of the protease in the molecular clone was confirmed by sequencing. The phenotypic significance of these individual amino acid substitutions was assessed by comparing the IC_{50} values of VB-11,328 and VX-478 for the mutant viruses against the parental HXB2 clone. The IC_{50} values were determined in an MT-4/MTT assay for both VB-11,328 and VX-478. The I50V mutant showed a moderate increase in IC_{50}

TABLE 2. Anti-HIV potency of VX-478 and VB-11-328 against HIV infectious molecular clones containing protease mutations

Mutant	IC_{50}[a] (nM) (ratio[b])	
	VB-11,328	VX-478
HXB2	29 (1)	82 (1)
L10F	23 (1)	80 (1)
L10F I84V	ND	198 (2)
M46I	27 (1)	86 (1)
I47V	13 (1)	120 (1)
I50V	86 (3)	177 (2)
I84V	30 (1)	124 (1)
I47V M46I	57 (2)	113 (1)
M46I I47V I50V	613 (20)	1115 (14)

[a] Determined in an MT-4/MTT assay; average of two to three assays.
[b] IC_{50} of HIV variants with respect to the wild-type (HXB2).

TABLE 3. Inhibition constants against HIV-1 protease mutants

Mutant	K_i (nM) (ratio[a])			
	VB-11,328	VX-478	Ro 31-8959[b]	L-735,524
Wild type	<0.1 (1)	0.6 (1)	0.8 (1)	1 (1)
L10F	<0.1 (1)	0.4 (1)	1.4 (1)	4 (4)
L10F I84V	2 (>22)	12 (20)	17 (21)	24 (24)
I84V	1 (>10)	14 (23)	10 (12)	20 (20)
M46I	0.1 (>1)	0.2 (1)	0.8 (1)	(4) (4)
I47V	0.2 (>2)	0.4 (1)	0.4 (1)	3 (3)
I50V	3 (>30)	50 (83)	17 (21)	10 (10)
M46I I47V I50V	14 (>140)	160 (270)	33 (41)	29 (29)

[a] Fold decrease in K_i with respect to the wild type.
[b] pH sensitive.

values of both compounds (Table 2). The triple mutant showed a 10- to 20-fold increase in IC_{50} values of VX-478 and VB-11,328, respectively, compared with the HXB2 control. Interestingly, the M46I and I47V single mutants showed essentially no increase in IC_{50} values of VB-11,328 and VX-478. This result suggests that there is a synergistic effect between the mutations at positions 46, 47, and 50 when all three mutations are present. The L10F and the I84V substitutions by themselves did not contribute to the resistant phenotype, but the double L10F I84V mutation did confer resistance to VX-478. The IC_{50} increases observed for the infectious clone mutants were in good agreement with the IC_{50} changes observed in the same assays for our passage 4 and 6 viruses for MT-4 cells. The inhibitory concentration values for the molecular clones were 5- to 10-fold lower (Table 2) than those determined for the passaged virus (Table 1). This difference may be due to viral strain variation and heterogeneity, use of different host cell lines (CEM-SS and MT-4), or use of different assay systems (p24 ELISA and MT-4 MTT dye uptake). A strong trend exists, however, between all of the virological data, suggesting that the triple mutant is the most resistant, followed by the I50V mutant.

Cross-resistance to other protease inhibitors. As expected from the genotypic analysis, the VB-11,328-resistant virus populations were highly cross resistant to VX-478, and similarly, the VX-478-resistant virus populations were cross resistant to VB-11,328 (Table 1). In addition, we were interested in exploring the question of cross-resistance between these resistant populations and other potent protease inhibitors that have been described. We observed no greater than a sixfold reduction in sensitivity between either the VB-11,328 or VX-478 resistant virus and the protease inhibitor L-735,524. No cross resistance was observed with Ro 31-8959 (Table 1). This finding suggests that VB-11,328 and VX-478 have different binding interactions with HIV protease compared with two other chemical classes of protease inhibitors.

Enzymology of mutant proteases. To confirm that the decreased sensitivity to VB-11,328 and VX-478 was due to a decrease in binding affinity for the inhibitors, we expressed enzymes containing the single substitutions L10F, M46I, I47V, I50V, and I84V or the triple mutant in *E. coli*. The mutant proteases were purified, and the K_i values were determined for VB-11,328, VX-478, Ro 31-8959, and L-735,524 (Table 3). In comparison with the wild-type protease, the L10F, M46I, and I47V mutations by themselves had minimal effects on binding affinity of all compounds tested. However, M46I and I47V together with the substitution of Val for Ile at residue 50, constituting the triple protease mutant, resulted in a greater than 100-fold increase in K_i for both VB-11,328 and VX-478. This shift was significantly greater than that obtained for the single I50V mutation. We observed 10- and 20-fold shifts in K_i for VB-11,328 and VX-478, respectively, for the I84V single mutant as compared with the wild-type protease. Finally, the K_i value for the L10F mutant demonstrates that this mutation does not have an effect on the binding of any of the compounds that we examined. Moderate levels of cross-resistance to L-735,524 and Ro 31-8959 were observed with I50V and I84V mutants and the triple mutant. These differences in K_i values do not result in significant shifts in the IC_{50} values of Ro 31-8959, as determined in the virus passage experiments (Table 1).

DISCUSSION

In this investigation, we have obtained HIV-1 variants with significant resistance to the structurally related N,N-disubstituted hydroxyethylamino sulfonamides VB-11,328 and VX-478. Passaging experiments with both compounds, using HIV-1_{IIIB} in CEM-SS cells, were comparable in duration, despite slight differences in the protocol, and were terminated at final drug concentrations of 2,560 and 3,200 nM for VB-11,328 and VX-478, respectively. The length of time required to achieve these drug concentrations is comparable to the length of time reported by Craig et al. (3) to select viral populations resistant to the protease inhibitor Ro 31-8959 at a concentration of 1,280 nM. This relatively slow rate of development of resistance to protease inhibitors contrasts sharply with the rapid generation of resistance observed by the same workers for the nonnucleoside inhibitor tetrahydroimidazo[4,5,1-*jk*] [1,4]benzodiazepin-2(1 *H*)-thione (TIBO).

The generation of overlapping mutations in our passage experiments with VB-11,328 and VX-478 was not surprising given the structural similarities of the two compounds (Fig. 2). As the selecting concentration was increased in both passage experiments, the L10F I84V mutant virus populations were replaced by variants with a more stable mutation at residue 50 (Table 1), suggesting either a possible growth advantage over HIV-1 isolates with a mutation at position 84 or negative interaction between the substitutions at residues 10 and 84. We are currently examining the replication kinetics of infectious clones with Val substitutions at residues 50 and 84 to understand the factors responsible for the shift in virus populations.

The infectious clone data and our kinetic analysis (Tables 2 and 3) demonstrate the importance of the I50V mutation in conferring resistance to VB-11,328 and VX-478. To date, the I50V substitution is unique to VB-11,328 and VX-478 resistance. No effect on antiviral potency was observed for the single I84V infectious clone. This finding contrasts with our

FIG. 3. Ribbon diagram of HIV-1 protease showing binding pocket interactions with VX-478. VX-478 is shown in light blue. The flap water molecule is shown in red and labeled Wat. The amino acid side chains subject to mutation during virus passage are Met-46/46′ (orange), Ile-47/47′ (white), Ile-50/50′ (yellow), and Ile-84/84′ (magenta) and are labeled on one monomer. Ile-50 (yellow) and Ile-84′ (magenta) side chains show the van der Waals surface.

enzymatic data, which show that the I84V mutant recombinant protease increases the K_i value approximately 10-fold. The reason for this discrepancy is not understood. It does appear that the L10F I84V double mutation (Table 2) affects inhibitor binding in the virus. The I84V mutation has been observed in selection experiments with the protease inhibitors RPI312 (8), ABT-538 (23), Ro 31-8959, and XM323 (38) and appears to be an important protease mutation for cross-resistance (2, 38).

We expect that mutations at residues Ile-84 and Ile-50 affect both inhibitor binding and substrate processing, given their central location in the active site. Ile-84 and Ile-50 are both at the center of the active site and are part of all subsites from S_2 to $S_{2'}$ (Fig. 3). The side chains of these residues are nearly coplanar with the two active-site Asp-25 and 25′ side chains. The C_δ atoms of Ile-84 and Ile-50 make hydrophobic interactions with the phenyl sulfonamide moieties in VB-11,328 and VX-478. Therefore, Ile-to-Val mutations could result in loss of hydrophobic interactions, resulting in reduced inhibitor binding (31). We are currently investigating the effects of the I84V and I50V mutations on a variety of synthetic *gag-pol* substrates to understand their effects on the catalytic efficiency of recombinant protease.

During late in vitro passages with VB-11,328 and VX-478, regardless of the selection protocol or host cell line, the M46I I47V I50V triple protease mutants were observed to arise against VB-11,328 and VX-478. The significance of the triple mutation in reducing the sensitivity of VB-11,328 and VX-478 was confirmed by site-directed mutagenesis of an HIV-1 infectious clone (Table 2). When M46I, and I47V were combined with I50V to form a triple mutant protease, the IC_{50} values increased 14- and 20-fold for VB-11,328 and VX-478, respectively. Although the inhibitory concentration values from our molecular clone data (Table 2) do not agree quantitatively with the inhibitory concentration values for the passaged virus (see Results and Table 1), a general trend exists whereby the triple mutant is the most resistant mutant, followed by the I50V mutant. This trend was also confirmed by our kinetic analysis, whereby the K_i values for the triple mutant were increased >100-fold for VB-11,328 and VX-478.

This work suggests that HIV-1 protease has the plasticity to sustain multiple sequence changes that result in reduced inhibitor binding but not elimination of catalytic competency. Although molecular modeling methods have been successful in identifying key amino acid residues in HIV-1 protease that influence drug sensitivity of substrate based inhibitors (21), cell culture experiments are necessary to predict the multiple amino acid substitutions that are advantageous for viral growth and escape from inhibition. Viral kinetic experiments suggest that compensatory mutations outside the active site often appear in conjunction with deleterious active-site mutations (3, 6, 8, 13, 15, 23, 27, 28). For example, M46I is frequently selected by the virus during in vitro passage experiments (13, 23, 38). Our molecular clone (Table 2) and kinetic (Table 3) data show that the M46I substitution is not directly responsible for drug resistance to the sulfonamide inhibitors. In passage experiments with A77003, Ho et al. (13) found that M46I appeared to improve the replication kinetics of a severely deficient Arg-8→Gln mutant virus. It appears that M46I is in an important region in the flap that influences substrate binding, and molecular dynamics simulations indicate that the M46I substitution favors a closed conformation relative to the wild-type enzyme when HIV protease binds substrate or inhibitor (23).

In our passage experiments with HIV-1_{IIIB} in the presence of VB-11,328 and VX-478, the L10F substitution was maintained stably throughout the selection process. This mutation has been observed in selection of HIV variants with reduced sensitivity to XM323 (38), but the molecular basis for this

change is unknown. Amino acid variation at position 10 occurs naturally in HIV-1 populations (41).

Cross-resistance profiles are valuable because they can identify distinct classes of protease inhibitors for possible clinical use in combination. Our cross-resistance data suggest that the structurally similar protease inhibitors VB-11,328 and VX-478 produce a pattern of resistance that is distinct from that obtained for Ro 31-8959 and L-735,524. In fact, we saw no cross-resistance to Ro 31-8959 and only a maximum of sixfold resistance to L-735,524 for the mutant viruses that we studied. The lack of cross-resistance to Ro 31-8959 is interesting, given that the triple mutant enzyme has a 40-fold decrease in K_i value relative to the wild-type enzyme. In preliminary studies, we observed that the K_i value for Ro 31-8959 decreases with pH, and thus some of the quantitative variance between the antiviral and biochemical data may be due to differences between enzyme assay conditions and the intracellular environment.

Although the in vitro data suggest that HIV-1 protease can sustain multiple sequence changes, little is known about the effects of these mutations on the replication capacity of the altered virus. This will be an area of study in the future, since there is evidence to suggest that small changes in replicative capacity may have dramatic effects on the evolution of virus populations (1). Our identification of novel HIV protease resistance mutations that arise upon selection against sulfonamide inhibitors highlights the need for a study of the population growth kinetics of HIV strains selected by various protease inhibitors (4).

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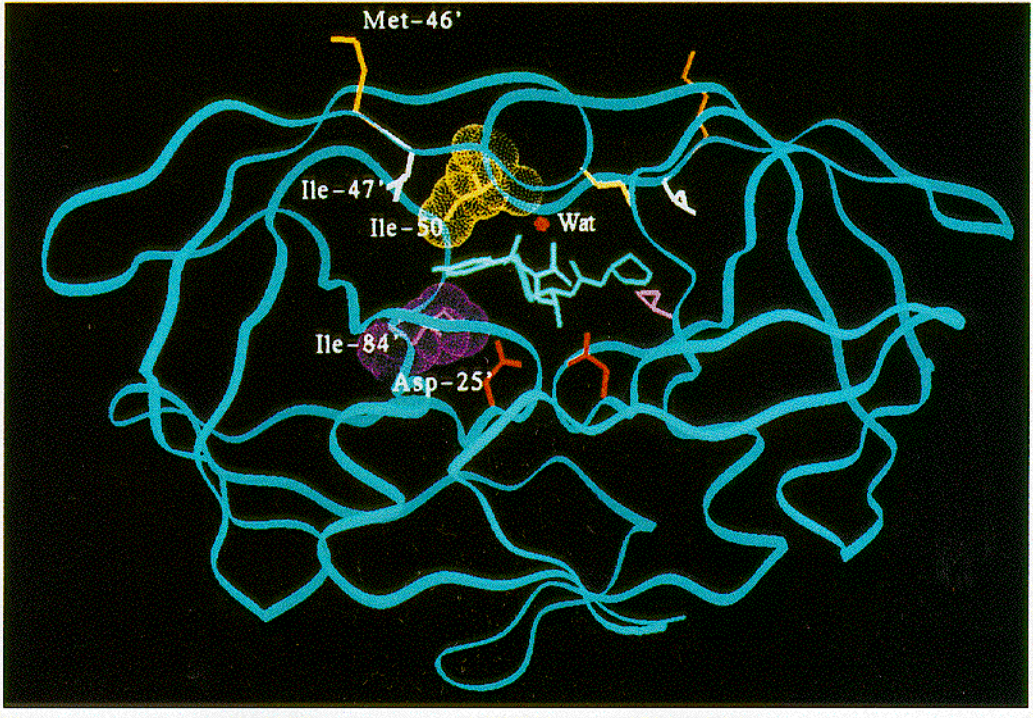
Met-46'
Ile-47'
Ile-50
Wat
Ile-84'
Asp-25'