ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1998, p. 2637–2644 Vol. 42, No. 10
0066-4804/98/$04.00+0
Copyright © 1998, American Society for Microbiology. All Rights Reserved.

Genotypic and Phenotypic Characterization of Human Immunodeficiency Virus Type 1 Variants Isolated from Patients Treated with the Protease Inhibitor Nelfinavir

A. K. PATICK,[1]* M. DURAN,[2] Y. CAO,[2] D. SHUGARTS,[3] M. R. KELLER,[1] E. MAZABEL,[1] M. KNOWLES,[1] S. CHAPMAN,[1] D. R. KURITZKES,[3] AND M. MARKOWITZ[2]

Agouron Pharmaceuticals, Inc., San Diego, California 92121[1]; Aaron Diamond AIDS Research Center, New York, New York 10016[2]; and University of Colorado Health Sciences Center, Denver, Colorado 80262[3]

Received 27 January 1998/Returned for modification 6 April 1998/Accepted 1 July 1998

Nelfinavir mesylate (formerly AG1343) is a potent and selective inhibitor of human immunodeficiency virus (HIV) protease approved for the treatment of individuals infected with HIV. Nucleotide sequence analysis of protease genes from plasma HIV type 1 (HIV-1) RNA revealed a unique aspartic acid (D)-to-asparagine (N) substitution at residue 30 (D30N) in 25 of 55 patients treated with nelfinavir for a median of 13 weeks. Although the appearance of D30N was occasionally associated with concurrent or sequential emergence of other changes (e.g., at residues 35, 36, 46, 71, 77, and 88), genotypic changes associated with phenotypic resistance to other protease inhibitors were not observed (e.g., at residues 48, 50, 82, and 84) or were only rarely observed (e.g., at residue 90). In phenotypic assays, viral isolates with high-level resistance to nelfinavir remained susceptible to indinavir, saquinavir, ritonavir, and amprenavir (formerly VX-478/141W94). Similar results were observed in phenotypic assays utilizing HIV-1 NL4-3, which contained the D30N substitution alone or in combination with substitutions at other residues (e.g., residues 46, 71, and 88). These data indicate that the initial pathway of resistance to nelfinavir is unique and suggest that individuals failing short courses of nelfinavir-containing regimens may respond to regimens containing other protease inhibitors.

The antiretroviral drugs currently approved for the treatment of individuals infected with human immunodeficiency virus (HIV) include nucleoside analogues and nonnucleoside inhibitors which target the viral reverse transcriptase (RT), as well as a third class of antiretroviral agents which target the viral protease. The HIV protease inhibitors approved to date include indinavir (MK-639), ritonavir (ABT-538), saquinavir (Ro 31-8959), and nelfinavir (AG1343) (30). Data from clinical trials have confirmed the utility of HIV protease inhibitors as important components of potent antiviral therapies and have indicated significant reductions in levels of HIV-1 RNA in plasma and increases in $CD4^+$ cells after treatment (3, 5, 10, 13, 27, 42, 49). However, the loss of suppression of virus replication in vivo is usually associated with the emergence of viral variants with reduced susceptibility to all three classes of drugs and all currently available protease inhibitors (7–9, 18, 20, 27, 31, 42, 43, 48). As a prerequisite for treating patients with sequential or combination protease inhibitor-containing regimens, an understanding of the susceptibility of protease-resistant variants to other protease inhibitors is essential. Current data indicate that broad phenotypic cross-resistance exists between HIV variants derived from patients treated with ritonavir and indinavir (7, 8, 31, 43). A subset of these HIV variants also demonstrate reductions in susceptibility to other protease inhibitors, including saquinavir and amprenavir. Reductions in susceptibility in isolates derived from patients treated with saquinavir have also been reported elsewhere (9). The broad cross-resistance observed among various protease-resistant HIV type 1 (HIV-1) variants is consistent with genotypic data, which show that these structurally unrelated protease inhibitors frequently select for the same amino acid substitutions at residues 82, 84, and 90 that interact either directly or indirectly with substrate or inhibitor binding (7–9, 27, 31, 42, 43).

Nelfinavir mesylate is a nonpeptidic inhibitor of HIV-1 protease that was discovered by protein structure-based design methodologies (22, 35). In vitro, nelfinavir has demonstrated potent activity against laboratory, clinical, and RT-resistant strains of HIV-1 and -2 and has produced additive-to-synergistic interactions when combined with other antiretrovirals (35, 36). In human clinical trials, nelfinavir has demonstrated safety and efficacy as monotherapy as well as in combination with either stavudine (d4T) or with zidovudine (ZDV) plus lamivudine (3TC) (6, 12, 15, 28, 29, 39, 41). We have previously characterized HIV-1 variants selected in vitro following serial passage of wild-type HIV-1 NL4-3 in the presence of increasing concentrations of nelfinavir (35). Following 22 serial passages, a variant (p22) was identified which had a sevenfold reduction in susceptibility to nelfinavir and which contained a previously undescribed aspartic acid (D)-to-asparagine (N) substitution at position 30 (D30N). To extend these in vitro studies, nucleotide sequence analysis was performed on HIV protease genes obtained from HIV-1 RNA in plasma of 55 patients enrolled in phase I/II nelfinavir dose-ranging studies (6, 12, 29, 38). To determine the potential for cross-resistance to nelfinavir, phenotypic assays were performed with HIV variants isolated both from patients treated with nelfinavir from clinical studies and in in vitro selection experiments. The relevance of specific genotypic changes was confirmed by per-

* Corresponding author. Mailing address: Department of Virology, Agouron Pharmaceuticals, Inc., 4245 Sorrento Valley Blvd., San Diego, CA 92121. Phone: (619) 622-3117. Fax: (619) 622-5999. E-mail: patick@agouron.com.

forming susceptibility assays with HIV-1 NL4-3 constructed to contain specific amino acid substitutions.

MATERIALS AND METHODS

Subjects. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from 55 patients enrolled in two phase I-to-II nelfinavir dose-ranging studies (6, 12, 29). Fifty-one patients received nelfinavir at doses ranging from 1,000 to 3,000 mg/day according to one of six dose regimens (500 mg twice a day [BID], 600 mg BID, 750 mg BID, 500 mg three times a day [TID], 750 mg TID, and 1,000 mg TID); four patients received nelfinavir at doses ranging from 1,500 to 3,000 mg/day according to one of three TID regimens (500, 750, and 1,000 mg TID) in combination with d4T (30 to 40 mg BID). Patients enrolled in these protocols initially received nelfinavir monotherapy for 4 to 8 weeks, after which time other antiretroviral therapies could be added. Plasma HIV-1 RNA was monitored by the branched-DNA (bDNA) Quantiplex HIV RNA assay (Chiron Corp., Emeryville, Calif.).

Compounds. Nelfinavir mesylate was synthesized at Agouron Pharmaceuticals, Inc. Other HIV protease inhibitors including indinavir, ritonavir, saquinavir, and amprenavir (formerly VX-478/141W94) were provided by Merck Research Laboratories, Abbott Laboratories, Roche Research Centre, and Vertex Pharmaceuticals, Inc., respectively.

Determination of HIV protease gene sequences. The nucleotide sequence of the protease gene from HIV-1 isolated from patient plasma or from virus-containing supernatants was determined by Professional Genetics Laboratory AB (Uppsala, Sweden). A guanidinium isothiocyanate extraction procedure was used to prepare virus RNA (vRNA) from plasma pelleted virus. cDNA was then synthesized from extracted vRNA by use of the First Strand cDNA Synthesis kit (Pharmacia Biotech AB, Uppsala, Sweden). cDNAs were used as templates to amplify the protease gene via a two-step PCR method which involved a primary PCR amplification and a second nested-PCR amplification. Purified PCR products were sequenced with the AutoRead Sequencing kit (Pharmacia Biotech AB). Sequencing reactions were run and analyzed on the A.L.F. DNA Sequencer (Pharmacia Biotech AB). Sequence analysis of mutation frequencies at specific base positions was semiquantitative; mutations which represented only 10 to 15% of the entire virus population were recorded and included in subsequent tabulations. Specific amino acid substitutions were identified by comparison of matched plasma vRNA samples obtained from patients prior to (baseline) and after initiation of nelfinavir therapy with the HIV-1 North American clade B protease sequence as a master consensus sequence (32).

Phenotypic analysis of HIV clinical isolates. HIV-1 clinical isolates were obtained following cocultivation of patient PBMCs with phytohemagglutinin (PHA)-stimulated PBMCs from HIV-seronegative donors. Drug susceptibility assays were performed by incubating 1,000 50% tissue culture infectious doses ($TCID_{50}$) of each isolate with 10^6 uninfected PHA-stimulated donor PBMCs for 1 to 2 h in the presence of interleukin-2 (21). Infected cells were then washed and resuspended and plated in duplicate onto a 96-well plate containing either fivefold dilutions of specific compounds (0.008 to 5 μM) or medium only. Levels of HIV p24 core antigen in cell-free supernatants were measured on day 7 with a commercial kit (Abbott Laboratories). Data from duplicate wells were averaged, and percent inhibition was calculated by comparison to that of the drug-free control wells. The 90% effective concentration (EC_{90}) was calculated as the concentration of drug that caused a decrease in the percentage of p24 produced in infected, drug-treated cells to 90% of that produced by infected, drug-free cells. The level of resistance was expressed as the ratio of the EC_{90}s obtained for isolates at the time of virologic relapse compared to baseline. Statistically significant resistance levels were determined by initially calculating an average ratio between each EC_{90} and the minimal EC_{90} for each isolate that had been tested more than once. An overall mean ratio for all isolates and 95% confidence intervals were determined; resistance levels of $\geq$fivefold were determined to be significant.

Analysis of HIV-1 NL4-3 recombinant variants. HIV-1 variant strains, containing defined mutations in the protease gene were constructed as previously described (17). Briefly, a 4,300-bp, *Sph*I-*Eco*RI fragment of the infectious molecular clone HIV-1 NL4-3 (1) was cloned into pALTER (Promega Corp., Madison, Wis.). Oligonucleotides containing the desired mutation(s) were synthesized (Perkin Elmer Corp., New Jersey, N.Y.) and used as primers to perform PCR site-directed mutagenesis (Stratagene Corp., La Jolla, Calif.) according to the manufacturer's instructions. The 4.3-kb fragment was isolated by agarose gel electrophoresis, purified, and reinserted into pNL4-3. After transformation into XL Blue, competent cells and recombinant colonies were confirmed by direct sequencing. Mutant pNL4-3 DNA was purified by QIAgen Corp. (Chatsworth, Calif.) and used to transform 293T cells via Lipofectamine (Gibco BRL, Gaithersburg, Md.) to produce infectious virus. For drug susceptibility assays, 500 $TCID_{50}$ of each virus was used to infect 5×10^5 MT-4 cells. Following a 2-h incubation, infected cells were washed and resuspended at 2×10^5 cells per ml in medium alone or medium containing fivefold dilutions of specific compounds (0.008 to 5 μM). Four days later, culture supernatants were removed and assayed for p24 antigen production. The EC_{90} was calculated as the concentration of drug that decreased the percentage of p24 produced in infected, drug-treated cells to 90% of that produced by infected, drug-free cells. Data (resistance level) were expressed as the ratio between the EC_{90} obtained for variant HIV-1 NL4-3 and the EC_{90} of wild-type HIV-1 NL4-3. Resistance levels of $\geq$threefold were determined to be significant.

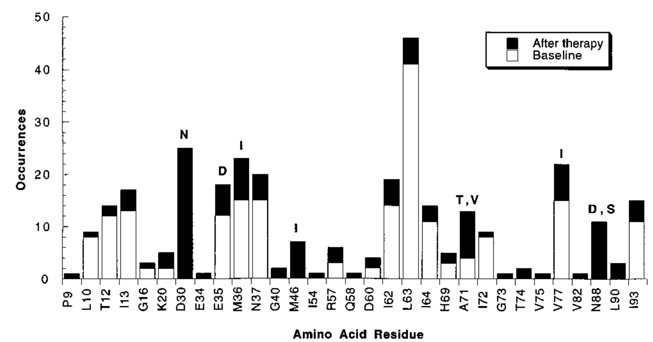

FIG. 1. Genotypic changes in HIV protease from patients treated with nelfinavir. Sequence analysis was performed on HIV protease genes obtained from matched plasma samples from 55 patients at baseline and after nelfinavir therapy. The numbers of occurrences of specific substitutions at individual amino acid residues that were detected in HIV protease at baseline, prior to nelfinavir therapy, and after nelfinavir therapy are indicated.

Statistical analysis. To determine the effect of baseline polymorphisms on the occurrence of the D30N substitution, the proportion of patients that acquired the D30N substitution was calculated for each of the two groups (polymorphisms absent or present). A test of the equality of the two proportions was conducted by Fisher's exact test.

RESULTS

Genotypic changes in plasma vRNA from patients treated with nelfinavir. To identify genotypic changes in HIV protease associated with virologic relapse, we performed nucleotide sequence analysis of plasma HIV-1 isolates from 55 patients enrolled in phase I/II dose-ranging studies of nelfinavir. Plasma samples for HIV-1 RNA sequencing were obtained at baseline and at the time of virologic failure, which was defined as the time of the initial increase in HIV-1 RNA level above the nadir. In some patients, additional isolates from later time points were also sequenced. The median duration of nelfinavir therapy at the time of genotype analysis was 13 weeks (range, 2 to 52 weeks). Amino acid substitutions in HIV protease that were detected in patients treated with nelfinavir occurred at 31 different residues (Fig. 1). Amino acid substitutions in HIV protease that occurred in >10% of patients occurred at residues 30, 35, 36, 46, 71, 77, and 88 (Fig. 1). The predominant amino acid substitution that was observed in isolates from 25 of the 55 patients was D30N. The N-to-D or serine (S) substitutions at residue 88 (N88D/S) were detected in isolates from 11 of the 55 patients. Seven of the isolates from 8 patients whose HIV protease contained the N88D substitution also contained the D30N substitution. A methionine (M)-to-isoleucine (I) substitution at residue 46 (M46I) was detected in 8 of 55 patients following nelfinavir therapy. Five of the eight isolates which contained the M46I substitution also contained the D30N substitution. In one of these patients, however, the M46I substitution reverted back to the baseline sequence in samples derived from latter time points despite continued nelfinavir therapy. A similar pattern of amino acid substitutions was observed in isolates from four patients who received nelfinavir in combination with d4T (data not shown).

Other substrate-inhibitor binding site amino acid substitutions that have been associated with phenotypic resistance to the other clinically relevant protease inhibitors were either not observed, e.g., glycine (G) to V at residue 48 (G48V), I50V, V82 phenylalanine(F)/T, and I84V, or were only rarely observed, e.g., leucine (L) to M at residue 90 (L90M), in 3 of 55

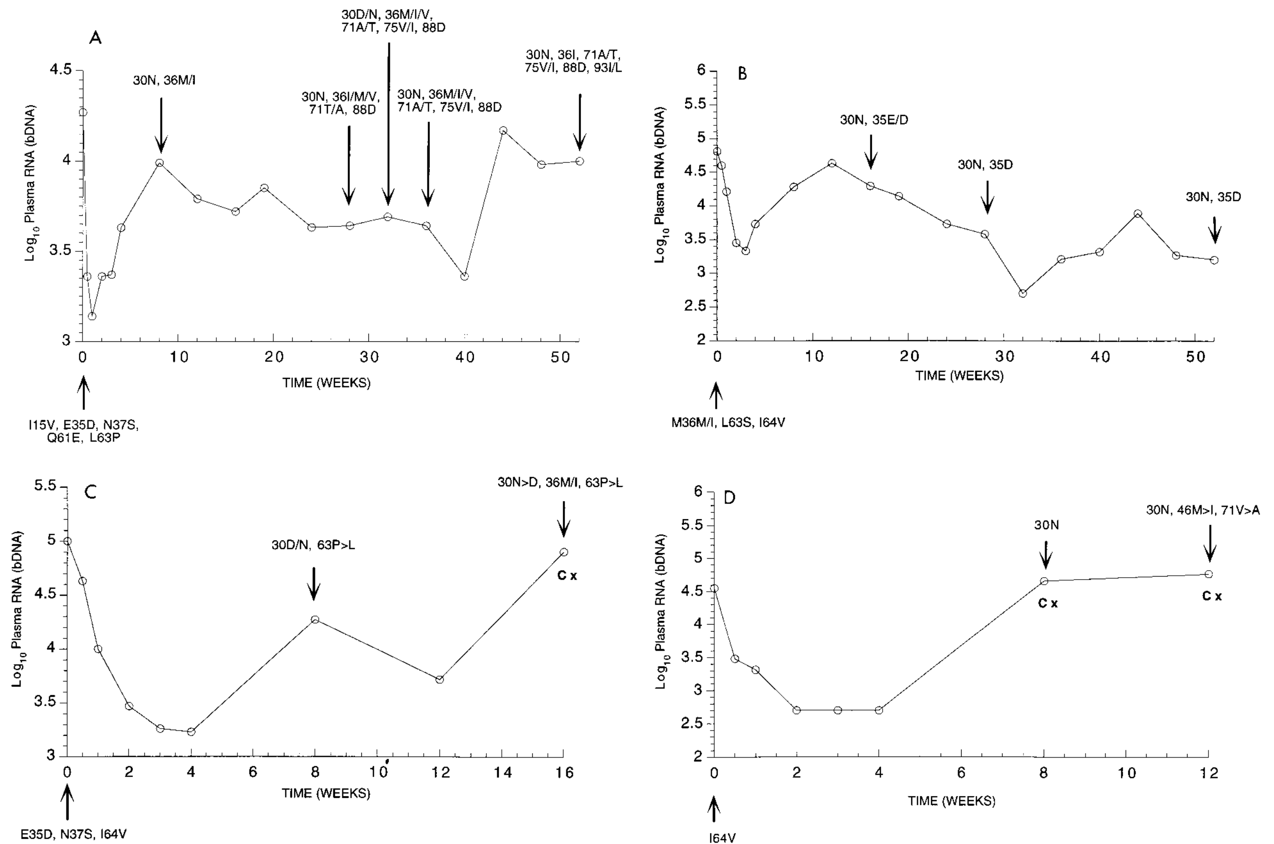

FIG. 2. Correlation of virological response ($\log_{10}$ of plasma HIV-1 RNA; bDNA copies/ml) with genotypic profiles in patients following treatment with nelfinavir monotherapy (750 mg BID) followed by d4T therapy at week 40 (A), nelfinavir monotherapy (500 mg BID) followed by d4T and 3TC therapy at weeks 18 and 38, respectively (B), nelfinavir monotherapy (600 mg BID) (C), and nelfinavir monotherapy (750 mg TID) (D). Amino acid substitutions at baseline and during therapy (in weeks) were identified based on comparison to the consensus sequence as described in Materials and Methods. Phenotype susceptibility assays were performed on patient isolates at specific times, denoted by Cx, as described in Materials and Methods.

patients during the time period studied (7, 20, 31, 33). A V82A substitution was detected in one patient on nelfinavir therapy. This patient however, had a baseline substitution of G48V and was receiving concurrent therapy with saquinavir. Patient isolates that did not contain the D30N substitution (30 of 55 patients) contained from one to four substitutions ($n = 20$) or carried no changes at all ($n = 10$) (see Fig. 3; data not shown). With the exception of the L90M and V82A substitutions detected in isolates from four of these patients, these substitutions occurred at residues that were identified as baseline polymorphisms in other patients (e.g., at residues 10, 13, 20, 35, 62, 63, 64, 77, and 93 [Fig. 1; see Table 3]) and/or were not associated with phenotypic resistance to nelfinavir (see Fig. 3).

Previous work has shown that in vitro, six additional passages of the p22 variant in significantly higher concentrations of nelfinavir resulted in the disappearance of virus containing the D30N substitution and the appearance of virus containing M46I/I84V substitutions (35). To determine if additional new genotypic changes in HIV-1 protease accumulate with prolonged exposure to nelfinavir, sequence analysis was performed on serial plasma vRNA samples collected from all 16 of the 25 patients whose isolates carried the D30N substitution and who remained on nelfinavir therapy for a median of 11 weeks (range, 4 to 44 weeks) beyond the time of initial virological failure (Fig. 2). The D30N substitution was found in association with a mean of two other amino acid substitutions (range, zero to five amino acid substitutions) in plasma HIV RNA samples obtained from patients after a median of an additional 8 weeks of nelfinavir. Although there was a trend toward the accumulation of additional changes, no statistically significant correlation was observed between the duration of nelfinavir therapy and the rate and number of accumulation of additional amino acid substitutions. Patients were just as likely to acquire as many as five additional changes following only 12 weeks of therapy (data not shown) as those who had received 52 weeks of therapy (Fig. 2A). In some cases, the D30N substitution was not accompanied by any additional changes (Fig. 2B) or was accompanied by reversion to the baseline sequence even after prolonged nelfinavir therapy (data not shown). Although the appearance of D30N was most often associated with substitutions of M36I, A71T, and N88D, the D30N substitution was stably maintained, and, significantly, no new substrate-inhibitor binding site amino acid substitutions, including I84V, were observed in any patients during continued nelfinavir therapy (Fig. 2A through D).

Phenotypic analysis of HIV variants selected by nelfinavir in vivo. To characterize the phenotype of HIV-1 isolates from the same nelfinavir-treated cohorts, 20 pairs of isolates were cultured from the PBMCs of 19 patients at baseline and after receiving nelfinavir therapy for periods ranging from 4 to 29 weeks. The critical role of the D30N substitution in nelfinavir resistance was inferred by the observation that all 10 clinical isolates which exhibited a significant reduction in susceptibility (5- to 93-fold increase in EC_{90}) to nelfinavir contained this

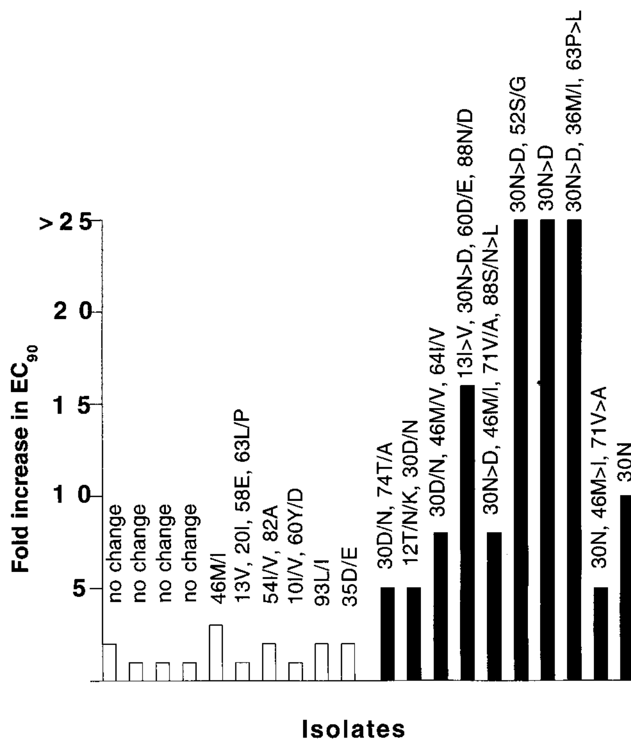

FIG. 3. Phenotypic characterization of HIV variants from patients treated with nelfinavir. HIV isolates were cultured from patient PBMCs at baseline and at various times after initiation of nelfinavir therapy. EC_{90}s were calculated from dose-response curves as described in Materials and Methods. Fold change was calculated by comparing the EC_{90} of nelfinavir for the isolate cultured after therapy to the EC_{90} of nelfinavir for the matched isolate cultured at baseline. Specific amino acid substitutions occurring after nelfinavir therapy are indicated and were identified by comparison of matched plasma HIV-1 RNA samples obtained from patients prior to (baseline) nelfinavir therapy as described in Materials and Methods.

substitution, whereas the isolates that lacked the D30N substitution did not show significant reductions in sensitivity (<fivefold) to nelfinavir (Fig. 3). Overall, the median EC_{90} for nelfinavir against 22 HIV-1 isolates obtained at baseline was 0.056 μM (range, 0.008 to 0.20 μM), whereas the median EC_{90} of 10 nelfinavir-resistant HIV-1 variants isolated after therapy was 0.68 μM (range, 0.04 to 2.5 μM). In addition to D30N, phenotypically resistant isolates also contained from zero to three other amino acid substitutions in protease (Fig. 2C and D and 3; Table 1). However, there did not appear to be any significant correlation between the number or pattern of these substitutions and nelfinavir susceptibility. As such, the concomitant presence of the N88D/S substitutions in two isolates did not increase the level of nelfinavir resistance above that observed for isolates containing the D30N substitution.

Cross-resistance of nelfinavir-resistant HIV variants. To evaluate potential cross-resistance to other protease inhibitors, nelfinavir-resistant isolates were examined for susceptibility to ritonavir, indinavir, saquinavir, and amprenavir (Table 1). Although HIV-1 variants demonstrated a 5- to 93-fold increase in EC_{90} to nelfinavir, significant reductions in susceptibility to other protease inhibitors tested were not observed. To confirm the role of the D30N substitution with reductions in susceptibility to nelfinavir and lack of cross-resistance to other protease inhibitors, similar susceptibility assays were performed with recombinant virus strains constructed to contain specific genotypic changes in HIV protease. HIV-1 NL4-3 containing the D30N substitution alone demonstrated a sixfold increase in EC_{90} to nelfinavir but remained susceptible to indinavir, ritonavir, saquinavir, and amprenavir (Table 1). In contrast, HIV-1 NL4-3 containing the single amino acid substitution of I84V demonstrated cross-resistance to all protease inhibitors tested, including nelfinavir. This latter observation was consistent with results obtained from susceptibility assays performed with the further-passaged nelfinavir-resistant variant p28, which also contained the I84V substitution.

The effects of other amino acid substitutions that appear in association with the D30N substitution were also tested. Recombinant virus strains containing either the M46I, A71V, or N88D substitution alone, however, remained sensitive to nelfinavir and/or other protease inhibitors tested (Table 2) (35). HIV-1 NL4-3 containing D30N/N88D, D30N/A71V, or D30N/M46I/A71V substitutions demonstrated 6- to 31-fold reductions in susceptibility to nelfinavir but remained susceptible to other protease inhibitors (Table 2). Similarly, an HIV variant selected in vitro (p22) and which contained both D30N and A71V substitutions also remained susceptible to indinavir, ritonavir, saquinavir, and amprenavir (Table 2). These results are also consistent with those observed with HIV variants isolated from patients treated with nelfinavir, in which the presence of substitutions at other amino acid residues (e.g., residues 36, 46, 52, 63, 71, and 74) in conjunction with D30N did not significantly alter the susceptible phenotype of these otherwise nelfinavir-resistant virus variants (Table 1).

Baseline polymorphisms do not predict the acquisition of D30N. During the course of this analysis, a significant degree of

TABLE 1. Susceptibility of nelfinavir-resistant HIV variants to other protease inhibitors[a]

Isolate (wk)	Genotypic change(s)[b]	EC_{90} (μM) (fold change)				
		Nelfinavir	Ritonavir	Indinavir	Saquinavir	Amprenavir
219 (13)	D30N>D, G52S/G	2.50 (93)	0.03 (<1)	0.04 (1)	0.03 (4)	0.04 (1)
222 (13)	D30N>D	1.00 (45)	0.01 (<1)	0.03 (<1)	0.03 (4)	0.03 (<1)
204 (12)	D30D/N, T74T/A	1.90 (5)	0.01	0.04	0.01	0.04
211 (16)	D30N>D, M36M/I, L63P>L	0.60 (60)	0.03 (3)	0.01 (1)	0.10 (1)	ND
228 (8)	D30N	0.20 (10)	0.01	0.03	0.01	0.04
228 (12)	D30N, M46M>I, A71V>A	0.90 (5)	0.09 (<1)	0.01 (<1)	0.10 (<1)	ND

[a] HIV was isolated from patient PBMCs at baseline and at various times after initiation of nelfinavir therapy (in weeks). Amino acid substitutions were identified by comparison of matched plasma vRNA samples obtained from patients prior to (baseline) and after initiation of nelfinavir therapy by using the consensus sequence as described in Materials and Methods. EC_{90}s were calculated from dose-response curves as described in Materials and Methods. Fold and change values (in parentheses) were calculated by comparing the EC_{90} of each drug for isolates cultured after therapy with the EC_{90} of each drug for the matched isolate cultured at baseline (data not shown). Values are derived from one experiment or are the means of two or more experiments. ND, not determined.

[b] Sequence analysis of amino acid substitutions was semiquantitative, e.g., protease gene sequences from HIV-1 isolate 219 from a patient treated with nelfinavir consists of 70 to 85% aspartic acid (N) and 15 to 30% asparagine (D) at residue 30 and approximately equal amounts of glycine (G) and serine (S) at residue 52.

TABLE 2. Susceptibility of HIV-1 variants to nelfinavir and other protease inhibitors

HIV-1 NL4-3 variant(s)[b]	EC_{90} (μM) (fold change) with the following compounds[a]:				
	Nelfinavir	Ritonavir	Indinavir	Saquinavir	Amprenavir
HIV-1 NL4-3	0.03	0.05	0.06	0.03	0.08
p22 (D30N/A71V)	0.19 (6)	0.09	0.07	0.01	0.04
p28 (M46I/I84V)	0.83 (28)	0.90 (18)	0.78 (13)	0.16 (5)	ND
D30N	0.18 (6)	0.01	0.01	0.01	0.02
N88D	0.02	0.01	0.01	0.03	0.02
I84V	0.13 (4)	0.80 (16)	0.64 (11)	0.09 (3)	ND
D30N/N88D	0.18 (6)	0.01	0.01	0.01	0.04
D30N/A71V	0.92 (31)	0.01	0.04	0.01	0.02
D30N/M46I/A71V	0.92 (31)	0.04	0.04	0.01	0.04

[a] Values in parentheses were calculated by comparing the EC_{90}s of wild-type HIV-1 NL4-3 and variant HIV-1 NL4-3 strains as determined in susceptibility assays with MT-4 cells as described in Materials and Methods. Fold changes (≥threefold) in EC_{90}s are indicated. Variant HIV-1 NL4-3 strains and wild-type HIV-1 NL4-3 were analyzed in parallel for sensitivity to all inhibitors tested. Values are derived from one experiment or are the means of two or more experiments. ND, not determined.

[b] Virus strains. HIV-1 NL4-3 represent wild-type HIV-1; p22 and p28 variants are HIV-1 NL4-3 strains isolated following 22 and 28 serial passages, respectively (35); D30N, N88D, I84V, D30N/N88D, D30N/A71V, and D30N/M46I/A71V represent recombinant HIV-1 NL4-3 strains containing HIV protease genes which were modified by oligonucleotide-mediated, site-directed mutagenesis to contain the indicated substitutions.

polymorphism was observed in the HIV-1 protease gene sequences obtained prior to the initiation of nelfinavir therapy. Differences from the clade B consensus sequence amino acids were noted at 37 of the 99 residues (data not shown). Substitutions in the HIV protease that occurred in >10% of patients were detected at amino acid residues 10, 12, 13, 15, 35, 36, 37, 41, 62, 63, 64, 72, 77, and 93 (Fig. 2; Table 3). Of note was the polymorphism identified at residue 63, which occurred in 41 of the 55 (75%) baseline isolates. Many of these substitutions were also observed following treatment with nelfinavir (Fig. 1 and 2) or have been reported following treatment with other protease inhibitors (7–9, 20, 27, 31, 42, 43). To determine if any of these baseline polymorphisms had predisposed to the emergence of nelfinavir-resistant variants containing the D30N substitution, isolates were initially classified according to whether they had a given polymorphism at baseline. The proportion of patients whose isolates acquired the D30N substitution was then calculated for each of the two groups. None of these polymorphisms was significantly associated with the subsequent acquisition of the D30N substitution during nelfinavir therapy (Table 3). Although genotypic analyses were performed at various time points after nelfinavir therapy, the lengths of duration of nelfinavir therapy were similar between the two groups, ranging from 28 to 368 days (median of 92 days) and from 28 to 346 days (median of 88 days) in patients who did and did not acquire the D30N substitution, respectively. These results suggest that these individual baseline polymorphisms were not associated with an increased risk of developing genotypic resistance to nelfinavir during the time period studied.

DISCUSSION

Although data from human clinical trials have described the effectiveness of HIV protease inhibitors in significantly reducing viral load and increasing CD4 cell counts, recent reports have described the emergence, in patients, of virus variants with reduced susceptibility to the protease inhibitor administered during therapy (7–9, 18, 20, 27, 31, 42, 43, 48). In many cases, these resistant virus variants have been shown to be cross-resistant to other structurally unrelated protease inhibitors (7–9, 29, 41). Therefore, an understanding of the genotypic and phenotypic changes associated with protease inhibitor resistance is essential.

Studies of the emergence of drug resistance in vitro with serial passage of HIV-1 in the presence of increasing concentrations of a given protease inhibitor (17, 19, 26, 33, 35, 45, 47) have revealed that phenotypic resistance can be attributed to a few specific amino acid substitutions that occur within highly conserved regions of the enzyme. These substitutions interact directly (e.g., residues 48, 50, 82, and 84) or indirectly (e.g., residue 90) with the substrate and/or inhibitor during binding. Results from in vitro serial passage studies have been somewhat predictive of genotypic changes observed in vivo, leading to resistance to individual protease inhibitors (20, 27, 31, 42, 43). Accordingly, sequence analyses of protease genes of HIV-1 strains isolated from patients enrolled in clinical trials of nelfinavir were also consistent with in vitro results (35). Analogous to the p22 variant, the predominant genotypic change observed in HIV-1 isolates from 55 patients treated with nelfinavir for periods of as many as 52 weeks was a D30N substitution. Significantly, some other amino acid substitutions which have been associated with resistance to other clinically relevant protease inhibitors (e.g., at residues 48, 50, and 82) (7, 20, 31, 33) were not detected in vitro and were also not detected in vivo. A substitution at residue 90 that was not detected in vitro was detected in 3 of the 55 patients studied. In contrast, the I84V substitution which was detected in vitro in a later-passaged nelfinavir-resistant variant (p28 [35]) was not detected in isolates obtained from nelfinavir-treated patients included in the present study. The long-term stability of the D30N substitution and the absence of the I84V substitution were evidenced by nucleotide sequence analysis of serial plasma HIV-1 isolates collected from patients whose initial isolates contained D30N and who continued on nelfinavir therapy for periods of as many as 44 weeks. Although we cannot exclude the possibility that viruses containing other substrate-inhibitor binding site mutations including the I84V substitution were present at frequen-

TABLE 3. Effect of baseline polymorphisms on occurrence of D30N substitution[a]

Amino acid position	No. of patients with D30N substitution (%)		P value[b]
	Polymorphism absent	Polymorphism present	
10	22/47 (46.8)	3/8 (37.5)	0.715
12	20/43 (46.5)	5/12 (41.7)	1.000
13	19/42 (45.2)	6/13 (46.2)	1.000
15	19/42 (45.2)	6/13 (46.2)	1.000
35	17/43 (39.5)	8/12 (66.7)	0.114
36	19/40 (47.5)	6/15 (40.0)	0.764
37	19/40 (47.5)	6/15 (40.0)	0.764
41	23/45 (51.1)	2/10 (20.0)	0.092
62	20/41 (48.8)	5/14 (35.7)	0.537
63	7/14 (50.0)	18/41 (43.9)	0.762
64	17/43 (39.5)	8/12 (66.7)	0.114
72	22/47 (46.8)	3/8 (37.5)	0.715
77	19/40 (47.5)	6/15 (40.0)	0.763
93	20/44 (45.5)	5/11 (45.5)	1.000

[a] Sequence analysis was performed on HIV protease genes obtained from plasma samples from 55 patients at baseline prior to nelfinavir therapy. Polymorphisms which occurred in >10% patients (≥6 changes/55 baseline sequences) were identified based on comparison with the consensus sequence as described in Materials and Methods. The proportion of patients that acquired the D30N substitution following nelfinavir therapy was then calculated for each of the two groups (polymorphism absent or present).

[b] A test of equality of the two proportions was conducted by Fisher's exact test.

cies too low to detect by the techniques utilized, these results suggest that viruses containing the D30N substitution must represent the most stable and competitively fit viral species in the presence of nelfinavir in vivo.

These observations have also been confirmed by a preliminary analysis of the protease genes from an additional 113 patients following 12 to 16 weeks of nelfinavir therapy (37). Although the median duration of nelfinavir therapy (13 weeks) in the present study may be considered short, preliminary analysis of HIV-1 isolates from an additional 18 patients treated with nelfinavir for a median of 55 weeks further support these findings (46). In the latter study, D30N and L90M substitutions were detected in 13 of 18 and 5 of 18 patients, respectively; substitutions at residue 48, 50, 82, or 84 were not detected.

In HIV-1 isolates from patients treated with nelfinavir, the D30N substitution was often accompanied by the concurrent or subsequent appearance of additional substitutions (e.g., at residues 35, 36, 46, 63, 71, 77, and 88) that have been described for HIV-1 variants isolated from patients treated with other protease inhibitors. In general, these substitutions occur at residues located in regions of the protease that are not directly involved with inhibitor and/or substrate binding. In some cases, these additional (secondary) substitutions have been shown to improve the growth of resistant isolates that has been negatively affected by primary resistance substitutions occurring in the substrate/inhibitor binding site of the enzyme (17, 40). Changes at these residues alone are not associated with reductions in susceptibility to protease inhibitors. However, accumulation of these secondary changes together with substitutions at critical substrate-inhibitor binding site residues has, in some cases, resulted in levels of resistance higher than those measured with substrate-inhibitor binding site changes alone (7, 8, 31, 33, 34).

Consistent with these findings are results from this and previous studies showing that viruses containing single changes at residues 46, 63, 71, and 88 are sensitive to nelfinavir as well as other protease inhibitors tested (26, 34, 35, 45). Furthermore, greater reductions in susceptibility to nelfinavir were detected in recombinant virus strains which contained the D30N substitution in combination with the A71V or M46I/A71V substitutions compared to virus strains that contained D30N alone. Despite the increased level of resistance to nelfinavir conferred by these additional substitutions, no reduction in susceptibility to other protease inhibitors was observed. This result is in contrast to that observed for viruses which have additional changes expressed in the genetic context of substitutions at other substrate-inhibitor binding site residues, e.g., 82 and 84. In these cases, increased reductions in susceptibility to individual protease inhibitors are accompanied by concomitant increases in levels of cross-resistance (7, 8, 31). In addition to changes that occur in the enzyme, other compensatory changes in gag or gag-pol polyprotein cleavage sites have been described (11), but the presence of such changes was not evaluated in the present study.

Consistent with other studies, a significant degree of polymorphism was detected in HIV-1 protease gene sequences from patients prior to nelfinavir therapy (23–25). The presence of pretreatment polymorphisms or compensatory substitutions in HIV protease prior to therapy raised the possibility that these viruses may be predisposed to more rapidly acquire drug resistance. However, no correlation was found between the presence of individual baseline polymorphisms and the acquisition of the D30N substitution. Although the possibility cannot be excluded that combinations of certain polymorphisms may lead to more rapid resistance, a larger data set would be required to perform these analyses due to the extensive heterogeneity in patterns of different polymorphisms that have been observed. Given the high mutation rate of HIV-1, incomplete suppression of virus replication alone should be considered the major factor that ultimately allows for the selection of drug-resistant HIV variants.

A structural basis for protease inhibitor resistance can be ascertained by analysis of the experimentally determined or modeled three-dimensional structures of the enzyme with bound inhibitors (2, 4, 11, 22, 50). In general, resistance can be attributed to a perturbation of specific hydrophobic or electrostatic interactions that occur between bound inhibitors and the enzyme. Crystallographic analyses which depict the binding of nelfinavir in the substrate-inhibitor binding site of HIV protease provide a structural basis for the relevance of the D30N substitution (2, 21). In this manner, the carboxylate oxygen of aspartic acid (D) located at residue 30 in the S2 subsite of the enzyme forms a hydrogen bond with the P2 phenylhydroxyl group of nelfinavir. However, asparagine (N) forms a much weaker hydrogen bond to the P2 substituent due to asparagine's lack of associated electrostatic charge and thus may destabilize nelfinavir binding. This interaction is unique among the approved protease inhibitors and may form the basis for the distinct resistance profile for nelfinavir. In a similar manner, both indinavir and ritonavir have been optimized to form strong hydrophobic interactions with the valine at residue 82 in the S3 subsite of the enzyme and are, therefore, most affected by a substitution at this residue. Accordingly, the most predominant change detected in resistant isolates from patients treated with these inhibitors occurs at residue 82 (7, 8, 31, 43). Although amino acid substitutions that occur at residues which interact with the substrate or inhibitor during binding can be easily rationalized, substitutions that occur at residues located outside the substrate-inhibitor binding site are more difficult to understand. It is postulated that these changes affect the stability or activity of the enzyme via long-range structural perturbations which restore viability to the protease and the virus (2, 4, 11, 44).

In summary, this study identified a unique amino acid substitution in the protease gene of HIV-1 isolates from patients failing nelfinavir-containing regimens. Isolates derived from these patients appear to retain in vitro susceptibility to other protease inhibitors. This finding has been confirmed in an analysis of seven HIV clinical isolates by recombinant virus phenotypic techniques (16). Overall, these findings suggest that patients failing a regimen containing nelfinavir might derive benefit from a subsequent regimen containing other protease inhibitors; however, confirmation by appropriate clinical studies is necessary. Although initial studies (14, 46) have shown that patients who failed on a nelfinavir-containing regimen and whose isolates contained predominantly a substitution at residue 30 have achieved an initial high level of suppression when switched to a ritonavir-saquinavir-containing regimen, further substantiation requires larger clinical trials.

ACKNOWLEDGMENTS

We thank Donna Setterberg and Rose Najarian for help in preparation of the manuscript and Richard Ogden and Karen Potts for critical reading of the manuscript.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Appelt, K.** 1997. Inhibitors of HIV-1 protease, p. 1–39. *In* P. Veerapandian (ed.), Structure-based drug design. Marcel Dekker, Inc., New York, N.Y.
3. **Carpenter, C. C. J., M. A. Fischl, S. M. Hammer, M. S. Hirsch, D. M.

Jacobsen, D. A. Katzenstein, J. S. G. Montaner, D. D. Richman, M. S. Saag, R. T. Schooley, M. A. Thompson, S. Vella, P. G. Yeni, and P. A. Volberding. 1997. Antiretroviral therapy for HIV infection in 1997. JAMA **277:**1962–1969.

4. **Chen, Z., H. B. Schock, D. Hall, E. Chen, and L. C. Kuo.** 1995. Three-dimensional structure of a mutant HIV-1 protease displaying cross-resistance to all protease inhibitors in clinical trials. J. Biol. Chem. **270:**21433–21436.
5. **Collier, A. C., R. W. Coombs, D. A. Schoenfeld, R. L. Bassett, J. Timpone, A. Baruch, M. Jones, K. Facey, C. Whitacre, V. J. McAuliffe, H. M. Friedman, T. C. Merigan, R. C. Reichman, C. Hooper, and L. Corey.** 1996. Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. N. Engl. J. Med. **334:**1011–1017.
6. **Conant, M., M. Markowitz, A. Hurley, D. Ho, J. Peterkin, and S. Chapman.** 1996. A randomized phase II dose range-finding study of the HIV protease inhibitor VIRACEPT® as monotherapy in HIV positive patients, abstr. Tu.B.2129. *In* Abstracts of the XI International Conference on AIDS, Vancouver, Canada.
7. **Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, and M. Shivaprakash.** 1995. *In vivo* emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature **374:**569–571.
8. **Condra, J. H., D. J. Holder, W. A. Schleif, O. M. Blahy, R. M. Danovich, L. J. Gabryelski, D. J. Graham, D. Laird, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, M. Shivaprakash, T. Yang, J. A. Chodakewitz, P. J. Deutsch, Randi Y. Leavitt, F. E. Massari, J. W. Mellors, K. E. Squires, R. T. Steigbigel, H. Teppler, and E. A. Emini.** 1996. Genetic correlates of *in vivo* viral resistance to indinavir, a human immunodeficiency virus type 1 protease inhibitor. J. Virol. **70:**8270–8276.
9. **Craig, J. C., I. B. Duncan, S. Gilbert, H. Jacobsen, R. Jupp, A. Moffatt, E. Race, N. A. Roberts, J. S. Mills, J. Mous, J. Sheldon, P. W. Tomlinson, and L. N. Whittaker.** 1996. Treatment with saquinavir (Invirase™) should leave the majority of patients the option to use other HIV proteinase inhibitors, p. 32. *In* Abstracts of the Fifth International Workshop on HIV Drug Resistance, Whistler, Canada.
10. **Danner, S. A., A. Carr, J. M. Leonard, L. M. Lehman, F. Gudiol, J. Gonzales, A. Raventos, R. Rubio, E. Bouza, V. Pintado, A. G. Aguado, J. Garcia De Lomas, R. Delgado, J. C. C. Borleffs, A. Hsu, J. M. Valdes, C. A. B. Boucher, and D. A. Cooper.** 1995. A short-term study of the safety, pharmacokinetics, and efficacy of ritonavir, an inhibitor of HIV-1 protease. N. Engl. J. Med. **333:**1528–1533.
11. **Erickson, J. W., and S. K. Burt.** 1996. Structural mechanisms of HIV drug resistance. Annu. Rev. Pharmacol. Toxicol. **36:**545–571.
12. **Gathe, J., Jr., B. Burkhardt, P. Hawley, M. Conant, J. Peterkin, and S. Chapman.** 1996. A randomized phase II study of VIRACEPT®, a novel HIV protease inhibitor, used in combination with stavudine vs. stavudine alone, abstr. Mo.B.413. *In* Abstracts of the XI International Conference on AIDS, Vancouver, Canada.
13. **Hammer, S. M., K. E. Squires, M. D. Hughes, J. M. Grimes, L. M. Demeter, J. S. Currier, J. J. Eron, J. E. Feinberg, H. H. Balfour, L. R. Deyton, J. A. Chodakewitz, and M. A. Fischl.** 1997. A controlled trial of two nucleoside analogues plus indinavir in persona with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. N. Engl. J. Med. **337:**725–733.
14. **Henry, K., E. Kane, H. Melroe, J. Simpson, A. Patick, and D. Winslow.** 1997. Experience with a ritonavir/saquinavir based regimen for the treatment of HIV-infection in subjects developing increased viral loads while receiving nelfinavir, abstr. I-204. *In* Program and Abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada. American Society for Microbiology, Washington, D.C.
15. **Henry, K., A. Lamarca, R. Myers, and S. Chapman for the Viracept Collaborative Study Group and Agouron Pharmaceuticals.** 1997. The safety of Viracept® (nelfinavir mesylate, NVR) in pivotal phase II/III double-blind randomized controlled trials as monotherapy and in combination with either d4T or AZT/3TC, abstr. 240. *In* Abstracts of the 4th Conference on Retroviruses and Opportunistic Infections, Washington, D.C.
16. **Hertogs, K., A. Patick, P. Schel, A. Van Cauwenberge, M. Markowitz, D. Kuritzkes, B. Anderson, and R. Pauwels.** 1997. Phenotypic resistance testing (PR-RT-Antivirogram™) of clinical HIV-1 isolates confirms the unique and different resistance pathway of nelfinavir, latebreaker abstr. 906. *In* Abstracts of the 6th European Conference on Clinical Aspects and Treatment of HIV Infection, Hamburg, Germany.
17. **Ho, D. D., T. Toyoshima, H. Mo, D. J. Kempf, D. Norbeck, C.-M. Chen, N. E. Wideburg, S. K. Burt, J. W. Erickson, and M. K. Singh.** 1994. Characterization of human immunodeficiency virus type 1 variants with increased resistance to a C2-symmetric protease inhibitor. J. Virol. **68:**2016–2020.
18. **Ives, K. J., H. Jacobsen, S. A. Galpin, M. M. Garaev, L. Dorrell, J. Mous, K. Bragman, and J. N. Weber.** 1997. Emergence of resistant variants of HIV *in vivo* during monotherapy with the proteinase inhibitor saquinavir. J. Antimicrob. Chemother. **39:**771–779.
19. **Jacobsen, H., K. Yasargil, D. L. Winslow, J. C. Craig, A. Krohn, I. B. Duncan, and J. Mous.** 1995. Characterization of human immunodeficiency virus type 1 mutants with decreased sensitivity to proteinase inhibitor RO 31-8959. Virology **206:**527–34.
20. **Jacobsen, H., M. Hänggi, M. Ott, I. B. Duncan, S. Owen, M. Andreoni, S. Vella, and J. Mous.** 1996. *In vivo* resistance to a human immunodeficiency virus type 1 proteinase inhibitor: mutations, kinetics and frequencies. J. Infect. Dis. **173:**1379–1387.
21. **Japour, A. J., D. L. Mayers, V. A. Johnson, D. R. Kuritzkes, L. A. Beckett, J.-M. Arduino, J. Lane, R. J. Black, P. S. Reichelderfer, R. T. D'Aquila, C. S. Crumpacker, The RV-43 Study Group, and The Aids Clinical Trials Group, Virology Committee Resistance Working Group.** 1993. Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **37:**1095–1101.
22. **Kaldor, S. W., V. J. Kalish, J. F. Davies II, B. V. Shetty, J. E. Fritz, K. Appelt, J. A. Burgess, K. M. Campanale, N. Y. Chirgadze, D. K. Clawson, B. A. Dressman, S. D. Hatch, D. A. Khalil, M. B. Kosa, P. P. Lubbehusen, M. A. Myesing, A. K. Patick, S. H. Reich, K. S. Su, and J. H. Tatlock.** 1997. Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. J. Med. Chem. **40:**3979–3985.
23. **Kozal, M. J., N. Shah, N. Shen, R. Yang, R. Fucini, T. C. Merigan, D. D. Richman, D. Morris, E. Hubbell, M. Chee, and T. R. Gingeras.** 1996. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. Nat. Med. **2:**753–759.
24. **Kozal, M., N. Leahy, J. Ross, N. Swack, and J. Stapleton.** 1997. Prevalence of protease inhibitor (PRI) and reverse transcriptase inhibitor (RTI) drug-resistance mutations in a rural Iowa HIV+ population: implication for treatment, abstr. 9. *In* Abstracts of the 4th Conference on Retroviruses and Opportunistic Infections, Washington, D.C.
25. **Lech, W. J., G. Wang, Y. L. Yang, Y. Chee, K. Dorman, D. McCrae, L. C. Lazzeroni, J. W. Erickson, J. S. Sinsheimer, and A. H. Kaplan.** 1996. *In vivo* sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. J. Virol. **70:**2038–2043.
26. **Markowitz, M., H. Mo, D. J. Kempf, D. W. Norbeck, T. Narayana Bhat, J. W. Erickson, and D. D. Ho.** 1995. Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor. J. Virol. **69:**701–706.
27. **Markowitz, M., M. Saag, W. G. Powderly, A. M. Hurley, A. Hsu, J. M. Valdes, D. Henry, F. Sattler, A. La Marca, J. M. Leonard, and D. D. Ho.** 1995. A preliminary study of ritonavir, an inhibitor of HIV-1 protease, to treat HIV-1 infection. N. Engl. J. Med. **333:**1534–1539.
28. **Markowitz, M., D. Winslow, Y. Cao, A. Hurley, R. O'Donavan, and B. Anderson.** 1997. Triple therapy with nelfinavir in combination with AZT and 3TC in 12 antiretroviral-naïve subjects chronically infected with HIV-1. *In* Abstracts of the 10th International Conference on Antiviral Research, Atlanta, Ga.
29. **Markowitz, M., M. Conant, A. Hurley, R. Schluger, M. Duran, J. Peterkin, S. Chapman, A. Patick, A. Henricks, G. J. Yuen, W. Hoskins, N. Clendennin, and D. D. Ho.** A preliminary evaluation of nelfinavir mesylate, an inhibitor of HIV-1 protease, to treat HIV infection. J. Infect. Dis., in press.
30. **McDonald, C. K., and D. R. Kuritzkes.** 1997. Human immunodeficiency virus type 1 protease inhibitors. Arch. Intern. Med. **157:**951–959.
31. **Molla, A., M. Korneyeva, Q. Gao, S. Vasavanonda, P. J. Schipper, H.-M. Mo, M. Markowitz, T. Chernyavskiy, P. Niu, N. Lyons, A. Hsu, G. R. Granneman, D. D. Ho, C. A. B. Boucher, J. M. Leonard, D. W. Norbeck, and D. J. Kempf.** 1996. Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat. Med. **2:**760–766.
32. **Myers, G., S. Wain-Hobson, L. E. Henderson, B. Korber, K.-T. Jeang, and G. N. Pavlakis.** 1993. Human retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, N.Mex.
33. **Partaledis, J. A., K. Yamaguchi, M. Tisdale, E. E. Blair, C. Falcione, B. Maschera, R. E. Myers, S. Pazhanisamy, O. Futer, A. B. Cullinan, C. M. Stuver, R. A. Byrn, and D. J. Livingston.** 1995. In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity of hydroxyethylamino sulfonamide inhibitors of HIV-1 aspartyl protease. J. Virol. **69:**5228–5235.
34. **Patick, A. K., R. Rose, J. Greytok, C. M. Bechtold, M. A. Hermsmeier, P. T. Chen, J. C. Barrish, R. Zahler, R. J. Colonno, and P.-F. Lin.** 1995. Characterization of a human immunodeficiency virus type 1 variant with reduced sensitivity to an aminodiol protease inhibitor. J. Virol. **69:**2148–2152.
35. **Patick, A. K., H. Mo, M. Markowitz, K. Appelt, B. Wu, L. Musick, V. Kalish, S. Kaldor, S. Reich, D. Ho, and S. Webber.** 1996. Antiviral and resistance studies of AG1343, an orally bioavailable inhibitor of human immunodeficiency virus protease. Antimicrob. Agents Chemother. **40:**292–297.
36. **Patick, A. K., T. J. Boritzki, and L. A. Bloom.** 1997. Activities of the human immunodeficiency virus type 1 (HIV-1) protease inhibitor nelfinavir mesylate in combination with reverse transcriptase and protease inhibitors against acute HIV-1 infection *in vitro*. Antimicrob. Agents Chemother. **41:**2159–2164.
37. **Patick, A. K., M. Duran, Y. Cao, T. Ho, P. Zhou, M. R. Keller, S. Chapman, R. Anderson, D. Kuritzkes, D. Shugarts, D. Ho, and M. Markowitz.** 1997. Genotypic analysis of HIV-1 variants isolated from patients treated with the

protease inhibitor nelfinavir, alone or in combination with d4T, AZT and 3TC, abstr. 10. *In* Abstracts of the 4th Conference on Retroviruses and Opportunistic Infections, Washington, D.C.

38. **Patick, A. K., D. Kuritzkes, V. A. Johnson, D. Shugarts, M. Bakhtiari, K. E. Potts, A. Farnsworth, R. Anderson, J. L. Koel, J. D. Hazelwood, C. D. Nail, M. Duran, M. Markowitz, D. Ho, and D. Richman.** 1997. Genotypic and phenotypic analyses of HIV-1 variants isolated from patients treated with nelfinavir and other HIV-1 protease inhibitors, abstr. 18. *In* Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.
39. **Powderly, W., M. Sension, M. Conant, A. Stein, and N. Clendeninn.** 1997. The efficacy of VIRACEPT® (nelfinavir mesylate, NFV) in pivotal phase II/III double-blind randomized controlled trials as monotherapy and in combination with d4T or AZT/3TC, abstr. 370. *In* Abstracts of the 4th Conference on Retroviruses and Opportunistic Infections, Washington, D.C.
40. **Rose, R. E., Y.-F. Gong, J. A. Greytok, C. M. Bechtold, B. J. Terry, B. S. Robinson, M. Alam, R. J. Colonno, and P.-F. Lin.** 1995. Human immunodeficiency virus type 1 viral background plays a major role in development of resistance to protease inhibitors. Proc. Natl. Acad. Sci. USA **93:**1648–1653.
41. **Saag, M., M. Gersten, Y. Chang, S. L. Greenberg, G. Yu, and N. J. Clendeninn.** 1997. Long term virological and immunological effect of the HIV protease inhibitor, Viracept (nelfinavir mesylate) in combination with zidovudine and lamivudine, abstr. *In* Abstracts of the Infectious Disease Society of America, San Francisco, Calif.
42. **Schapiro, J. M., M. A. Winters, F. Stewart, B. Efron, J. Norris, M. J. Kozal, and T. C. Merigan.** 1996. The effect of high-dose saquinavir on viral load and $CD4^+$ T-cell counts in HIV-counts in HIV-infected patients. Ann. Intern. Med. **124:**1039–1050.
43. **Schmit, J.-C., L. Ruiz, B. Clotet, A. Raventos, J. Tor, J. Leonard, J. Desmyter, E. DeClercq, and A.-M. Vandamme.** 1996. Resistance-related mutations in the HIV-1 protease gene of patients treated for one year with the protease inhibitor ritonavir (ABT-538). AIDS **10:**995–999.
44. **Schock, H. B., V. M. Garsky, and L. C. Kuo.** 1996. Mutational anatomy of an HIV-1 protease variant conferring cross-resistance to protease inhibitors in clinical trials. J. Biol. Chem. **271:**31957–31963.
45. **Smidt, M. L., K. E. Potts, S. P. Tucker, L. Blystone, T. R. Steibel, W. C. Stallings, J. J. McDonald, D. Pillay, D. D. Richman, and M. L. Bryant.** 1996. A mutation in human immunodeficiency virus type 1 protease at position 88, located outside the active site, confers resistance to the hydroxyethylurea inhibitor sc-55389a. Antimicrob. Agents Chemother. **41:**515–522.
46. **Tebas, P., E. Kane, M. Klebert, J. Simpson, W. G. Powderly, and K. Henry.** 1998. Virologic responses to a ritonavir/saquinavir containing regimen in patients who have previously failed nelfinavir, abstr. 510, p. 175. *In* Abstracts of the 5th Conference on Retroviruses and Opportunistic Infections, Chicago, Ill.
47. **Tisdale, M., R. E. Myers, B. Maschera, N. R. Parry, N. M. Oliver, and E. D. Blair.** 1995. Cross-resistance analysis of human immunodeficiency virus type 1 variants individually selected for resistance to five different protease inhibitors. Antimicrob. Agents Chemother. **39:**1704–1710.
48. **Vella, S., C. Galluzzo, G. Giannini, M. F. Pirilo, I. Duncan, H. Jacobsen, M. Andreoni, L. Sarmati, and L. Ercoli.** 1996. Saquinavir/zidovudine combination in patients with advanced HIV infection and no prior antiretroviral therapy: $CD4^+$ lymphocyte/plasma RNA changes, and emergence of HIV strains with reduced phenotypic sensitivity. Antiviral Res. **29:**91–93.
49. **Williams, I. G.** 1996. Use of protease inhibitors. J. HIV Combin. Ther. **1:**4–7.
50. **Wlodawer, A., and J. W. Erickson.** 1993. Structure-based inhibitors of HIV-1 protease. Annu. Rev. Biochem. **62:**543–585.