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Phenotypic Hypersusceptibility to Multiple Protease Inhibitors and Low Replicative Capacity in Patients Who Are Chronically Infected with Human Immunodeficiency Virus Type 1

Javier Martinez-Picado,[1]* Terri Wrin,[2] Simon D. W. Frost,[3] Bonaventura Clotet,[1] Lidia Ruiz,[1] Andrew J. Leigh Brown,[4] Christos J. Petropoulos,[2] and Neil T. Parkin[2]

Affiliations: IrsiCaixa Foundation, Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain[1]; ViroLogic, Inc., South San Francisco, California[2]; University of California, San Diego, California[3]; and University of Edinburgh, Edinburgh, Scotland, United Kingdom [4]

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Increased susceptibility to the protease inhibitors saquinavir and amprenavir has been observed in human immunodeficiency virus type 1 (HIV-1) with specific mutations in protease (V82T and N88S). Increased susceptibility to ritonavir has also been described in some viruses from antiretroviral agent-naïve patients with primary HIV-1 infection in association with combinations of amino acid changes at polymorphic sites in the protease. Many of the viruses displaying increased susceptibility to protease inhibitors also had low replication capacity. In this retrospective study, we analyze the drug susceptibility phenotype and the replication capacity of virus isolates obtained at the peaks of viremia during five consecutive structured treatment interruptions in 12 chronically HIV-1-infected patients. Ten out of 12 patients had at least one sample with protease inhibitor hypersusceptibility (change ≤0.4-fold) to one or more protease inhibitor. Hypersusceptibility to different protease inhibitors was observed at variable frequency, ranging from 38% to amprenavir to 11% to nelfinavir. Pairwise comparisons between susceptibilities for the protease inhibitors showed a consistent correlation among all pairs. There was also a significant relationship between susceptibility to protease inhibitors and replication capacity in all patients. Replication capacity remained stable over the course of repetitive cycles of structured treatment interruptions. We could find no association between in vitro replication capacity and in vivo plasma viral load doubling time and CD4$^+$ and CD8$^+$ T-cell counts at each treatment interruption. Several mutations were associated with hypersusceptibility to each protease inhibitor in a univariate analysis. This study extends the association between hypersusceptibility to protease inhibitors and low replication capacity to virus isolated from chronically infected patients and highlights the complexity of determining the genetic basis of this phenomenon. The potential clinical relevance of protease inhibitor hypersusceptibility and low replication capacity to virologic response to protease inhibitor-based therapies deserves to be investigated further.

Antiretroviral treatment of human immunodeficiency virus type 1 (HIV-1) infection does not successfully suppress HIV replication in all patients (5, 14, 16, 20). Continued viral replication in the presence of selective drug pressure leads to antiretroviral agent resistance, hindering the efficacy of subsequent treatment. Both phenotypic and genotypic assays are available to monitor drug resistance, and their results have been incorporated into the management of HIV-1-infected individuals in the last few years (13, 27). Different prospective studies support at least short-term clinical utility of HIV resistance testing in treatment-experienced patients (2–4, 7, 33).

The routine use of highly reproducible phenotypic assays (24) revealed that many clinical HIV-1 isolates exhibited significant hypersusceptibility (defined as a change in the 50% inhibitory concentration versus the reference of ≤0.4-fold) to the non-nucleoside reverse transcriptase inhibitor class (12, 31, 34). This phenomenon is associated with previous nucleoside analogue treatment and with an increase in the efficacy of salvage antiretroviral regimens including non-nucleoside reverse transcriptase inhibitors (12, 31, 32, 34). In chronically infected antiretroviral-experienced patients, amprenavir hypersusceptibility is associated with the N88S mutation in protease, which is not seen in drug-naïve patients (26, 35). Several mutations in protease have been correlated with increased susceptibility to each protease inhibitor in wild-type viruses (23). A more recent study also showed that ritonavir hypersusceptibility in HIV from recently infected, untreated patients is not associated with a particular single mutation but with combinations of amino acids at polymorphic sites and that the same genotypes which confer hypersusceptibility to protease inhibitors also confer low in vitro replication capacity (15).

The present study examines the existence of chronically HIV-1-infected patients who harbor viral isolates with a hypersusceptible phenotype to multiple protease inhibitors along with a low in vitro replication capacity.

MATERIALS AND METHODS

Study population and specimens. The samples analyzed were obtained during a structured treatment interruption study conducted at the Hospital Universitari Germans Trias i Pujol, Badalona, Spain. Full details of the selection criteria and antiretroviral treatments are given elsewhere (28, 29). In brief, 12 HIV-1-infected patients who had CD4$^+$ cell counts >600 cells/μl and a CD4/CD8 ratio of >1 sustained for a minimum of 6 months and who had plasma HIV-1 RNA levels below 50 copies/ml for at least 2 years before study entry were scheduled to interrupt their antiviral therapy in an intermittent manner. These criteria were chosen in order to enroll patients with well-conserved immunity and long-term

* Corresponding author. Mailing address: IrsiCaixa Foundation Hospital Germans Trias i Pujol, Ctra. de Canyet, s/n 08916 Badalona, Spain. Phone: 34 93 4656374. Fax: 34 93 4653968. E-mail: javiermp@ns.hugtip.scs.es.

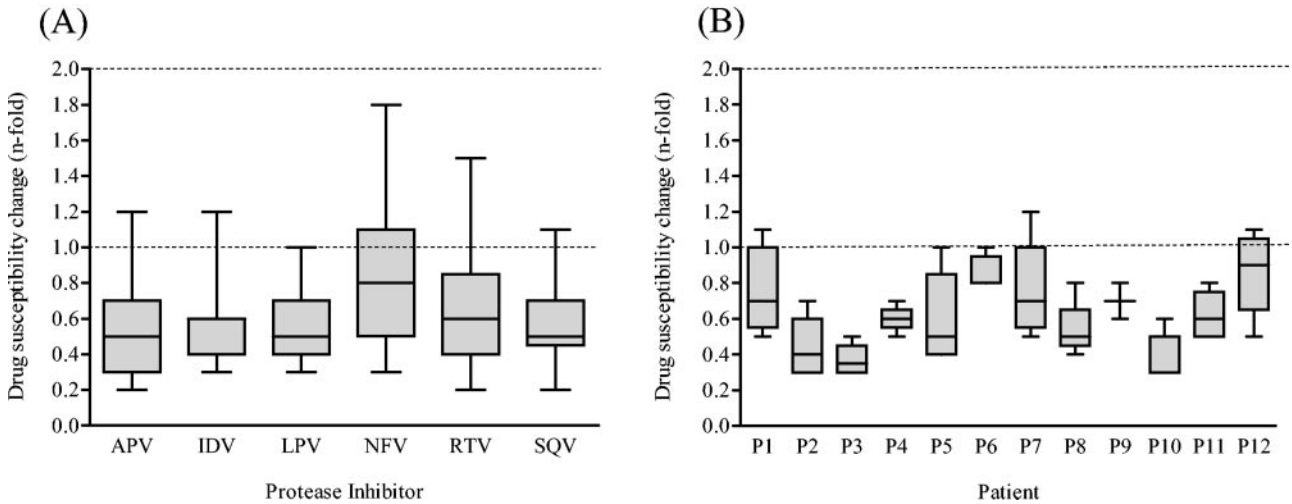

FIG. 1. Box-plot representation of phenotypic susceptibility to protease inhibitors. (A) Values for all patients grouped by protease inhibitor. A total of 61 determinations were performed for each protease inhibitor except for lopinavir, which had 53. (B) Values for all protease inhibitors grouped by patient. Five to six determinations were obtained for each patient except for patient 9, who had only two because plasma viral load did not rebound after some of the treatment discontinuations. For each box, the center line represents the median change, the box boundaries define the 25th and 75th percentiles, and the whiskers define the 10th and 90th percentiles. Change values are relative to HIV-1_{NL4-3}. APV, amprenavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir.

viral suppression. Treatment interruptions during the first four cycles lasted for a maximum of 30 days, or until plasma viral loads reached levels higher than 3,000 copies/ml in two consecutive determinations, after which highly active antiretroviral treatment was resumed for approximately 90 days until the next structured treatment interruption. In all cases, this resulted in suppression of plasma viremia to less than 50 copies/ml prior to the next interruption. At the fifth treatment interruption, patients remained off treatment for more than 12 months. Treatment regimens were fairly homogenous among patients and did not include non-nucleoside reverse transcriptase inhibitors.

Viral sequence analysis. HIV-1 RNA isolated from patient plasma samples was reverse transcription-PCR amplified and genotyped using population-based sequencing for the protease and the reverse transcriptase at the peak of viremia during each structured treatment interruption. Phylogenetic analyses of HIV-1 RNA sequences were performed to exclude cross-contamination. All samples were determined to belong to subtype B.

Drug susceptibility assay. A rapid recombinant assay was used to measure the drug susceptibility of the patients' viral isolates (HIV PhenoSense, ViroLogic) (24). This assay involves the construction of resistance test vectors, which are comprised of a pool of recombinant HIV-1 containing *gag* (3′ end from p7), protease, and reverse transcriptase sequences derived from the virus sample that is being evaluated. Resistance test vectors also contain a luciferase reporter gene replacing *env* to monitor a single round of virus replication. Susceptibility of resistance test vectors to a panel of antiretroviral drugs was compared to a reference vector containing the protease and reverse transcriptase sequences derived from HIV-1_{NL4-3}. Two independent measurements of each viral isolate were obtained.

Replication capacity assay. Replication capacity was measured using a modified version of the PhenoSense drug susceptibility assay (6, 10). The relative replication capacity of the virus was determined by measuring the amount of luciferase activity produced 72 h after infection in the absence of drug. Replication capacity is expressed as the percentage of the luciferase activity produced by the vectors containing patient-derived *gag-pol* sequences compared to the luciferase activity from vectors containing the HIV-1_{NL4-3} *gag-pol* reference sequences (100%). Replication capacity measurements were normalized for differences in transfection efficiencies by monitoring the luciferase activity generated in transfected cells. Two replicates were performed for each sample.

Association between viral growth rate, CD4, CD8, and replication capacity. Viral growth rates based on plasma viral load doubling time, $CD4^+$ and $CD8^+$ T-cell counts, and CD4/CD8 ratio during each structured treatment interruption cycle had been previously calculated for the four first interruptions in the study (9) and have been compared here with the replication capacity of each patient isolate at each interruption.

Genetic basis of hypersusceptibility. Decision tree models describing the genetic basis for hypersusceptibility to protease inhibitors in primary HIV-1 strains were applied to our samples as previously described (15). The number of samples defined as hypersusceptible with or without mutations at each position were also compared using Fisher's exact test (23).

Statistical analysis. Statistical analyses were performed using the R language (http://www.r-project.org) and the GraphPad Prism version 4 (GraphPad Software, San Diego, CA). The correlation analysis of susceptibility among different protease inhibitors and also between protease inhibitor susceptibility and replication capacity was estimated using Spearman′s rank correlation test. All *P* values were two-tailed.

RESULTS

Protease inhibitor susceptibility and genotype. The drug susceptibility and *pol* sequences of 61 viral isolates derived from 12 patients were evaluated. For lopinavir, only 53 viral isolates were included in the analysis. The median change in the 50% inhibitory concentration for the different protease inhibitors ranged from 0.5-fold for amprenavir to 0.8-fold for nelfinavir, with nelfinavir being the only drug with a 75% percentile slightly higher than 1 (Fig. 1A). Although the median protease inhibitor change (except nelfinavir) previously reported for HIV-1 lacking any drug-selected mutations is also less than 1-fold (22), the values determined here were even lower than this. Using a definition of hypersusceptibility of a change 0.4-fold or lower, 23 (38%) isolates were hypersusceptible to amprenavir, 21 (34%) to ritonavir, 11 (18%) to indinavir, 9 (17%) to lopinavir, 12 (20%) to saquinavir, and 7 (11%) to nelfinavir. Ten out of 12 patients had at least one sample hypersusceptible to ≥1 protease inhibitor. The median change in protease inhibitor susceptibility for each patient ranged from 0.3-fold for patient 3 to 0.9-fold for patient 12 (Fig. 1B).

We also compared the median susceptibility values for drugs in each of the three antiretroviral classes for which we had data. Susceptibility to protease inhibitors (median = 0.60) was significantly lower than susceptibility to nucleoside reverse transcriptase inhibitors (median = 0.84, $P < 0.0001$, Wilcoxon matched-pair test) and non-nucleoside reverse transcriptase

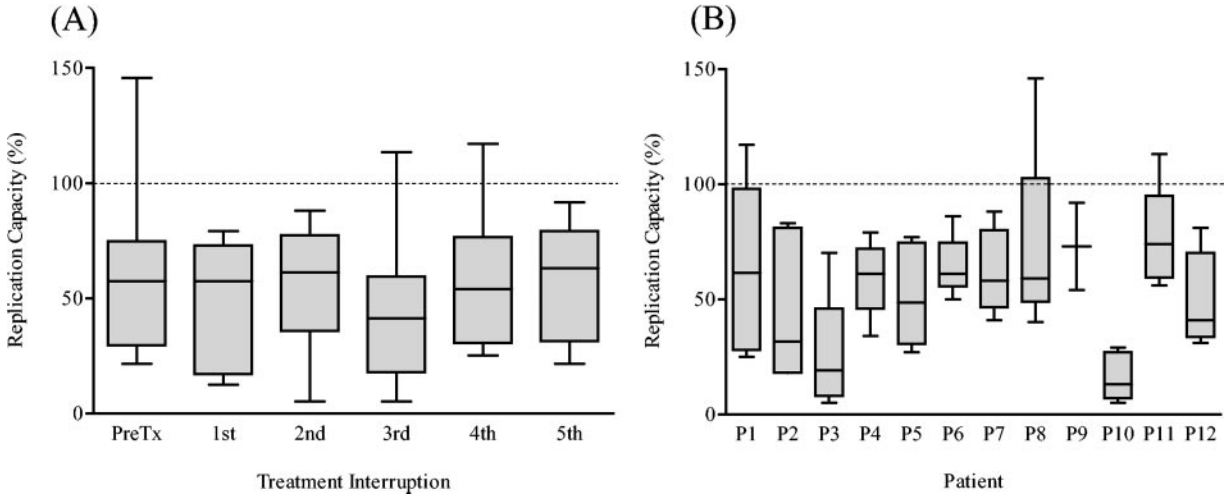

FIG. 2. Box-plot representation of the replication capacity values measured as a percentage of the patient's virus with respect to the reference HIV-1_{NL4-3} (100%). (A) Values grouped by pretreatment determination (PreTx) and peak of viremia at each structured treatment interruption. Nine to 11 values are included per time point. (B) Values grouped by patient. Five to six determinations were obtained for each patient except for patient 9, who had only two because plasma viral load did not rebound after some of the treatment discontinuations.

inhibitors (median = 1.13, $P < 0.0001$). Therefore, the hypersusceptibility phenomenon is confined to the protease inhibitor class.

None of the patient isolates contained primary protease inhibitor-associated mutations (mutations 24, 30, 32, 46, 47, 48, 50, 54, 82, 84, 88, and 90) over the course of the study, in agreement with phenotypic data: no viral isolate had a fold change value above the PhenoSense assay biological cutoff (22).

Low replication capacity. The median replication capacity for all of the patient isolates was 57.6% (mean, 53.1%), with 25 and 75% percentiles of 29.7 and 72.0%, respectively. Patients whose samples had the lowest replication capacity (patients 2, 3, and 10) were the same than those with the lowest protease inhibitor change. Median replication capacity for all patients grouped by number of structured treatment interruptions ranged from 41 to 63% (Fig. 2A); the median of all values grouped by patient ranged from 13 to 74% (Fig. 2B). No significant changes in replication capacity were observed over the course of repetitive interruptions.

Correlation between protease inhibitor susceptibility and replication capacity. Correlation analysis of log-transformed drug susceptibility and replication capacity values were significantly positive for all protease inhibitors tested. Amprenavir showed the highest correlation with replication capacity ($\rho = 0.65$, $P < 0.0001$), while lopinavir susceptibility had the lowest correlation ($\rho = 0.44$, $P = 0.0011$). However, the relationship was better fitted by the quadratic curves shown in Fig. 3 than by straight lines, with the exception of saquinavir, for which there was no significant difference between the models.

Correlation between protease inhibitor susceptibilities. Pairwise protease inhibitor susceptibility analysis showed high correlation values among all pairs of protease inhibitors tested (Table 1). Amprenavir and ritonavir had the highest correlation ($\rho = 0.92$, $P < 0.0001$), while indinavir and lopinavir had the lowest ($\rho = 0.66$, $P < 0.0001$).

Correlation between replication capacity and growth rate during structured treatment interruptions. Plasma viral load doubling times during each structured treatment interruption did not correlate with replication capacity (Fig. 4), and neither did absolute $CD4^+$ or $CD8^+$ cell counts or the $CD4^+/CD8^+$ ratio.

Genotypic predictors of hypersusceptibility. Decision tree models previously built to analyze the genotypic predictors of hypersusceptibility to ritonavir in viral strains isolated from patients with primary infection (15) did not predict hypersusceptibility in the viral isolates used in this study. Individual mutations positively associated with hypersusceptibility to all protease inhibitors tested were R41K and I93L, with the exception of I93L to nelfinavir. Mutations negatively associated with protease inhibitor hypersusceptibility (i.e., the mutation is less prevalent in hypersusceptible samples) were L10V, I13V, and L19P/I for amprenavir; I64L/V for indinavir; and T12P/S/I, I13V, L19P/I, and I64L/V for ritonavir.

DISCUSSION

The present study describes the existence of viral isolates that are hypersusceptible to multiple protease inhibitors and have low replication capacity in patients with chronic HIV-1 infection. The cohort presented here was selected based on preservation of CD4 cells and excellent response to highly active antiretroviral treatment to perform studies of structured treatment interruption (8, 9, 25, 28, 29). The drug susceptibility and replication capacity study was done retrospectively without excluding any patient, and therefore hypersusceptibility to protease inhibitors and low replication capacity were not expected in advance.

Hypersusceptibility to specific protease inhibitors has previously been associated with single mutations that confer resistance to other drugs. In particular, HIV-1 bearing an artificially introduced substitution, V82T, in protease showed increased susceptibility to saquinavir (19), although this has not yet been confirmed in clinical isolates. N88S, a mutation that confers resistance to nelfinavir, has been shown to cause hypersusceptibility to amprenavir in both laboratory viruses and clinical isolates (26, 35). Hypersusceptibility to ritonavir has recently

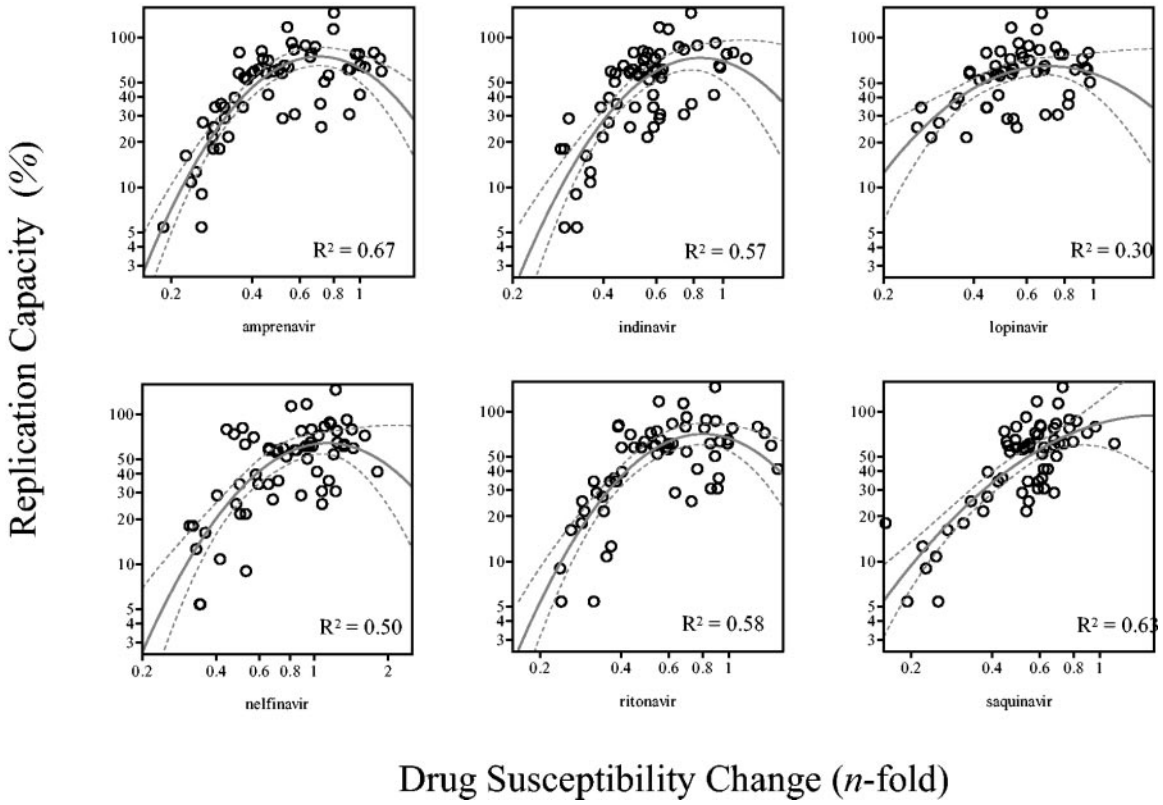

FIG. 3. Relationship between susceptibility to protease inhibitors and replication capacity. A total of 61 determinations were performed for each protease inhibitor except for lopinavir, which had 53. R^2 indicates the percentage of variability of the independent variable explained by the dependent variable.

been described in up to 12% of 182 cases of untreated primary infected patients (15).

Here we extend the observation to protease inhibitor hypersusceptibility in isolates from chronically HIV-1-infected patients who do not show specific protease inhibitor resistance-associated mutations. Interestingly, increased susceptibility in this cohort was common to all protease inhibitors tested, with 10 out of 12 patients having at least one sample being hypersusceptible to at least one protease inhibitor. Therefore, the overall increases in drug susceptibility in the study cohort cannot be assigned to specific patients. Moreover, the median drug susceptibility for the six protease inhibitors tested was 0.2-fold lower than the median drug susceptibility of almost 3,000 wild-type viruses from the ViroLogic database (22), indicating that intrinsic virus characteristics are responsible for the hypersusceptible phenotype.

The hypersusceptibility phenomenon in the present cohort

TABLE 1. Pairwise correlations of susceptibility change[a]

Protease inhibitor comparison	ρ	*P*
Amprenavir vs. indinavir	0.78	<0.0001
Amprenavir vs. lopinavir	0.89	<0.0001
Amprenavir vs. nelfinavir	0.79	<0.0001
Amprenavir vs. ritonavir	0.92	<0.0001
Amprenavir vs. saquinavir	0.82	<0.0001
Indinavir vs. lopinavir	0.66	<0.0001
Indinavir vs. nelfinavir	0.84	<0.0001
Indinavir vs. ritonavir	0.84	<0.0001
Indinavir vs. saquinavir	0.82	<0.0001
Lopinavir vs. nelfinavir	0.67	<0.0001
Lopinavir vs. ritonavir	0.85	<0.0001
Lopinavir vs. saquinavir	0.81	<0.0001
Nelfinavir vs. ritonavir	0.90	<0.0001
Nelfinavir vs. saquinavir	0.78	<0.0001
Ritonavir vs. saquinavir	0.84	<0.0001

[a] A total of 61 determinations were performed for each inhibitor except lopinavir, which had 53.

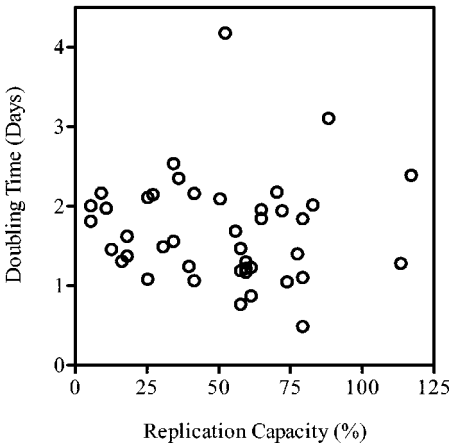

FIG. 4. Correlation between replication capacity and plasma viremia doubling time during therapy discontinuations. The analysis included a total of 61 determinations.

has only been observed for protease inhibitors but not for nucleoside reverse transcriptase inhibitor and non-nucleoside reverse transcriptase inhibitors, suggesting that technical artifacts that lead to such results are unlikely. In addition, the mean replication capacity of recombinant viruses containing the 3′ end of *gag*, the protease, and the reverse transcriptase coding regions of all these isolates was only 53.1% (median, 57.6%), significantly lower than the expected average for wild-type viruses (100%).

The strong correlation found among susceptibility values for all the protease inhibitors tested is striking. The correlation between protease inhibitor susceptibility and replication capacity was also remarkable. The Spearman correlation coefficient for ritonavir was 0.6 ($P < 0.0001$), similar to the 0.5 observed for primary HIV strains (15). Correlation analyses at the peak of viremia for the first, second, and third structured treatment interruption separately retained statistical significance (data not shown). In a large group of clinical samples lacking protease inhibitor resistance mutations, there was a significant correlation between replication capacity and protease inhibitor susceptibility (21). However, about 12% of the samples in that study had either protease inhibitor hypersusceptibility without low replication capacity or did not have protease inhibitor hypersusceptibility in spite of having low replication capacity, indicating that replication capacity might not be the only determinant of protease inhibitor hypersusceptibility (21).

It seems unlikely that the structured treatment interruption had a direct effect on replication capacity, since the replication capacity values did not change significantly over time. In fact, pretreatment replication capacity values, when available, were not different from those at the last structured treatment interruption. This would reflect that hypersusceptibility and low replication capacity in this patient cohort are biological characteristics linked to the patients rather than to the treatment strategy. Of note, based on protease, reverse transcriptase, and *env* genotypes, all viruses were subtype B (data not shown). Eleven patients had R5 tropic viruses, and patient 8 harbored dual tropic viruses (17); patients 5, 6, 8, and 9 were heterozygous for CCR5Δ32 (17). None of these characteristics could be directly associated with hypersusceptibility or low replication capacity. However, it is plausible that the selection of the studied cohort, based on high and sustained $CD4^+$ T-cell counts, among other characteristics (25), might reflect reduced viral pathogenesis, which in turn has been associated with reduced replication capacity (11).

Of note, $CD4^+$ and $CD8^+$ T-cell counts and the CD4/CD8 ratios at the peak of viremia of each structured treatment interruption could not be correlated with the replication capacity values. In turn, the good response to highly active antiretroviral treatment in these patients might be due to the increased drug-susceptible phenotype of the viral variants analyzed. Although the data shown here are restricted to samples from the cohort described, other samples from chronically HIV-infected patients also showed hypersusceptibility to protease inhibitors as well as low replication capacity values in the absence of protease inhibitor-associated mutations. For example, a patient isolate with drug susceptibility change values of <0.35-fold to all protease inhibitors tested and a replication capacity of 2% was recently observed (data not shown).

The genotypic basis of hypersusceptibility to protease inhibitors was explored by using the decision tree models built for ritonavir hypersusceptibility in primary HIV strains (15). However, such models could not predict any combination of amino acids at polymorphic sites or reduced replication capacity. This result might indicate that the genotypic basis of hypersusceptibility to protease inhibitor is different in primary and chronic infection. Alternatively, it could suggest that such genotypic determinants are so variable that they would change among study groups (23). Although our analysis was based on protease alone, the inclusion of the 3′-end *gag* region in the data did not improve the performance of the model in primary HIV strains (15). A recent report suggests that reduced replication capacity in a wild-type population of HIV-1 could be explained in part by mutations in p6 *gag* that occur at specific sites that serve critical roles in binding to cellular proteins required for viral budding (1). Of note, one of the *gag* mutations that were significantly associated with low replication capacity values (position 483, at the LYP motif that binds to AIP1) was also included among the genotypic determinants of hypersusceptibility to protease inhibitors and low replication capacity of HIV-1 strains in primary infection (1, 15).

Since different patients' samples had different in vitro replication capacity values, we intended to correlate them with in vivo growth rates during structured treatment interruptions. However, no correlation was found, most likely because the effects of other genes such as *env* probably dwarf the variation in replication capacity due to *pol*.

The clinical benefit of the hypersusceptibility phenomenon when using protease inhibitor-based therapies has not been investigated thoroughly. One preliminary study reported an association between increased susceptibility to amprenavir and response to amprenavir-containing regimens (30). Two patients of our cohort who were treated with lamivudine, stavudine, and indinavir developed the M184V mutation in reverse transcriptase after the second treatment interruption. Nevertheless, their plasma viremia returned to undetectable levels after restarting the same therapeutic regimen, even when the virus was phenotypically resistant to lamivudine (18). In both cases, the rebounding virus containing the M184V mutation was hypersusceptible to all protease inhibitors tested. We speculate that hypersusceptibility to indinavir could have been responsible for the virologic response in these patients.

This analysis is limited to a subset of viruses derived from a small number of selected subjects. However, virus isolates from the same patients showed consistently enhanced susceptibility to the protease inhibitors tested. Unfortunately, the prevalence and the identification of the viral characteristics linked to hypersusceptibility to protease inhibitors in patient-derived viruses are not straightforward. In contrast to hypersusceptibility to non-nucleoside reverse transcriptase inhibitors, hypersusceptibility to multiple protease inhibitors is not necessarily associated with prior antiretroviral treatment.

In summary, hypersusceptibility to multiple protease inhibitors as well as low replication capacity can be found in recombinant viruses derived from chronically infected patients. However, the genetic basis of these phenomena remains elusive. A treatment strategy based on repetitive controlled treatment interruptions did not change the phenotype and replication capacity values and did not correlate with virus growth rates

after treatment discontinuation. The underlying mechanism of protease inhibitor hypersusceptibility is unknown. It has been proposed that variation in protease function is directly responsible for variations in fitness among strains in primary HIV infection (15). However, the impact of protease inhibitor hypersusceptibility on virologic response to protease inhibitor-based therapies remains to be investigated.

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