Baseline Human Immunodeficiency Virus Type 1 Phenotype, Genotype, and RNA Response after Switching from Long-Term Hard-Capsule Saquinavir to Indinavir or Soft-Gel–Capsule Saquinavir in AIDS Clinical Trials Group Protocol 333

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AIDS Clinical Trials Group protocol 333 was an open-label trial of a switch from saquinavir (SQV) hard capsules (SQVhc) to indinavir (IDV) or saquinavir soft-gel capsules (SQVsgc) after >48 weeks of prior treatment with SQVhc. Eighty-nine subjects received IDV or SQVsgc or continued to receive SQVhc and continued unchanged treatment with non–protease-inhibitor antivirals for 8 weeks. Subjects receiving SQVhc then switched treatment to IDV. Baseline drug susceptibility and protease gene sequencing were done; 12 codons related to IDV and SQV resistance were analyzed. After 112 weeks (median) of SQVhc, the fall in human immunodeficiency virus (HIV) type 1 RNA level from baseline was significantly greater with IDV and was inversely correlated with the number of protease substitutions. The number of substitutions also correlated with baseline CD4 cell count, HIV-1 RNA level, SQV experience, and drug susceptibility. Substitution at codon 10, which occurred only in isolates with ⩾2 substitutions, was associated with blunted RNA response. IDV IC_{50} correlated with HIV-1 RNA response after the switch to IDV but added little predictive power once the genotype was considered.

Saquinavir (SQV) was the first of a new class of antiretrovirals, the human immunodeficiency virus (HIV) protease inhibitors. This agent has been shown to be well tolerated and to have in vivo anti-HIV activity [1]. The original formulation of SQV, a hard capsule (INVIRASE; Hoffmann–La Roche, Nutley, NJ), had low bioavailability [2] but still conferred antiretroviral activity in AIDS Clinical Trials Group (ACTG) protocol 229, a clinical trial of triple-combination therapy [3]. In 1996, a new formulation of a soft-gel capsule (FORTOVASE; Hoffmann–La Roche), designed to have improved oral bioavailability, was evaluated. In 1996, another antiretroviral protease inhibitor, indinavir (IDV) sulfate (CRIXIVAN; Merck, West Point, PA), was approved by the US Food and Drug Administration. IDV therapy had been shown to result in substantial increases in CD4 cell counts and suppression of viral replication [4–6] in HIV-infected persons. Combination therapy of IDV or SQV plus nucleoside analogue reverse-transcriptase (RT) inhibitors further enhanced virus suppression.

As with RT inhibitors, in vitro and in vivo susceptibility may decrease with continued use of the HIV protease inhibitors in regimens that do not fully suppress HIV replication. HIV-1 isolates with reduced susceptibility to SQV have been selected in vitro. These isolates have shown primarily mutations leading to amino acid substitutions in the protease gene at codon 48

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Subjects signed an informed consent approved by the institutional review board of each participating unit before enrollment, in keeping with human experimentation guidelines of the US Department of Health and Human Services.

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(glycine to valine) and codon 90 (leucine to methionine) [3], although mutations at additional sites, including amino acid substitutions at codons 10, 46, 82, and 84, have been selected in vivo by SQV [7, 8]. Increasing drug resistance has been seen with the accumulation of multiple mutations in the HIV-1 protease gene. Mutations at multiple loci may contribute to IDV resistance (10, 46, 63, 82, 84, 90, and others) [9, 10]. Decreased in vitro susceptibility to SQV of isolates from persons receiving IDV has been reported elsewhere [9, 10]. Similarly, viruses exhibiting reduced susceptibility to IDV have been isolated from SQV-treated patients [11]. Although such resistance is demonstrable in vitro, there had been no clinical trials examining antiretroviral activity with the sequential use of SQV followed by IDV or whether SQV efficacy could be improved with softgel–capsule SQV (SQVsgc), the newer, more bioavailable formulation of SQV. Therefore, we undertook a phase II, randomized, open-label clinical trial (adult ACTG protocol 333) to address the short-term virologic and immunologic responses of hard-capsule SQV (SQVhc)–experienced subjects to either IDV or SQVsgc.

The primary objective of ACTG 333 was to determine if, after prolonged use (>48 weeks) of SQVhc, recipients experienced a decrease in plasma HIV-1 RNA level after substitution of SQVhc with IDV or SQVsgc. This 1996 trial was done early in our understanding of development of resistance to protease inhibitors. Because the subjects changed only their protease inhibitor, the study design allowed an estimate of the incremental antiviral activity of IDV or SQVsgc when substituted for SQVhc. The antiviral activity of the single new agent was used to assess the predictive power of the baseline protease genotype and baseline in vitro drug susceptibility (phenotype) for the in vivo antiviral response.

Methods

Study design. ACTG protocol 333 was a multicenter, randomized, phase II, open-label trial. Patients were randomized to 1 of 3 arms: 8 weeks of SQVhc, followed by IDV; 8 weeks of SQVsgc, followed by crossover to IDV, if there was no HIV-1 RNA response; or 8 weeks of IDV, followed by crossover to SQVsgc, if there was no HIV-1 RNA response.

Drug doses were SQVhc, 600 mg every 8 h; SQVsgc, 1200 mg every 8 h; and IDV, 800 mg every 8 h. In all study arms, subjects continued their concurrent non–protease-inhibitor antiretrovirals. HIV-1 RNA was measured at weeks 2, 4, 6, 8, 16, and 24. Virus isolates were obtained for drug susceptibility testing and genotyping studies at baseline, week 8, and week 24. Subjects randomized to SQVhc had additional visits at weeks 10, 12, 14, and 16 after a switch to IDV at week 8. For this study, a virologic response was defined as a decline in the mean HIV-1 RNA level ($\log_{10}$ copies/mL) from weeks 4, 6, and 8 to below the baseline value.

Study population. Subjects were ⩾18 years old and had HIV-1 infection documented by a licensed EIA with a confirmatory immunoblot assay. Subjects had taken SQVhc, ⩾1800 mg/day, for ⩾48 weeks (cumulatively) and were naive to all other protease inhibitors and nonnucleoside RT inhibitors. They had been receiving a stable antiretroviral regimen, including SQVhc, for 8 weeks before entry and could have any HIV-1 RNA level. Other eligibility criteria were the following levels: hemoglobin, ⩾9.1 g/dL for men and ⩾8.9 g/dL for women; neutrophils, ⩾750/mm^3; platelets, ⩾50,000/mm^3; alanine aminotransferase and aspartate aminotransferase, ⩽5.0 times the upper limit of normal (ULN); serum creatinine, ⩽1.5 times the ULN; serum bilirubin, ⩽1.5 mg/dL. Women of childbearing age had to have a negative result on serum β–human chorionic gonadotropin testing within 14 days before study entry.

Exclusion criteria included use of interferon, interleukin, or other experimental therapies within 30 days of entry; therapy for acute infections within 14 days of entry; active substance abuse, psychological issues, or medical instability that might prevent the proper observation of safety or activity of the study treatment; pregnancy or breast-feeding; and systemic chemotherapy for malignancy within 8 weeks of study entry.

The study was designed to enroll 144 subjects (48 in each arm); this would have provided 80% power to detect a difference in HIV-1 RNA level of 0.70 $\log_{10}$ copies/mL between any 2 arms. Subjects were stratified by virus load (⩾50,000 or <50,000 copies/mL at screening) and by the number of nucleoside analogue RT inhibitors in their entry antiretroviral drug regimen (0–1 or ⩾2). However, because of increasing concerns over single-drug substitution in patients receiving combination therapy, an interim analysis of the initial 8 weeks of HIV-1 RNA data from the first 72 subjects was done. The ACTG review board recommended that the study be closed to accrual (at 89 subjects) and that all subjects taking SQV be offered IDV plus substitution of their nucleoside analogue RT inhibitors. This report describes the antiviral and immunologic responses during the 8 weeks after the protease inhibitor substitution in these 89 subjects.

Population sequencing. HIV-1 RNA was extracted from patient plasma samples, and genes were amplified in a nested RT–polymerase chain reaction (PCR) [12–14]. The pool of amplified protease genes was sequenced with dye primers and an automated DNA sequencer (PE Applied Biosystems, Foster City, CA) by use of procedures recommended by the manufacturer.

Independent clone sequencing. Total viral RNA was prepared from patient plasma and was amplified by multiple nested RT-PCR reactions. Reverse transcription was done with primer P8, d(TA AATCTGACTTGCCCAATTCAATTTT). First-strand cDNA was divided into 12 equal aliquots, and each was subjected to first-round PCR in a thermal cycler (model 9600; Perkin-Elmer, Norwalk, CT) with primers P8 and P7, d(AGACCAGAGCCAACAGCCCCAC), for 35 cycles of 15 s at 94°C, 1 min at 61°C, and 2 min at 72°C. Each was amplified in a second round with primers P9, d(ACG CGUACUAGUAGCCGATAGACAAGGAACTGTA), and P10, d(AUGGAGAUCUCUCAGGATGGAGTTCATAACCCAT), for 35 cycles of 15 s at 94°C and 10 min at 52°C. Molecular cloning and sequencing with dye terminators were done by use of a modification of the method described elsewhere [9, 10].

Comparison of sequencing results. Sequence data generated by the population and independent clone sequencing methods were compared. In all analyses, sequences judged to be mixtures of the wild type (clade B consensus) and mutant were considered to be mutant. In instances of disagreement of analyzed residues between

Table 1. Characteristics of study subjects at baseline in study of treatment with indinavir (IDV), hard-capsule saquinavir (SQVhc), and soft-gel–capsule saquinavir (SQVsgc) and extent of follow-up.

Characteristic	Total (n = 89)	Treatment arm: SQVhc (n = 29)	SQVsgc (n = 30)	IDV (n = 30)
Enrolled in ACTG 229	33 (37)	10 (34)	10 (33)	13 (43)
Sex, % male	89	90	87	90
Race				
White non-Hispanic	64 (72)	21 (72)	23 (77)	20 (67)
African American	11 (12)	2 (7)	3 (10)	6 (20)
Weeks of prior SQVhc				
Median	112	112	107	119
25%–75%	77–125	79–126	78–121	69–157
⩾2 NRTIs at baseline	76 (85)	24 (83)	26 (87)	26 (87)
Weeks of prior NRTI, median	236	273	181	254
CD4 cells/mm^3				
Median	240	263	231	229
25%–75%	144–320	174–307	110–313	157–379
RNA level, $\log_{10}$ copies/mL				
Median	4.10	4.06	4.33	3.92
25%–75%	3.35–4.74	3.37–4.85	3.36–4.69	3.14–4.74
RNA level <500 copies/mL	10 (11)	2 (7)	4 (13)	4 (13)
Protease gene substitutions, median no.	2	2	2	2
No./total tested (%) with syncytium-inducing phenotype	13/48 (27)	3/16 (19)	5/15 (33)	5/17 (29)
No. completing first 8 weeks	84	26	29	29

NOTE. Data are no. of subjects (%), except where noted. ACTG, AIDS Clinical Trials Group; NRTI, nucleoside analogue reverse-transcriptase inhibitor.

the 2 sequencing methods, the samples were resequenced and the results compared again. All initial discrepancies between the 2 methods were resolved by this repeated sequencing.

Interpretation of genotypes. The HIV-1 protease gene was fully sequenced, but only amino acid substitutions at protease residues 10, 20, 24, 46, 48, 54, 71, 73, 82, 84, 88, and 90 were analyzed, because of their recognized association with resistance to SQV and/or IDV. When a mutation or wild-type–mutation mixture was detected that would lead to a substitution of an amino acid at these positions, a mutation was scored. All analyses of genotype refer to these 12 selected protease codons only.

Susceptibility testing. The same amplified fragments used for population sequencing were used to generate recombinant viruses to determine drug susceptibility [15]. This assay was done with the HIV-1 recombinant stock virus at MOIs of 0.002 and 0.006 in the presence of increasing drug concentrations [16]. All IC_{50}s reflect the geometric mean of a drug susceptibility assay done in duplicate.

Plasma collection and HIV-1 RNA quantification. Blood was collected into EDTA tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) and separated into plasma and mononuclear cells by ficoll-hypaque gradient centrifugation within 4–6 h of collection, per ACTG virology consensus methods [17]. CD4 cell counts were determined by standard flow cytometric methods [18]. Blood plasma was stored at −70°C for shipment to the University of Washington for storage and batch HIV-1 RNA quantification.

The level of HIV-1 RNA in EDTA-anticoagulated blood plasma was assayed (Amplicor HIV Monitor; Roche Molecular Systems, Branchburg, NJ) [19, 20]. Both the manufacturer's and the ACTG's Virology Quality Assurance Program (VQAP) HIV-1 RNA standards were run with each assay, and the testing laboratory was certified by the VQAP as HIV-1 RNA assay proficient.

Statistical analyses. The t test was used to compare uncensored continuous variables, and Fisher's exact test was used to compare categorical variables. The analysis of change in HIV-1 RNA level excluded patients with unquantifiable HIV-1 RNA (<500 copies/mL) at baseline.

HIV-1 RNA values below the threshold of quantification create left-censoring of HIV-1 RNA values. Standard methods of right-censoring were adapted to analyze left-censored data [21]. Differences between groups were compared by use of the log rank test, and medians were calculated from the Kaplan-Meier estimate. The mean change in $\log_{10}$ HIV-1 RNA level from baseline was related to predictors by use of a censored-data linear model with a normal error distribution [22]. The residual distribution compared favorably with the expected distribution. The method of Wei and Johnson [23] was used to pool log rank tests over weeks 4, 6, and 8.

Results

Baseline characteristics and extent of follow-up. Characteristics of the 89 study subjects at baseline are shown in table 1. Thirty-seven percent had been enrolled originally in ACTG 229 [3]. The median prior SQVhc experience was 112 weeks. Prior nucleoside analogue RT inhibitor use was also extensive. Of the patients taking RT inhibitors, ~50% were taking zidovudine plus lamivudine, and 19% were receiving zidovudine plus zalcitabine. The median HIV-1 RNA level was 4.1 $\log_{10}$ copies/mL, and the median CD4 cell count was 240 cells/mm^3. Baseline characteristics were distributed evenly across arms. Five (6%) of the 89 enrolled subjects did not complete the initial

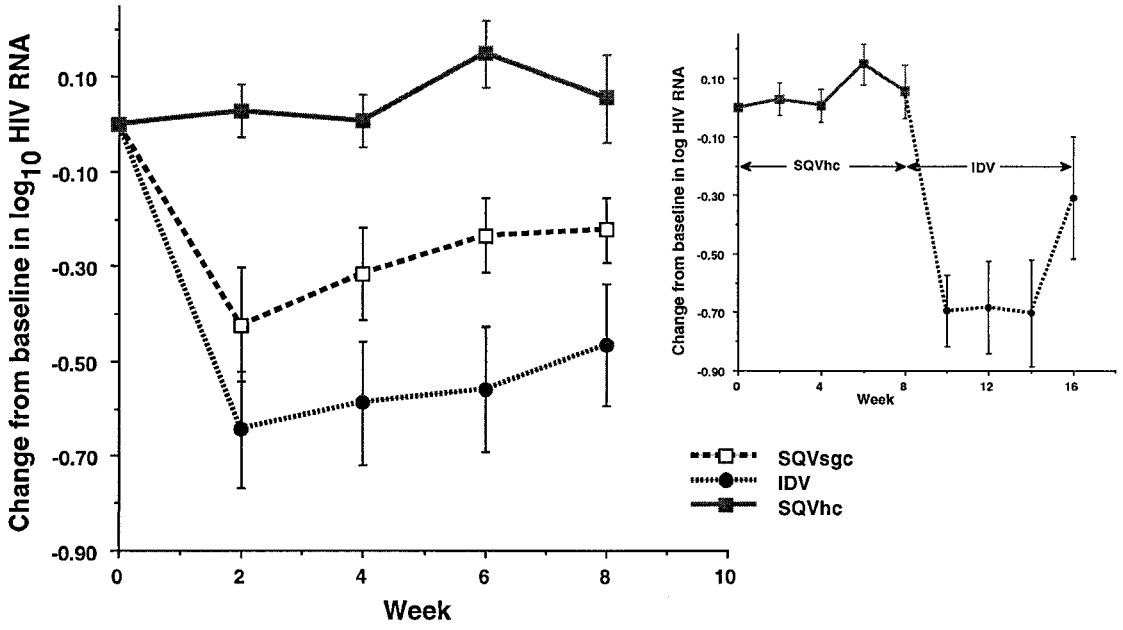

Figure 1. Mean change human immunodeficiency virus (HIV) type 1 RNA level (log_{10} copies/mL) during first 8 weeks of trial of indinavir (IDV), hard-capsule saquinavir (SQVhc), and soft-gel–capsule saquinavir (SQVsgc). SE bars are shown. *Insert,* Weeks 0–16 for subjects in SQVhc arm who switched to IDV at week 8. In this analysis, 500 copies/mL was used as RNA copy number for subjects with undetectable HIV-1 RNA (<500 copies/mL).

8 weeks of the study: 3 were in the SQVhc arm, and 1 each were in the other 2 arms. All 26 SQVhc recipients who switched to IDV at week 8 completed 8 weeks of IDV therapy.

Virologic and immunologic response. Figure 1 shows the mean change in RNA level over the first 8 weeks of the trial. In this analysis, 500 copies/mL was used as the RNA copy number for subjects with <500 copies/mL. By study arm, the week 4, 6, and 8 average change in HIV-1 RNA level from baseline was +0.07 log_{10} copies/mL (1.2-fold increase) for SQVhc, −0.24 log_{10} copies/mL (1.7-fold reduction) for SQVsgc, and −0.57 log_{10} copies/mL (3.7-fold reduction) for IDV. Comparison of responses in the 3 arms (*t* test) during the first 8 weeks of treatment showed significant differences between the SQVhc and IDV arms ($P < .001$) and between the SQVhc and SQVsgc arms ($P < .001$). The mean change in RNA level during the first 8 weeks in the IDV arm and the first 8 weeks of subsequent IDV treatment in the SQVhc arm (study weeks 8–16), called "combined IDV arms," was −0.54 log_{10} copies/mL. The difference between the combined IDV arms and the SQVsgc arm was also statistically significant ($P = .05$). About half the subjects reached their HIV-1 RNA nadir at week 2. Subjects treated with IDV were more likely to achieve a viral RNA level of <500 copies/mL (49%) than were subjects who received SQVhc (4%; $P < .001$) or subjects who received SQVsgc (8%; $P < .001$).

The mean changes in CD4 cell counts at weeks 4, 6, and 8 were −3.3, +29.8, and +13.1 cells/mm^3 for the SQVhc, SQVsgc, and IDV arms, respectively (figure 2). For subjects in the SQVhc arm who switched to IDV at week 8, the change in CD4 cell count from week 8 to 16 was +8.3 cells/mm^3. The only significant difference among the CD4 cell responses was between the SQVsgc and SQVhc arms ($P = .01$; *t* test).

Relationship of baseline genotype and baseline subject characteristics. At study baseline, 81 (91%) of the 89 subjects had levels of plasma-associated HIV-1 RNA sufficient for amplification for sequencing. Comparison of the sequences derived by the 2 different methods, population sequencing and sequencing by multiple independent molecular clones [9, 10], showed 96% concordance at the protease codons analyzed. The distribution and frequencies of single and multiple substitutions are shown in table 2. Codon 90 was altered most frequently, with 59% of subjects having an amino acid substitution at this position, followed by codons 71 (47%) and 10 (40%). Only 10% of subjects carried isolates with a substitution at codon 48, which is thought to be commonly selected by SQV. The median number of protease amino acid substitutions per subject was 2, but 25% of the subjects had no substitutions detected. Of subjects with a single substitution, the most common was at codon 90 (7 [47%] of 15). Of subjects with 2 substitutions, the most common pair was codons 90 and 71 (6 of 12), and, in subjects with 3 substitutions, the most common substitutions were at codons 90, 71, and 10 (9 [56%] of 16). Because of the

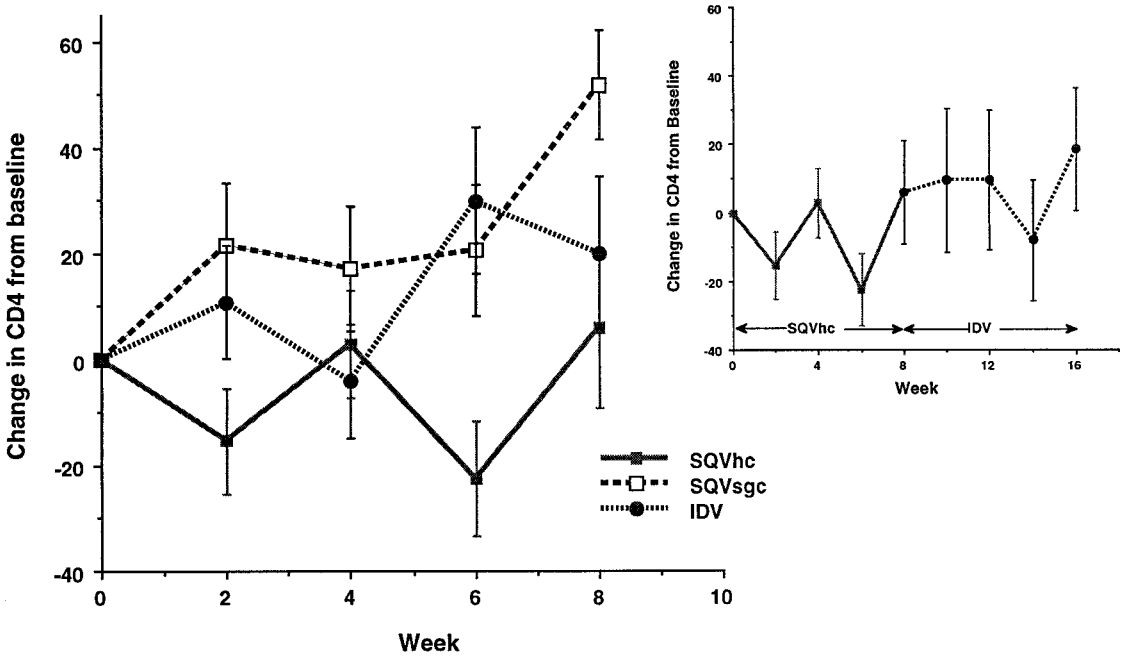

Figure 2. Mean change in CD4 cell count during first 8 weeks of trial of indinavir (IDV), hard-capsule saquinavir (SQVhc), and soft-gel–capsule saquinavir (SQVsgc). SE bars are shown. *Insert,* Weeks 0–16 for subjects in SQVhc arm who switched to IDV at week 8.

lack of isolates with substitutions at position 24 and only 1 subject with a substitution at position 88, no further analyses of these codons were done.

The relationship between the total number of select protease substitutions and duration of SQV use, baseline CD4 cell count, and plasma HIV-1 RNA level is shown in the box plots of figure 3. There was a significant association between the number of amino acid substitutions and longer duration of SQV use ($P < .001$), lower CD4 cell count ($P < .001$), and higher baseline virus loads ($P < .001$).

Genotype and virologic response. Comparison of the effect of amino acid substitutions on HIV-1 RNA response was applied separately for the combined IDV arms and SQVsgc arm. Table 3 relates the number of protease amino acid substitutions at the 12 select positions to change in HIV-1 RNA level (from the censored-data linear model) 4, 6, and 8 weeks after the change to IDV therapy. Increasing numbers of substitutions were associated with smaller HIV-1 RNA responses ($P < .01$). This result was driven primarily by differences in response among patients with 0 or 1 substitution versus those with ⩾2 total substitutions ($P < .001$). The relationship is also evident from the number of subjects whose HIV-1 RNA level was suppressed to <500 copies/mL. Fifteen (75%) of 20 IDV recipients with 0 or 1 amino acid substitution responded with HIV-1 RNA below the limit of quantification (<500 copies/mL) during the first 8 weeks, compared with 2 (9%) of 22 subjects with ⩾2 substitutions.

The viral RNA responses of subjects who had wild-type virus at each specific position were compared to those of subjects who had a substitution at each select position. Table 4 shows the mean change in HIV-1 RNA level from baseline for the 51 IDV-treated subjects (combined arms) and the 28 SQVsgc-treated subjects, with genotype data. The presence of a substitution or a combination of substitutions at codons 10, 20, 48, 82, 84, or 90 was significantly associated with a blunted week 8 response to IDV treatment. The presence of a substitution or a combination of substitutions at codons 10, 73, or 84 was significantly associated with a blunted week 8 response to SQVsgc treatment. Notably, neither codon 48 nor 90 substitutions were significantly associated with blunted response to SQVsgc treatment.

The independent effect of a substitution at each of the select codons on HIV-1 RNA response to treatment was difficult to isolate because the substitutions most often appeared in combinations. The presence of a combination that included a substitution at codon 10 (which never occurred alone; see table 2) was highly statistically significant. A virus with a substitution at codon 10 was associated with an average blunting of an IDV response of ~1 $\log_{10}$. This analysis showed that codon 10 status remained predictive in the presence of all other codon changes.

Phenotype and virologic response. In vitro drug susceptibility (drug phenotype) to IDV and SQV was tested for 76 baseline isolates in which viral RNA could be amplified for population sequencing. The distribution of IDV and SQV IC_{50}s

Table 2. Distribution of protease substitutions, by total number of substitutions.

1 Substitution (n = 15)	2 Substitutions (n = 12)	3 Substitutions (n = 16)	4 Substitutions (n = 9)	5 Substitutions (n = 7)	6 Substitutions (n = 2)
90 (7)	71 + 90 (6)	10 + 71 + 90 (9)	10 + 71 + 73 + 90 (3)	10 + 20 + 71 + 73 + 90 (2)	10 + 20 + 71 + 73 + 82 + 90 (1)
71 (5)	20 + 90 (3)	10 + 20 + 90 (1)	10 + 71 + 84 + 90 (3)	10 + 20 + 71 + 84 + 90 (1)	10 + 48 + 54 + 71 + 82 + 90 (1)
20 (3)	10 + 90 (2)	10 + 20 + 82 (1)	10 + 48 + 71 + 82 (1)	10 + 46 + 48 + 82 + 90 (1)	
	73 + 90 (1)	10 + 48 + 54 (1)	20 + 71 + 82 + 90 (1)	10 + 48 + 54 + 71 + 82 (1)	
		10 + 48 + 82 (1)	10 + 48 + 82 + 90 (1)	10 + 48 + 54 + 71 + 90 (1)	
		46 + 71 + 90 (1)		10 + 54 + 71 + 73 + 90 (1)	
		71 + 73 + 90 (1)			
		71 + 88 + 90 (1)			

NOTE. Data are codon and codon combinations having substitutions (no. of isolates); 20 isolates had no substitutions.

for all subjects by total number of substitutions appears in figure 4. The mean IC_{50}s for IDV and SQV are compared for each isolate. There was a close correlation between the SQV and IDV IC_{50}s (Spearman correlation, .80; $P < .001$). Higher baseline HIV-1 RNA values were associated with higher SQV IC_{50}s ($P < .01$). The total number of protease substitutions was also positively associated with both the IDV and SQV IC_{50} ($P < .001$, both comparisons).

There was a significant relationship with increasing susceptibility to IDV and larger decreases in HIV-1 RNA levels for subjects in the combined IDV arms pooled at weeks 4 (P = .03), 6 (P = .01), and 8 (P = .03). In the censored data linear model, a 5-fold difference in IDV IC_{50} was associated with an average difference of -0.69, -0.97, and -0.77 $\log_{10}$ copies/mL change in HIV-1 RNA level from baseline to weeks 4, 6, and 8, respectively.

Multivariate models incorporating other factors were used to examine the predictive power of baseline in vitro susceptibility on changes in viral RNA level. The analysis found that IDV IC_{50} was consistently associated with a change in viral HIV-1 RNA level when it was the sole predictor variable. A 5-fold reduction in IDV IC_{50} was associated with an HIV-1 RNA response of -0.77 $\log_{10}$ copies/mL at week 8 ($P < .05$). We also fitted models that simultaneously examined the predictive effect of phenotype, genotype, and other baseline factors on viral RNA. The number of protease substitutions (0 or 1 vs. ≥ 2) or codon 10 status remained highly predictive, but IDV IC_{50} did not. Subjects with 0 or 1 protease substitutions had a change in HIV-1 RNA level of -1.38 $\log_{10}$ copies/mL ($P < .01$), even after we controlled for IDV IC_{50}. The effect of IDV IC_{50} was consistently markedly reduced. After adjusting for genotype, a 5-fold reduction in IDV IC_{50} was associated with a change in HIV RNA level of $+0.05$ $\log_{10}$ copies/mL at week 8. This suggests that the unadjusted association between IDV response and IDV IC_{50} resulted from the association between IDV IC_{50} and the number of protease substitutions (or codon 10 status). We find that the genotypic measurements distinguished different HIV RNA profiles. Once that genotype is known, in vitro IDV IC_{50} consistently added no predictive information.

Similar censored-data linear models were also applied to changes in HIV-1 RNA in the SQVsgc recipients. The model with SQV IC_{50} as a predictor did not show a significant relationship with change in HIV-1 RNA level after the switch to SQVsgc (data not shown). The modest virologic effects of addition of SQVsgc and the smaller number of SQVsgc subjects restricted the study's capability to demonstrate this relationship.

Discussion

Subjects enrolled in ACTG 333 had received SQVhc therapy for >48 weeks and were taking a stable drug regimen for 8 weeks before entering the trial. For the first 8 weeks, the study permitted substitution of only the protease inhibitor. This study design allowed an estimate of the incremental antiviral activity of IDV or SQVsgc when substituted for SQVhc. The antiviral activity from the single new agent then was used to assess the predictive power of the baseline protease genotype and baseline in vitro drug susceptibility phenotype for subsequent HIV-1 RNA responses. Our study showed that, in highly SQV-experienced subjects, there was a significant relationship between the number of protease substitutions at baseline and duration of prior SQV use, baseline CD4 cell count, baseline HIV-1 RNA level, and susceptibility to SQV and IDV.

With a switch from SQVhc, the decline in HIV-1 RNA level during the first 8 weeks was significantly greater for IDV than for SQVsgc and for SQVsgc than for SQVhc. However, CD4 cells significantly increased only in the SQVsgc recipients, compared with the SQVhc recipients, despite the smaller decrease in viral RNA level with SQVsgc. This discordant rise in CD4 cell count with SQV is similar to that seen in ACTG 229 [3]. The short time of follow-up and the large variability in CD4 cell counts may have limited our ability to detect longer-term trends or differences among the arms.

The significance of a substitution at codon 10 on HIV-1 RNA response is surprising, because this is not in the active site of the protease enzyme and has been shown previously to have only subtle effects on resistance to either SQV or IDV. Among the IDV recipients with <2 substitutions in the protease, the virus was uniformly wild type at position 10 (22/22). If the isolate had ≥ 2 substitutions in protease, it was likely (21/26) to have a substitution at position 10. Because of this close relationship between codon 10 status and the total number of substitutions, it was not possible to distinguish whether the

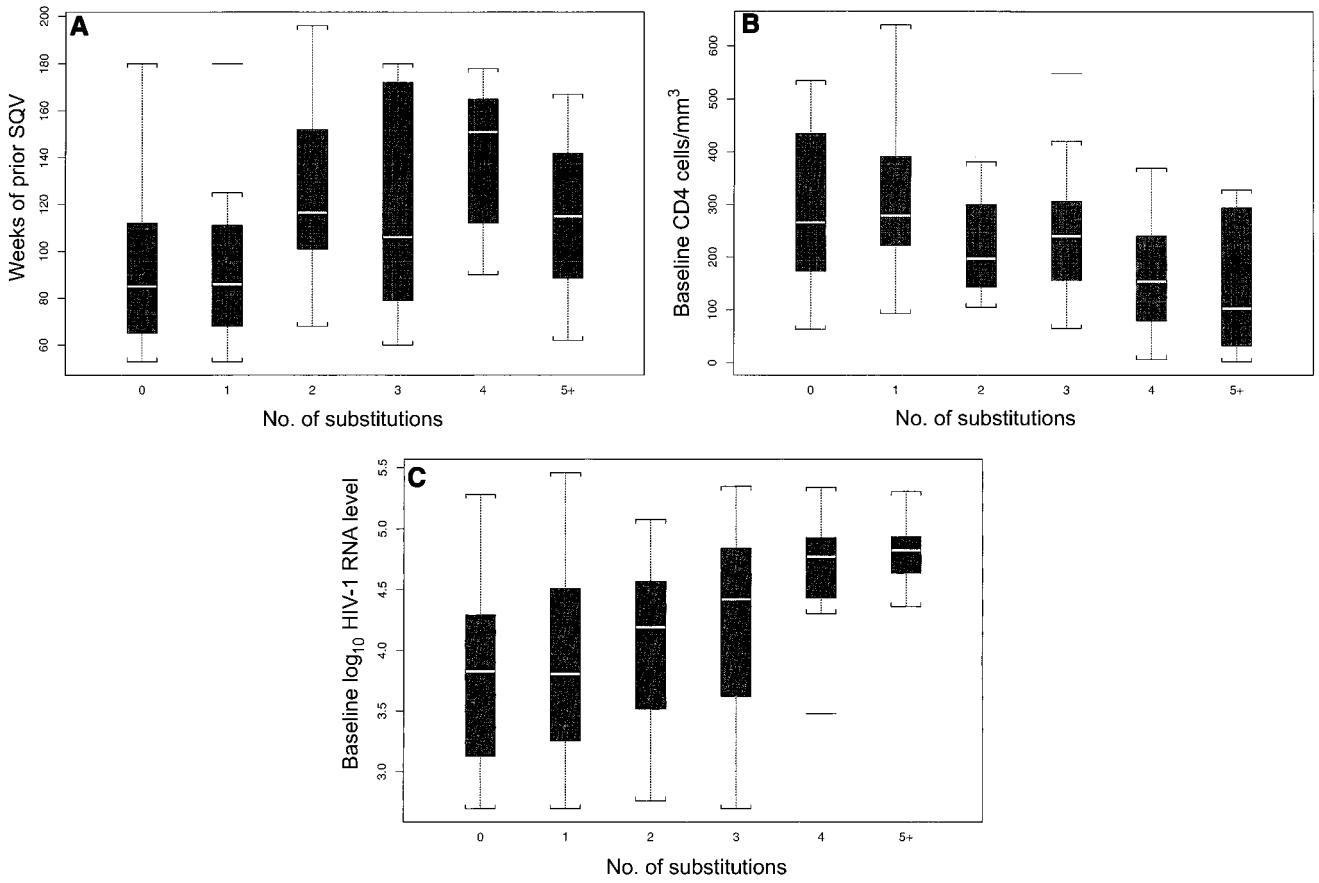

Figure 3. Number of protease substitutions versus *(A)* duration of prior saquinavir (SQV), *(B)* CD4 cell count/mm^3, and *(C)* baseline human immunodeficiency virus (HIV) type-1 RNA level ($\log_{10}$ copies/mL).

codon 10 substitution itself or the presence of ⩾2 protease substitutions drove the apparent difference in HIV-1 RNA response to IDV. Neither variable was significant in regression models that included the other variable as a predictor of viral RNA response.

Among IDV recipients, those with 0 or 1 protease substitution at entry had HIV-1 that was wild type at protease codon 10 and had a greater decrease in HIV-1 RNA level than did subjects with ⩾2 protease substitutions, who usually carried a substitution at position 10 and had a poorer virologic response ($P < .0001$). The IC_{50}s of IDV and SQV were highly correlated with each other. Decreased susceptibility to SQV was associated with a higher baseline viral RNA level. Although decreased phenotypic drug susceptibility to IDV was associated with a blunted viral RNA response to the drug, once the viral genotype was known, the degree of IDV drug susceptibility added little predictive power for the virologic response.

The virologic response to the switch of protease inhibitors in this trial was significantly less than that seen with the addition of protease inhibitor monotherapy in drug-naive patients [24]. It is unlikely that this limited antiviral activity in our study was due to the short 8-week period of observation, because half the IDV and SQVsgc subjects reached their viral RNA nadir at week 2, and <10% of subjects reached their nadir after study week 6. This observation is most likely due to cross-resistance between SQV and IDV. Evidence for cross-resistance, as well as the predictive value of protease genotyping, was suggested by the better virologic response (⩾1.5 $\log_{10}$ copies/mL) of the ACTG 333 subjects who had no substitutions in codons associated with the drug resistance in the viral protease, which is

Table 3. Number of protease substitutions and change in human immunodeficiency virus (HIV) type 1 RNA level after switch to indinavir in combined indinavir arms.

No. of substitutions	No. of subjects	Change in RNA, $\log_{10}$ copies/mL		
		Week 4	Week 6	Week 8
0	15	−1.29 (0.26)	−2.18 (0.50)	−1.72 (0.42)
1	8	−1.59 (0.35)	−2.37 (0.55)	−1.14 (0.49)
2	6	−0.71 (0.35)	−0.53 (0.40)	−0.35 (0.50)
3	11	−0.53 (0.25)	−0.90 (0.30)	−0.52 (0.35)
4	6	−0.58 (0.33)	−0.41 (0.37)	−0.23 (0.47)
5 or 6	5	−0.85 (0.35)	−0.80 (0.45)	−0.44 (0.57)

NOTE. Data are model-based mean (SE).

Table 4. Change in human immunodeficiency virus type 1 RNA level after week 8, by baseline genotype.

	Indinavir (combined arms)			Soft-gel–capsule saquinavir		
Codon	*n*	Change in RNA, $\log_{10}$ copies/mL	*P*	*n*	Change in RNA, $\log_{10}$ copies/mL	*P*
10, WT	28	−1.65	<.001	20	−0.40	.04
10, Mutant	23	−0.49		8	−0.14	
20, WT	45	−1.14	.02	23	−0.31	.79
20, Mutant	6	−0.37		5	−0.34	
46, WT	50	−1.04	.79	27	−0.31	.93
46, Mutant	1	−1.68		1	−0.43	
48, WT	46	−1.14	.02	25	−0.34	.26
48, Mutant	5	−0.37		3	−0.14	
54, WT	48	−1.04	.50	26	−0.34	.10
54, Mutant	3	−1.37		2	−0.05	
71, WT	26	−1.25	.20	16	−0.38	.16
71, Mutant	25	−0.93		12	−0.22	
73, WT	44	−1.12	.21	26	−0.34	.02
73, Mutant	7	−0.63		2	+0.15	
82, WT	47	−1.15	.03	24	−0.35	.16
82, Mutant	4	−0.22		4	+0.16	
84, WT	49	−1.10	.04	27	−0.33	.02
84, Mutant	2	−0.07		1	+0.23	
90, WT	23	−1.61	.002	10	−0.41	.19
90, Mutant	28	−0.69		18	+0.18	

NOTE. Method of Wei and Johnson [23] was used to pool log rank tests over weeks 4, 6, and 8. WT, wild type.

comparable to the response seen in protease inhibitor–naive subjects [24]. Although only 4 SQVsgc recipients had virus isolates with none of the select protease substitutions, the HIV-1 RNA response of these subjects was also much larger than the virologic response in subjects with more protease substitutions.

The combination of a relatively low median baseline HIV-1 RNA level and the use of the standard Roche Amplicor HIV-1 Monitor assay resulted in a number of subjects with virus levels below the lower limit of quantification (500 copies/mL). The frequency of unquantifiable HIV-1 RNA complicated the statistical analysis and required highly structured models. Those models assumed that baseline HIV-1 RNA was independent of subsequent change and that log change in HIV-1 RNA level followed a normal distribution. These assumptions appeared to fit the data well, but the analysis involves some extrapolation beyond the range of the changes in HIV-1 RNA level observed in the study.

Because of the known problems with identification of genotypic mixtures [25], 2 sequencing strategies were used in 2 separate highly experienced sequencing laboratories to derive the protease sequence. Both methods had limitations. Neither method could obtain amplifiable sequences from subjects with very low virus loads, and low-level mixtures were difficult to resolve. Nevertheless, because of use of a team consensus approach and resequencing of specimens for which there was a lack of agreement, our sequences are likely to be more reliable than those generated by any single method.

Although the whole protease coding sequence was analyzed, we prospectively selected only 12 of the 99 amino acid positions in the protease for our analysis. These 12 positions were chosen because they have been implicated in resistance to either SQV or IDV [7, 9, 10, 26, 27]. Substitutions in other protease positions could have affected our interpretation of the virologic response and genotype. For example, one active-site protease substitution not included in our list, V32I, has been reported in some patients after IDV treatment [9, 10]. However, this substitution has not been associated with selection by SQV therapy, and, consistent with that observation, no V32I substitutions were seen in our study. Substitution of codon 63, implicated in resistance to both SQV and IDV, was not analyzed because of its high prevalence (>50%) in protease inhibitor–naive patients [28], and the significance of its presence would be difficult to interpret. Accordingly, we believe that these 12 residues represent the most easily interpretable codons that could potentially affect the response to IDV and SQV.

In our analysis, subjects with mixtures of mutant and wild virus codons were considered to have a mutant genotype. With respect to amino acid residues 24, 46, 48, 54, 73, 82, 84, and 90, none of which occur in protease inhibitor–naive subjects at any appreciable frequency, the appearance of a substitution at any of these 8 positions, even when present in a minority of the population, strongly suggests the evolution of resistance. Amino acid substitutions at the remaining residues, 10, 20, and 71, occur as natural genetic polymorphisms, with <10% prevalence in untreated patients [28], and do not appear to engender measurable resistance when occurring alone. However, these substitutions are associated with reduced susceptibility to IDV when other substitutions are present [9, 10].

The relationship between the baseline HIV-1 RNA level and CD4 cell count, the number of protease substitutions, and drug susceptibility adds additional support to the potential usefulness of antiretroviral susceptibility and genotype sequencing assays. As expected, the longer the duration of SQV use, the more protease substitutions were found. The more protease substitutions, the higher the baseline HIV-1 RNA level and the lower the baseline CD4 cell count. Moreover, the more SQV resistant the baseline virus, the higher the baseline virus load.

A major potential role for sequencing of the protease gene will be for the prediction of virologic and clinical response to a new antiretroviral drug regimen in patients with prior protease inhibitor experience. Our study showed that most subjects with virus isolates that contained 0 or 1 protease substitution at relevant protease codons had a good short-term virologic response to IDV, whereas subjects with $\geq$2 substitutions did not. However, the large variety of substitutions and small number of subjects with each combination of substitutions made the prediction of virologic response from a specific combination of substitutions unreliable. Nevertheless, the close association between a substitution at protease position 10 and the occurrence of multiple protease substitutions in these subjects may allow for the reliable prediction of poor virologic response to IDV or SQV, if a position 10 substitution is selected by prior SQV therapy. However, there is no evidence from other studies that

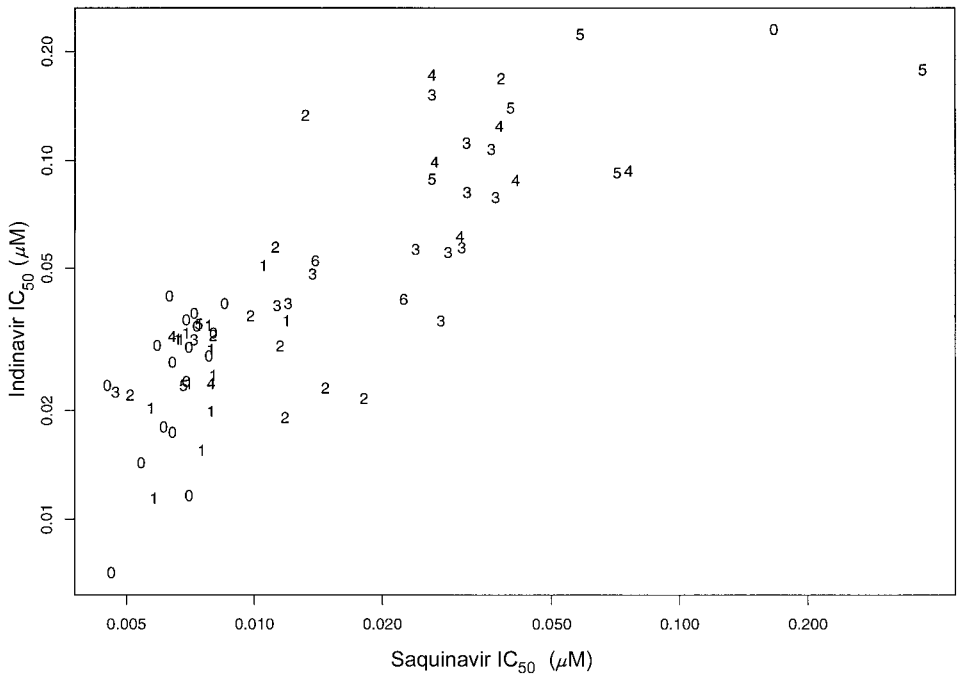

Figure 4. Indinavir IC_{50} versus saquinavir IC_{50} for each baseline isolate. No. of codon substitutions for each isolate is indicated by nos. on plot.

the presence of a single substitution at residue 10 alone had any measurable effect on either viral phenotype or on virologic responses to protease inhibitor therapy.

These data should not be extrapolated to predict virologic response to other combinations or sequences of protease inhibitors, for several reasons. First, the constellation of protease substitutions in our study was selected during long-term use of SQVhc. Although the current formulation of SQV, SQVsgc, has been shown to select a similar spectrum of amino acid substitutions in a clinical setting, it is possible that the relative distribution of these might be altered from that seen in this study, because of increased potency and selective pressure [29]. Second, our recombinant virus drug susceptibility assay is similar to commercially available phenotypic assays, but it is not identical. Direct comparisons of these assays are not available. Last, the predictive power of the genotype and drug susceptibility–phenotype assays was based on the HIV-1 RNA data for a relatively small number of subjects (~50) who switched from SQVhc to IDV. Nevertheless, these limitations do not change the significant relationships between baseline characteristics, virologic response, and genotype and drug susceptibility–phenotype assays that we have observed.

Because the prediction of virologic response in patients with multiple protease substitutions is problematic, HIV-1 drug susceptibility testing is being used increasingly [30]. Our study showed a relationship between drug susceptibility and the number of protease substitutions and between drug susceptibility and virologic response. This relationship between drug susceptibility and virologic response might be clinically useful, but the phenotypic assay's true value will depend on its reproducibility. The drug susceptibility of our isolates fell within a relatively narrow range. The mean IDV IC_{50} of isolates with no protease substitutions and good virologic response was only 2–3-fold lower than that of virus with multiple substitutions from subjects with poor virologic response. This variation is within the range of normal assay variability, and it remains to be seen whether other phenotypic assays with better precision and reproducibility might have greater clinical predictive value. Changes in the IC_{50} of 2–3-fold must be reproducibly detected for this assay to prove useful for individual patient management.

Although drug susceptibility was predictive of virologic response, the predictive power of the genotype included all the prognostic information of drug susceptibility (i.e., the IDV IC_{50} added little prognostic information once the genotype was known). This is not the usual view of these assays and further emphasizes the need for careful prospective validation of both the genotype and drug susceptibility–phenotype and assays before their widespread clinical use. Genotype changes are thought to exert their effect by making the virus more drug resistant, and thus the prognostic information of genotype should be reflected in the drug susceptibility phenotype. However, our study found that, at a given level of drug susceptibility, the genotype still greatly influenced the virologic response. Furthermore, our data suggest that substitutions in the protease can have an effect on virologic response in a manner that is

not detected by in vitro drug susceptibility assays. The relationship between drug susceptibility phenotype, genotype, and clinical response requires further study.

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