Ordered accumulation of mutations in HIV protease confers resistance to ritonavir

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Analysis of the HIV protease gene from the plasma of HIV-infected patients revealed substitutions at nine different codons selected in response to monotherapy with the protease inhibitor ritonavir. Mutants at valine-82, although insufficient to confer resistance, appeared first in most patients. Significant phenotypic resistance required multiple mutations in HIV protease, which emerged subsequently in an ordered, stepwise fashion. The appearance of resistance mutations was delayed in patients with higher plasma levels of ritonavir. Early mutants retained susceptibility to structurally diverse protease inhibitors, suggesting that dual protease inhibitor therapy might increase the duration of viral suppression.

Until recently, chemotherapies for treatment of the acquired immunodeficiency syndrome (AIDS) used inhibitors of the human immunodeficiency virus (HIV) reverse transcriptase. The clinical benefit of these agents is modest and short-lived because of drug-related toxicity and the rapid emergence of resistant strains of HIV (ref. 1). The clinical effects of inhibitors of another viral enzyme, HIV protease, are actively being investigated, and three of these agents have been recently licensed for clinical use. Ritonavir (ABT-538) is a potent and selective inhibitor of HIV protease with high oral bioavailability and sustained plasma concentrations in humans[2]. Phase I/II clinical trials with ritonavir monotherapy demonstrated a rapid decline in mean HIV plasma RNA to ~1% of baseline levels with a concomitant increase in circulating CD4 cells[3–6]. However, over time and as an inverse function of drug exposure, virological responses to treatment waned, and after 32 weeks of treatment, only patients receiving the highest dose of ritonavir (600 mg twice a day (b.i.d.)) maintained a mean plasma HIV RNA reduction >0.8 log and a >100 median CD4 cell increase[3]. Phase III studies using that dose demonstrated marked delay in the progression of HIV-related disease and prolonged survival in patients with advanced AIDS (ref. 7). In order to understand the basis for the rebound in viral RNA observed at suboptimal doses of ritonavir, we have genotypically examined the HIV protease sequences from 48 patients representing eight different phase II dose groups. Here we report that the gradual loss of antiviral activity observed during monotherapy with ritonavir is linked to the stepwise, ordered accumulation of mutations in the protease target. Viral variants cross-resistant to a battery of protease inhibitors selected by monotherapy with another protease inhibitor have been described[8]. However, neither the nature of the selection pathway leading to those mutants nor their temporal relationship to loss of viral suppression was examined. We have found that decreasing susceptibility to ritonavir requires the stepwise accumulation of multiple mutations in HIV protease. Furthermore, we report that the rate at which resistance mutations appear is inversely related to the plasma levels of ritonavir. These results suggest that effective suppression of the replication of the early variants in the resistance pathway offers the best strategy for prolonging antiviral efficacy *in vivo*. Phenotypic analysis has revealed that viral strains of intermediate susceptibility to ritonavir retain sensitivity to structurally diverse protease inhibitors. The duration of viral suppression by ritonavir might therefore be extended by combination with other protease inhibitors with distinct and similarly well-characterized resistance pathways.

Genotypic analysis of HIV selected by ritonavir *in vivo*

In order to understand the basis of diminished virological and immunological responses to ritonavir, we selected a subset of 41 patients who had experienced either an incomplete suppression of plasma RNA or a rebound from maximal suppression as measured by the branched DNA assay[9] (limit of detection 10^4 copies per milliliter). In addition, we selected a subset of seven patients in whom maximal viral suppression continued through the sampling period. The HIV protease gene in 196 samples from these patients (2–9 sequences per patient) before and during ritonavir therapy was examined by population sequencing of the polymerase chain reaction (PCR) product derived from plasma viral RNA. To delineate the subset of mutations selected by exposure

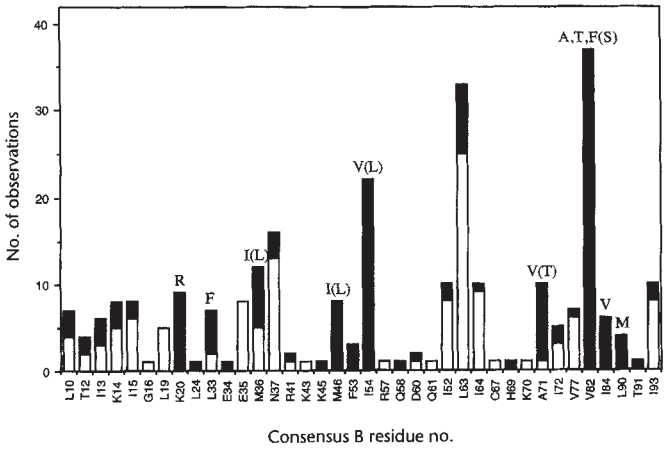

Fig. 1 Variation of the HIV protease sequence in patients exposed to ritonavir. The baseline and final sequences from 42 patients were compared with the consensus B HIV protease sequence[10]. *white bars*, Variations from the consensus B sequence at a given amino acid position that appeared in the baseline samples. *black bars*, Variations from the consensus B sequence that appeared during ritonavir therapy but were not present in the baseline sequence from that patient. The identity of the predominant mutations are shown at the top of the bar as single-letter amino acid symbols (minor variants shown in parentheses).

to ritonavir, the pretreatment and last on-treatment sequences from 42 patients were examined for variation relative to the consensus B sequence[10] (Fig. 1). Pretreatment sequences for the remaining six patients were unavailable. The following variations were observed predominantly (>50% of the total number of variations) or exclusively after treatment with ritonavir: K20R, L33F, M36I/L, M46I/L, I54V, A71V/T, V82A/F/T/S, I84V and L90M. Mutations at these nine consensus sites were judged to be the result of drug selection (see Methods section). In contrast, variations from the consensus B sequence that existed in >50% of patients at baseline or that reverted to the consensus B sequence in a significant number of patients (for example, residue 63) were not attributed to drug exposure. The sequences of all of the 41 patients who experienced either viral rebound or less than maximal suppression contained one or more consensus mutations. Only wild type sequences were observed at the above nine positions in 5 out of 7 patients with continued maximal suppression. The remaining 2 patients with $<10^4$ copies per ml of plasma viral RNA displayed a V82A or I84V mutation, respectively, in the final sequence. Subsequently, those patients discontinued therapy so that RNA data were not available to determine whether diminution of viral suppression was about to occur.

Although the variation in virtually every patient differed in detail, we observed a consistent pattern wherein multiple mutations accumulated in an ordered fashion. The frequency of mutations at the above nine positions in 81 unique sequences representing 43 patients is catalogued in Table 1. Mutation at residue 82 was highly associated with the initial loss of antiviral effect: no rise in plasma RNA following initial suppression was observed without variation at 82 as either a single mutation or part of multiple mutation pattern. This pathway was not predicted by *in vitro* passage with ritonavir, in which mutation at position 84 was noted before mutation at residue 82 (ref. 11). The initial mutation at position 82 represents a single base change from GTC (V) to either GCC (A) or TTC (F). Further mutation to 82T (ACC) and 82S (TCC), which requires a double point mutation from wild type, was not observed before detection of 82A or 82F. Following or in concert with mutation at residue 82, the following variations were observed most frequently: I54V>L (41/81 sequences, 25 out of 43 patients), A71V>T (26 sequences, 14 patients) and M36I>L (26 sequences, 13 patients). Mutations at the remainder of the above nine positions occurred with lower frequency and, in most samples, were found with at least two of the primary mutations above. The most common double and triple mutations were at positions 82/54 (7 out of 17 sequences) and 82/54/71 (6 out of 17 sequences), respectively. Of the 26 sequences with four or more mutations, all varied at position 82, only two lacked variation at residue 54, and all but three varied at either residue 71 or 36. Each of the five last-mentioned sequences contained I84V and/or M46I mutations, but not K20R. Notably, the L90M mutation[12] occurred the least frequently (4 out of 43 patients) and was always accompanied by at least two additional mutations. Taken together, these results identify an ordered pattern of mutations that emerge in response to ritonavir therapy, beginning with the outgrowth of single mutants at position 82.

To analyze further the apparent stepwise appearance of mutations, we examined multiple sequences from individual patients. Patient 124, who was treated with a less than maximally effective

Table 1 Analysis of plasma HIV protease sequences during ritonavir treatment

No. of mutations	No. of sequences	V82A/T/F	I54V	A71V/T	M36I/L	I84V	K20R	M46I/L/V	L33F	L90M
1	21	20	0	1	0	0	0	0	0	0
2	17	16	7	3	4	2	0	2	0	0
3	17	17	10	7	7	1	2	3	2	2
4	15	15	13	6	7	6	4	4	3	2
5	9	9	9	8	6	3	5	3	1	1
6	2	2	2	1	2	2	2	1	0	0
Total	81	79	41	26	26	14	13	13	6	5
No. of patients*	43	42	25	14	13	7	9	11	5	4

Sequences from each patient were analyzed for the presence of consensus mutations selected by ritonavir, as defined in Fig. 1. Sites showing a mixture of the consensus amino acid and mutant amino acid were recorded as mutants. Multiple occurrences of the same set of mutations in individual patients were recorded only in the first instance. Single mutations that were observed both during and before the initiation of ritonavir therapy were not recorded.
*Total number of patients represented for each sequence.

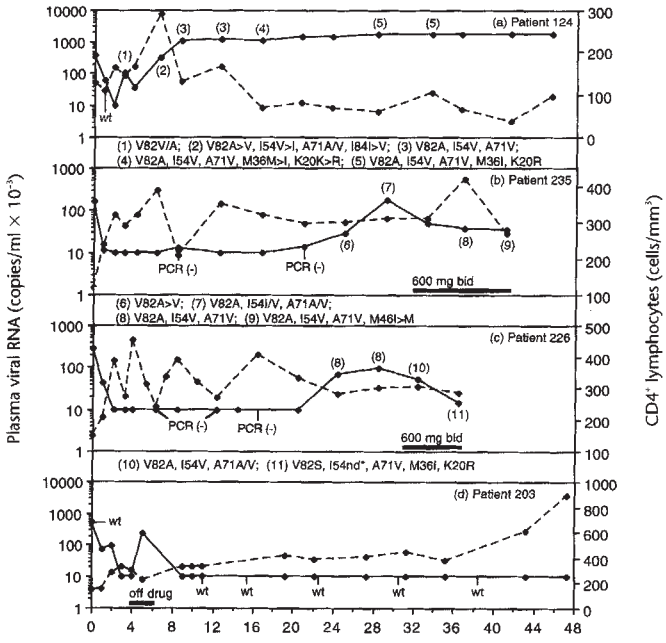

Fig. 2 Correlation of virological and immunological response to mutation patterns for individual patients. *solid lines*, Plasma RNA (measured using the branched DNA assay), *dashed lines*, CD4 levels. HIV protease sequences that varied from the pretreatment, baseline sequence at the consensus residues defined in Fig. 1 are listed: (a) patient 124 (400 mg b.i.d.), (b) patient 235 (500 mg b.i.d.), (c) patient 226 (500 mg b.i.d.), (d) patient 203 (600 mg b.i.d.). Wild-type sequences were defined as the absence of consensus resistance mutations that did not preexist in the baseline sequence. Samples labeled PCR negative refer to the sequencing procedure, not to the plasma RNA quantification protocol. The dose of ritonavir in patients 235 and 226 was escalated to 600 mg b.i.d. where indicated. *Amino acid identity at position 54 of this sequence was not determined.

dose of ritonavir (400 mg b.i.d.), experienced a rebound in plasma viral RNA as early as week 3, concomitant with the appearance of V82A (Fig. 2*a*). By week 8.5, the V82A, I54V, A71V triple mutant was the predominant viral species. Although the plasma viral load had rebounded beyond pretreatment levels, two additional mutations (M36I and K20R) appeared. The emergence of variants from patients 235 and 226, who received a higher dose of ritonavir (500 mg b.i.d.), was substantially delayed (Fig. 2, *b* and *c*). Following the eventual rebound in plasma HIV RNA, the dose was elevated to 600 mg b.i.d., and additional mutations beyond the intermediate V82A, I54V, A71V triple mutants emerged in both patients. In patient 235, alanine remained the predominant residue at position 82, whereas further mutation to serine was observed in patient 226. In each of the patients who experienced a loss of antiviral effect, the rebound from maximal suppression of plasma viral RNA was associated with the appearance of resistance mutations. By contrast, in patient 203 (600 mg b.i.d.), none of the above nine mutations appeared over 39 weeks of therapy, the latest point at which sequence information was obtained (Fig. 2*d*). After 47 weeks, plasma viral load was reduced from 530,000 to below the 10^4 HIV-RNA copies per milliliter detection limit[3], and CD4 cells increased from 150 to 890 cells per mm^3.

Phenotypic analysis of HIV selected by ritonavir *in vivo*

To elucidate the effect of the above mutations on the susceptibility of HIV to ritonavir, we phenotypically characterized HIV isolated from patients receiving ritonavir therapy using peripheral blood mononuclear cell (PBMC) cultures (Table 2). In analogy to previous work with indinavir[8], the loss of sensitivity to ritonavir generally correlated with the number of consensus mutations in the protease sequence. Thus, cultures containing predominantly single V82 mutations, representing initial time points following rebound of viral RNA from maximal suppression, exhibited only a small decrease in sensitivity. Significant (7- to 10-fold) resistance was observed with isolates from patients 129, 131 and 235, containing three to four mutations. Finally, isolates from patients with prolonged suboptimal exposure to ri-

Table 2 Susceptibility of patient isolates to a panel of HIV protease inhibitors

			EC_{50} (nM) (fold over pretreatment)				
Patient no.	Day	Resistance mutations in sequence	Ritonavir	Saquinavir	Indinavir	VX-478	Nelfinavir
104	21	82A	46 (2)	1 (1)	44 (1)	30 (1)	47 (2)
104	28	82T	67 (3)	2 (1)	41 (1)	30 (1)	57 (3)
224	110	33L/F, 82T/S	79 (3)	3 (1)	31 (4)	ND	ND
224	190	20K/N/R, 33L/F, 36M/I, 54V/M, 82S	496 (17)	7 (1)	33 (4)	ND	ND
313	57	(36M/I), 82F*	64 (5)	1 (1)	6 (1)	12 (1)	23 (6)
313	85	36M/I, 54V/I, 82A/F/T/S	274 (23)	4 (2)	69 (8)	27 (3)	15 (4)
131	88	36I, 54V/I, 82V/A	163 (9)	5 (1)	31 (4)	3 (1)	67 (10)
131	200	20K/R, 36I, 54V/I, 82A	731 (41)	11 (2)	34 (4)	4 (2)	86 (12)
129	84	36I, 54V, 71V, 82T	203 (8)	3 (3)	55 (4)	7 (2)	34 (8)
129	140	20K/R, 36M/I, 54V, 71V/A, 82T	677 (28)	1 (1)	117 (8)	12 (4)	55 (14)
235	276	54V/I, 71V, 82A, 90L/M	66 (7)	7 (2)	48 (3)	8 (1)	24 (2)

Variation only at the consensus residues identified in Fig. 1 is shown. Fold loss of sensitivity was calculated by comparison to the susceptibility of baseline (pretreatment) isolate for the same patient (data not shown). Median effective concentration (EC_{50} values) represent either single or the average of two determinations except for those of ritonavir, which represent the average of 2–11 determinations.

*This sample was sequenced both by population and clonal analysis. Population analysis revealed a mixed population at position 36; 5 out of 5 clones showed only the 82F-resistant mutation.

tonavir (for example, from patients 224, 313, 131 and 129) contained four to five mutations and displayed a high-level (≥20-fold) resistance. To further understand the effect of individual mutations on the phenotypic susceptibility of HIV to ritonavir, we constructed HIV-1 molecular clones containing one or more of the above consensus mutations. The activity of ritonavir against each mutant clone was evaluated in MT4 cells using cytopathic effect as an end point[13] (Table 3). The median effective concentration (EC_{50} values) for clones containing single V82A, T or F or I84V mutations did not differ significantly from the pNL4-3 parent strain. In contrast, the V82S single mutant and V82T, I54V double mutant, which occur later during therapy, displayed 6- and 10-fold loss of drug sensitivity, respectively. Clones reconstructed from patient 129 containing multiple mutations (E35D, M36I, I54V, A71V, V82T and K20R, E35D, M36I, I54V, A71V, V82T, respectively) were markedly resistant to ritonavir in both MT4 cells and PBMC cultures (Table 3). In analogy to previous observations[8], however, clones with multiple consensus mutations that lacked variation at amino acid 82 did not display high-level resistance (data not shown). Taken together, these results indicate that the early mutants selected by ritonavir retain considerable sensitivity to the drug, and that selection of additional mutations confers increasing levels of resistance.

Correlation of selection rate with plasma levels of ritonavir

To more fully understand the factors influencing the development of resistance to ritonavir, we measured the *in vivo* selection rate of individual patients using a retrospective analysis. We selected the 13 patients for whom ≥4 viral sequences, including baseline, and day 21 pharmacokinetic profiles were available. The number of consensus resistance mutations (from the nine above) within those sequences was plotted versus time after initiation of therapy to provide an estimate of the *in vivo* selection rate. No relationship was observed between the selection rate and any of the following: baseline plasma viral RNA, baseline CD4 or maximum CD4. However, an inverse correlation between *in vivo* selection rate and trough plasma concentrations (C_{min}) was clearly evident (Fig. 3, $R^2 = 0.58$) A similar relationship was observed with total drug exposure (area under curve (AUC),

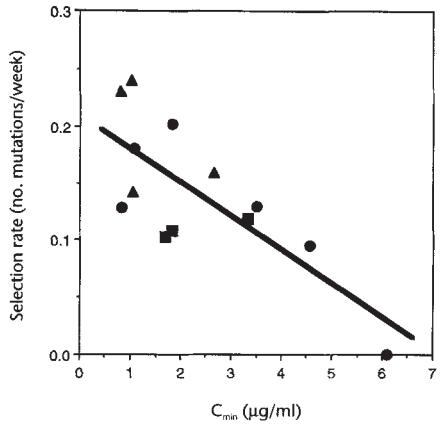

Fig. 3 Relationship of *in vivo* selection rate and minimum ritonavir concentration. Each dot represents one patient. ▲, 400 mg b.i.d.; ■, 500 mg b.i.d.; ●, 600 mg b.i.d.

$R^2 = 0.51$, data not shown). The selection rate correlated less well with dose. For example, several patients receiving ritonavir at 600 mg b.i.d. displayed lower C_{min}, concomitant with higher selection rate, than the three patients receiving a 500 mg b.i.d. dose. The patient with the highest C_{min} (patient 203, Fig. 2*d*) did not select any of the consensus resistance mutations, and experienced continued viral suppression. These results indicate that the maintenance of high drug levels may be critical for impeding or preventing the development of resistance to ritonavir.

Cross-resistance of HIV-selected by ritonavir *in vivo*

In order to assess the cross-resistance of mutant HIV selected by ritonavir, we examined the sensitivity of the patient isolates to the protease inhibitors saquinavir (Ro 31-8959)[12], indinavir (MK-639)[8], VX-478 (ref. 14), and nelfinavir (AG 1343)[15] (Table 2). In each case resistance to ritonavir was more significant than that to any of the other inhibitors. Although precise, quantitative characterization was confounded by the heterogeneous nature of the isolates, those containing only the initially selected mutations retained sensitivity similar to (less than or equal to one-fourth) the corresponding baseline (pretreatment) isolate. Apparent cross-resistance to indinavir and nelfinavir was observed with some of the isolates containing multiple mutations. In contrast, no significant cross-resistance to either saquinavir or VX-478 was noted. A similar trend was observed with the mutant viral clones (Table 3). The V82T, I54V double mutant and the highly ritonavir-resistant multiple mutants displayed cross-resistance to both indinavir and nelfinavir, but retained wild-type sensitivity to saquinavir and VX-478. Clones containing G48V or L90M mutations, selected by saquinavir[12], exhibited wild-type susceptibility to ritonavir. The low degree of

Table 3 Susceptibility of molecular clones to a panel of HIV protease inhibitors

	EC_{50} (nM) (fold resistance)				
Virus clone	Ritonavir	Saquinavir	Indinavir	VX-478	Nelfinavir
wt (pNL4-3)	71	16	53	78	26
V82A	140	10	61	56	30
V82F	57	10	54	48	17
V82T	86	5	55	40	13
V82S	410 (6)	15	82	ND	ND
I84V	140	29	41	ND	ND
V82T, I54V	613 (9)	20	241 (5)	191	80 (3)
G48V	110	43 (3)	91	ND	ND
L90M	93	28	64	ND	ND
wt (HXB2)	68	11	32	68	43
E35D, M36I, I54V, A71V, V82T	1170 (17)	25	250 (8)	43	150 (3)
K20R, E35D, M36I, I54V, A71V, V82T	1410 (21)	17	190 (6)	40	180 (4)
K20R, E35D, M36I, I54V, A71V, V82T*	350 (32)	ND	ND	ND	ND

*Assay in PBMCs using p24 antigen as end point; EC_{50} for wild-type (HXB2) virus was 11 nM. Abbreviations: wt, wild-type; ND, not determined.

cross-resistance to saquinavir and VX-478 is consistent with the distinct mutation patterns following *in vitro* selection[11,12,14].

Discussion

HIV protease inhibitors are promising new agents for the chemotherapy of AIDS. In contrast to previous regimens of reverse transcriptase inhibitors, therapies using a protease inhibitor can produce a profound and durable suppression of plasma viral RNA accompanied by substantial elevation of CD4 cells[3,4,16–18]. Informed clinical use of protease inhibitors requires elucidation of the determinants of resistance development *in vivo*. Understanding the patterns of mutations selected by unique agents may facilitate the rational choice of protease inhibitor combination regimens. Furthermore, information regarding the degree of susceptibility of one inhibitor to mutant HIV selected by another inhibitor may be important for guiding treatment decisions for protease inhibitor experienced patients. The *in vivo* selection of variants with reduced susceptibility to the protease inhibitors saquinavir[12] and indinavir[8] have recently been reported. We present here the results of our studies on the *in vivo* genotypic and phenotypic changes in HIV following monotherapy with ritonavir.

In patient plasma, HIV undergoes rapid turnover[5,6,19]. To analyze the dynamic response of the HIV quasispecies to ritonavir treatment, we therefore used population sequencing of the HIV protease gene amplified from plasma virus. We identified mutations at nine positions within HIV protease selected as a result of suboptimal exposure to ritonavir. Initial variation at position 82 was consistently observed, regardless of time on therapy, followed most frequently by the ordered accumulation of mutations at positions 54, 71 and 36, respectively. We observed multiple mutations only in HIV from patients who experienced either a lack of complete suppression of plasma viral RNA or a rebound from undetectable levels. Furthermore, those viral strains with a sufficient number of amino acid changes to confer high-level resistance were uniformly preceded by strains containing only a subset of those changes. The initial outgrowth of V82 single mutants suggests that the incompletely suppressed replication of those preexisting[20], marginally resistant strains provides the primary mechanism for further selection. The set of common mutations present in all of the resistant strains examined, which were collected from diverse patient populations, is highly unlikely to occur in the absence of selective pressure. Each amino acid substitution occurring as a result of a single nucleotide change will be present in approximately $1/10^4$ particles[20]. Mutants that contain simultaneously four or more of these mutations therefore occur spontaneously in fewer than 1 out of 10^{16} particles, a number well in excess of the total particles estimated over the lifetime production of virus in infected individuals[5,19]. Our results argue against the outgrowth of viral strains harboring multiple resistance mutations that exist before initiation of therapy with ritonavir. Instead, it is likely that such highly resistant variants are the result of *de novo* generation and selection through the ongoing replication of incompletely suppressed HIV.

Our analysis identifies those amino acid residues in HIV protease that emerge in response to ritonavir therapy. As the viral population represents the set of most replication-competent species under the pressure of a given drug exposure, individual mutations may contribute to viral fitness either by directly reducing the affinity of the active site for the inhibitor and/or by enhancing the growth kinetics of the viral quasispecies[8,21,22]. Of the nine residues judged to contribute significantly to resistance to ritonavir, only V82 and I84 interact directly with the inhibitor in the active site[2,11]. The pivotal role of variation at V82 both as the initial mutation *in vivo* and the basis for diminution of sensitivity by multiple mutants is presumably a consequence of this direct interaction. The mechanism by which subsequent mutations augment resistance remains undefined. The L63P mutation has been reported to enhance the replication kinetics of V82F, I84V double and L10R, M46I, V82T, I84V quadruple mutants[8,11]. Although variation at position 63 occurred during ritonavir therapy, >50% heterogeneity at that position was present at baseline and reversion to 63L was also observed in four patients. Thus, the subtle role that mutations at highly variable positions such as L63 may play in conferring greater fitness to the HIV population in individual patients was not discernible through our analysis of this cohort.

We analyzed both virus isolated from patients and mutant molecular clones to examine the relationship between genotypic selection and phenotypic susceptibility. Qualitatively, we observed a correlation between the number of consensus mutations and the degree of resistance to ritonavir. A similar relationship has been noted in studies on indinavir resistance[8]. Previous *in vitro* studies using p24 production as an end point have shown the activity of ritonavir toward single V82F and I84V clones to be one-fourth and one-eighth the activity directed toward the corresponding wild-type clone[11]. Similarly, a 3.4-fold difference between wild-type HIV and a single L90M mutant in susceptibility toward saquinavir has been reported[23]. We used the cytopathic effect of HIV in MT4 cells to examine the susceptibility of mutant clones. In that assay, only small differences between the EC_{50} values of single mutants and wild-type HIV were observed. A similar absence of change in susceptibility beyond experimental error was noted with single mutant clones by Condra *et al.*[8]. Because the outgrowth of viral variants *in vivo* represents a combination of drug susceptibility and growth kinetics, a true estimate of the fitness of mutant HIV strains in the presence of inhibitors must await *in vitro* assays that adequately address both variables simultaneously. Our studies using both patient isolates and molecular clones suggest that the initial mutants selected by ritonavir are highly susceptible to structurally diverse protease inhibitors. However, the incidence of cross-resistance to indinavir and nelfinavir by HIV selected in response to prolonged ritonavir therapy may be significantly higher than to saquinavir or VX-478. This observation is consistent with the mutation patterns selected by each agent *in vitro*[11,14,15,23] and/or *in vivo*[8,12] and may be useful in developing strategies for dual protease inhibitor therapies.

The inverse relationship between the rate of resistance development *in vivo* and the exposure of patients to ritonavir offers insight into the optimal use of HIV protease inhibitors in therapy. A related study has shown high doses of saquinavir to be qualitatively associated with a lower incidence in the appearance of mutations[16]. Our results indicate that the constant maintenance of plasma levels that are suppressive of preexisting, marginally resistant mutant strains is critical for blocking the incremental emergence of more highly resistant multiple mutants. Intermittent dosing, poor patient compliance or pharmacokinetic variation between individuals may profoundly affect the rate of resistance development and the duration of response. Furthermore, resistance generated on suboptimal regimens may compromise the effect of higher drug doses. In patients 235 and 226 (Fig. 2, *b* and *c*), elevation of the ritonavir dose to 600 mg

b.i.d. after the appearance of multiple variants in the viral population was insufficient to completely block further replication or the selection of additional mutations. In contrast, exposure to high plasma concentrations of ritonavir early in therapy can block the emergence of resistant mutants in some patients (for example, patient 203). Therapeutic regimens should therefore be designed to rapidly achieve and maintain highly suppressive plasma concentrations.

Our results show that the early variants on the resistance pathway display substantial sensitivity to ritonavir. Higher plasma concentrations are associated with a more durable antiviral response and with a delay in the appearance of resistance mutations. Accordingly, combinations of potent HIV protease inhibitors that lack cross-resistance to the initial variants on the resistance pathway should prevent or significantly delay the onset of high-level resistance. The initial mutants selected *in vivo* by ritonavir and saquinavir[12] are distinct. Early variants selected by one agent retain sensitivity to the other inhibitor. Thus, reciprocal blockade of the early steps on the resistance pathway with ritonavir-saquinavir cotherapy may extend the duration of maximum antiviral activity for both agents. The *in vivo* antiviral activity of saquinavir appears to be compromised by poor oral pharmacokinetics[24]. However, plasma levels of saquinavir are significantly enhanced by coadministration with ritonavir (D.J.K., unpublished results). Exploration of the benefit of combination therapy with ritonavir/saquinavir is therefore under way.

Methods

Patient selection. Patients were retrospectively selected from two double-blind, randomized, placebo-controlled phase I/II studies of ritonavir monotherapy[3,4]. The distribution of patients from study M93-112 and its extension phase M94-169 (ref. 3) was as follows: 300 mg b.i.d. (five patients), 400 mg b.i.d. (eight patients), 500 mg b.i.d. (eight patients), 600 mg b.i.d. (seven patients). The distribution from study M93-134 (ref. 4) was as follows: 200 mg t.i.d. (eight patients), 300 mg t.i.d. (eight patients), 200 mg q.i.d. (three patients), 300 mg q.i.d. (one patient). Patients were specifically selected to represent all of the dose groups of both studies and a broad range of responses to therapy, including rapid, moderate or delayed loss of suppression of viral RNA as well as continued maximal suppression. Sequences (two to nine sequences per patient) were selected in order to represent a broad range of time points on therapy. Baseline sequences were obtained either before initiation of therapy or on day one of therapy.

Genotypic analysis of patient sequences. A total of 196 plasma samples from 48 HIV-infected individuals undergoing treatment with ritonavir were sequenced by ProGen Lab AB (Uppsala, Sweden). Viral RNA was extracted from patient samples and amplified, first by reverse transcriptase-PCR (RT-PCR) using an HIV-1 specific primer, and then nested PCR using one 5'-biotinylated primer. After strand separation using Dynal paramagnetic beads, population sequencing was performed using the Pharmacia Autoread Sequencing reactions kit followed by electrophoresis with the Pharmacia A.L.F. Autosequencer. Variation of HIV protease codons 1–99 was analyzed relative to the HIV-1 consensus B sequence[10]. Mutations at individual positions were judged to be selected in response to ritonavir therapy based on the following criteria: (1) variation during treatment accounted for >50% of the total number of variations from the consensus B sequence, (2) variation appeared with ≥10% incidence on treatment, and (3) reversion from a preexisting variant to the consensus B sequence was not observed.

Determination of *in vivo* selection rate. Thirteen patients from Study M93-112 were selected based on the following criteria: (1) ≥4 available plasma sequences, including baseline, spanning a minimum of 8 weeks of treatment in which drug was administered at least 80% of the time period; (2) available ritonavir exposure data after 3 weeks of ritonavir treatment. The exposure to ritonavir was calculated as the 12-h (one dose interval) area under the plasma ritonavir concentration-time profile with linear trapezoidal rules. For each patient, the appearance in the population of consensus resistance mutations, as defined in Fig. 1, that did not preexist in the baseline sequence was plotted versus the time from initiation of treatment. Individual slope values representing the *in vivo* selection rate were calculated using linear best-fit analysis.

Phenotypic analysis of patient HIV. Protease inhibitors were synthesized according to literature methods. Saquinavir was obtained from Roche Laboratories. Viruses were isolated by cocultivation of patient PBMCs with 72-h PHA-stimulated PBMCs from healthy donors. Drug susceptibility assays were performed on both pretreatment and on-treatment isolates according to the AIDS Clinical Trials Group (ACTG) consensus protocol[25] using serial twofold dilutions of the test compounds. Inhibition of p24 antigen production was monitored by ELISA and expressed as percent of control (no inhibitor) antigen for that isolate. Total genomic DNA from infected PBMCs was amplified by PCR using primers for HIV protease and sequenced directly.

Analysis of mutant HIV molecular clones. Single mutant viruses were constructed at specific sites within the protease coding region by PCR using mismatched pairs of primers. Proviral clones were constructed by subcloning of the 599-bp *ApaI–BsmBI* fragment containing the mutated protease gene into a cassette shuttle vector (pNL4-3). For the construction of multiple mutant clones, RNA was isolated from patient plasma samples, amplified by RT-PCR using primers for HIV protease, cloned into a TA vector and sequenced. The mutant protease gene fragments thus obtained were cotransfected with a HXB2 protease deletion clone[26]. After 48 h the supernatants were assayed for p24 expression, and where positive, used to infect MT4 cells. The complete sequence of the protease gene from the mutant viral population was confirmed by DNA sequencing. For drug susceptibility assays, viral supernatants were propagated, titered and utilized in MT4 cells according to the method of Pauwels *et al.*[13]. Alternately, drug susceptibility in PBMC cultures was evaluated as described above. Differences in sensitivity ≥3-fold were judged to be significant.

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