MAJOR ARTICLE

Failure of Treatment with First-Line Lopinavir Boosted with Ritonavir Can Be Explained by Novel Resistance Pathways with Protease Mutation 76V

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Background. Virological failure of first-line antiretroviral therapy based on lopinavir boosted with ritonavir (lopinavir/r) has rarely been associated with resistance in protease. We identified a new genotypic resistance pathway in 3 patients who experienced failure of first-line lopinavir/r treatment.

Methods. Viral protease and the C-term part of Gag were sequenced. The observed mutations were introduced in a reference strain to investigate impact on protease inhibitor susceptibility and replication capacity.

Results. A detailed longitudinal analysis demonstrated the selection of the M46I+L76V protease mutations in all 3 patients. The L76V conferred a solitary 3.5-fold increase in one-half the maximal inhibitory concentration to lopinavir but severely hampered viral replication. Addition of M46I, which did not confer any lopinavir resistance on its own, had a dual effect. It partly compensated for the loss in replication capacity and increased the one-half maximal inhibitory concentration to above the lower clinical cutoff (11-fold). Analysis of a large clinical database (>180,000 human immunodeficiency virus [HIV] sequences) demonstrated a significant association (Spearman ρ, 0.93) between the increased presence of L76V in clinical samples (0.5% in 2000 to 3.4% in 2006) and lopinavir prescription over time.

Conclusions. The HIV protease substitution L76V, in combination with M46I, confers clinically relevant levels of lopinavir resistance and represents a novel resistance pathway to first-line lopinavir/r therapy.

Introduction of protease inhibitors (PIs) has proven to be a powerful concept in the treatment of human immunodeficiency virus (HIV) infection. The viral protease is required for cleavage of the viral precursor Gag and GagPol polyproteins, resulting in mature infectious viruses. Development of PI resistance occurs frequently and usually represents a step-wise process [1, 2]. Initially, mutations in the substrate-binding pocket of the protease are selected, leading to reduced binding of the PI (i.e., resistance) and the natural Gag and GagPol substrate, which affects viral replication [3–6]. To compensate for loss of replication capacity (RC), additional mutations in the viral protease that improve processing efficiency of resistant protease are selected [3, 6, 7]. Furthermore, compensatory changes have been iden-

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tified in the Gag substrate of the viral protease [7–10]. These changes were thought to adapt the virus to the drug resistant viral protease, and were considered to be compensatory changes that do not directly affect PI resistance. However, we have demonstrated that Gag cleavage site (CS) changes are able to reduce PI susceptibility and represent an alternative PI resistance mechanism [11].

Two strategies have been developed to prevent development of resistance in PI-naive patients. First, novel PIs with high potency to known single- and double-resistant mutants have been developed. Second, levels of PIs in the plasma of patients have been increased by boosting with ritonavir, a cytochrome P4503A4 enzyme inhibitor [12]. Consequently, high levels of PI resistance are needed to achieve virological failure. For lopinavir boosted with ritonavir (lopinavir/r), the lower clinical cutoff is 9–10-fold [13]. In general, to achieve such levels of resistance, >2 protease resistance mutations are required, resulting in a high genetic barrier to resistance development.

In a clinical study of lopinavir/r salvage therapy, baseline isolates harbored multiple protease mutations and demonstrated 5–6-fold lopinavir resistance. After subsequent initiation of lopinavir/r salvage therapy, additional protease substitutions were acquired [14]. Apparently, accumulation of protease mutations during prior PI therapy compromised the genetic barrier to lopinavir resistance.

This observation is in sharp contrast with absence of selection of lopinavir resistance, as observed in antiretroviral therapy–naive patients. Two large studies involving >750 patients who initiated lopinavir/r therapy in combination with 2 nucleoside reverse-transcriptase inhibitors demonstrated that infrequently observed cases of virological failure could not be attributed to resistance in protease [15, 16]. Only 2 cases of primary lopinavir resistance have been published. One case report describes an HIV-1 subtype C–infected patient treated with lopinavir/r and 2 nucleoside reverse-transcriptase inhibitors [17]. At baseline, this patient harbored a virus with the protease polymorphism M36I, and during lopinavir/r therapy, substitutions at codons L33F, I54V, and V82A were acquired. The baseline polymorphism M361 is associated with PI resistance [18] and may have enabled accumulation of additional substitutions. A second case report describes a patient who was treated with several reverse-transcriptase inhibitors. One was switched to lopinavir/r; subsequently, all reverse-transcriptase inhibitor therapy was stopped, and the patient continued to receive lopinavir/r monotherapy [19]. Resistance analysis demonstrated acquisition of protease substitutions V32I, M46M/I, and I47A and high-level lopinavir resistance. Analysis of genotypic and phenotypic resistance profiles of clinical samples in a large database demonstrated a low prevalence (0.03%) of the I47A substitution. This substitution was always associated with at least 2 other protease mutations and high levels of lopinavir resistance [19]. The publication of just 2 case reports indicates that generation of primary resistance to lopinavir/r in vivo is difficult but not impossible.

In the present study, we describe 3 antiretroviral therapy–naive patients who experienced failure of first-line lopinavir/r therapy. Remarkably, genotypic resistance analysis demonstrated selection of the M46I and L76V protease substitutions in all 3 patients. We investigated the effect of these mutations on resistance and RC and report a novel lopinavir resistance pathway.

MATERIAL AND METHODS

Plasma Viral RNA Analysis

Viral RNA was isolated from 200–1500 μL of plasma [20]. Subsequently, the isolated viral RNA was used to reverse transcribe and amplify viral protease and the C-terminus of Gag (including the p2/NC, NC/p1, and p1/p6 CS) [21, 22]. The polymerase chain reaction (PCR) product was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems International) and ViroSeq V2 HIV-1 sequencing kit (Abbott) [22]. Furthermore, the isolated viral RNA was used to reverse transcribe, amplify, and sequence the N-terminus of reverse transcriptase [21, 22].

Generation of Site-Directed Mutants

A set of site-directed mutants (SDMs) containing $\geqslant$1 of the following mutations was generated: M46I, L76V, and V82A. Therefore, PCR was performed on wild-type HXB2 plasmid DNA with the Expand High Fidelity kit (Roche Diagnostics GmbH) with use of primers 5'-p2, 3'-prot2 [23], and a third primer, which was either protM46I 5′-GGA-AAC-CAA-AAA-TAA-TAG-GG-3′, protL76V 5′TAG-GTA-CAG-TAG-TAG-TAG-GAC-3′, or protV82A 5′-TTC-AAT-TAT-GTT-GGC-AGG-TGT-3′. Subsequently, the PCR products were used to generate recombinant virus. The HXB2 variant containing the A431V NC/p1 mutation was generated previously [11].

Viral Culture

Cells. MT2 and SupT1 cells were maintained in RPMI 1640 with L-glutamine (BioWhittaker) supplemented with 10% fetal bovine serum (Biochrom AG) and 10 μg/mL of gentamicine (Gibco). 293T cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% fetal bovine serum and 10 μg/mL of gentamicine. All cells were passaged twice weekly.

Generation of recombinant virus. The viral DNA fragment including the C-terminal portion of Gag and protease was cloned into an HXB2 reference strain, as described elsewhere [23]. Subsequently, the isolated plasmid was used to generate recombinant virus by transfection of 293T cells.

Drug susceptibility analysis. The infectious virus titer (me-

dian tissue culture infective dose) was determined using end point dilutions in MT2 cells. Drug susceptibility of the viruses was determined at least in duplicate with use of the multiple-cycle MTT assay [24].

Viral replication capacity analysis. In each recombinant virus batch, p24 was determined by enzyme-linked immunosorbent assay (Aalto Bioreagent), and viral replication experiments were performed by infecting 2.0×10^6 SupT1 cells with 100 ng of p24 of each recombinant virus batch. Cultures were maintained for 14 days, and each day, 2×150 μL of cell-free viral supernatant was taken for p24 analysis.

Plasma Lopinavir Concentration Analyses

Plasma lopinavir concentrations were assessed by reversed-phase high performance liquid chromatography, essentially as described elsewhere [25]. The lower limit of detection was 0.05 mg/L.

Phylogenetic Analyses

HIV sequences corresponding to the C-term part of Gag and protease were aligned to a reference sequence from the Los Alamos database (http://www.hiv-web.lanl.gov) with use of Clustal software(http://megasoftware.net). Neighbor-joining phylogenetic trees were constructed from 669 gap-stripped nucleotides with use of Mega software, version 4.0 (http://megasoftware.net), the maximum composite likelihood model, and γ-distributed rates (γ parameter, 0.5). Bootstrap analysis was performed using the same methods (1000 replicates). A bootstrap value >70% was interpreted as support for a specific cluster.

Database Analyses

Frequency of L76V was surveyed in the Quest Diagnostics clinical laboratory database of >180,000 samples submitted for protease and reverse transcriptase sequencing from 2000 through 2007. This database provides a sampling of resistance mutations in US patients under care with access to drug resistance testing. Because clinical treatment histories are not available in this database, direct correlation of mutation frequency changes to specific PI regimens is not possible. Therefore, predicted antiretroviral resistance, as determined by the Quest Diagnostics resistance algorithm, was used as a surrogate marker for treatment [26].

PI prescription use data was acquired from Verispan (http://www.versispan.com). Covariation between L76V and 62 other protease amino acid substitutions at 37 drug resistance–associated codons was analyzed for 24,721 HIV-1 subtype B sequences with predicted PI resistance genotyped from 2003 through 2006. Positions with mixed amino acid substitutions that included the wild-type subtype B amino acid were excluded from the analysis. The significance of the calculated binomial correlation coefficient (phi) was assessed by χ^2 analysis at a false discovery rate of 0.01 to correct for 62 multiple comparisons.

RESULTS

Selection of M46I and L76V Protease Substitutions During Lopinavir/r Therapy

We investigated the evolution of resistance in 3 therapy-naive patients who experienced failure of lopinavir/r treatment. To unravel the mechanism of virological failure, genotypic analysis of the viral protease and C-term part of Gag was performed.

Patient B. Antiretroviral therapy (lopinavir/r, lamivudine, and zidovudine) was initiated in 2004 (figure 1*A*). A few months later, lamivudine was replaced by abacavir because of polyneuropathy. After a year of successful therapy (plasma HIV RNA level, <50 copies/mL), low-level viremia (HIV RNA level, 50–300 copies/mL) was observed for 18 months, despite sufficient lopinavir levels in plasma (figure 1*A*). Analysis of viral sequences after 131 weeks (sample B2) indicated infection with HIV-1 subtype B. The transient appearance of I10I/L and of mixtures at 3 other protease resistance–associated codons (M46M/I, L76L/V, and V82V/A) was observed (figure 1*A*) [18]. Several weeks later (sample B3), a minor population with the I54I/V substitution was observed. Analysis of the C-term part of Gag revealed the A431V substitution in the NC/p1 CS and, later, on a minor population with codon P453P/L substitution in the p1/p6 CS (figures 1*A* and 2*A*). Furthermore, several other substitutions in the C-terminal part of Gag and protease were observed. In the N-term part of reverse transcriptase, the resistance-associated substitutions K65R and V75I were detected. Subsequently, therapy was switched to lamivudine, zidovudine, efavirenz, and tenofovir, which resulted in viral suppression (plasma HIV RNA level, <50 copies/mL).

Patient K. Antiretroviral therapy (lopinavir/r, lamivudine, and stavudine) was initiated in 2003 (figure 1*B*). After brief virological success (plasma HIV RNA level, <50 copies/mL), the viral RNA load rebounded to pretherapy levels (1×10^5 copies/mL). At the time of viral rebound, poor lopinavir plasma concentrations were detected (figure 1*B*). Analysis of viral sequences indicated infection with HIV-1 subtype K and selection of the protease substitutions M46M/I and L76V (sample K2), followed by the V82A substitution (sample K3). In addition, the protease resistance–associated L33F and Q58Q/E and a few other protease and Gag substitutions were observed (figures 1*B* and 2*B*) [18]. In the N-term part of reverse transcriptase, the resistance-associated M184V substitution was detected. After 2 years, therapy was switched to abacavir, efavirenz, and tenofovir, which resulted in a brief reduction in viral RNA load.

Patient M. Antiretroviral therapy (lopinavir/r, lamivudine, and zidovudine) was initiated at the beginning of 2004 (figure 1*C*). During 2 years of successful therapy, a transient increase in plasma viral RNA concentration was observed (figure 1*C*)

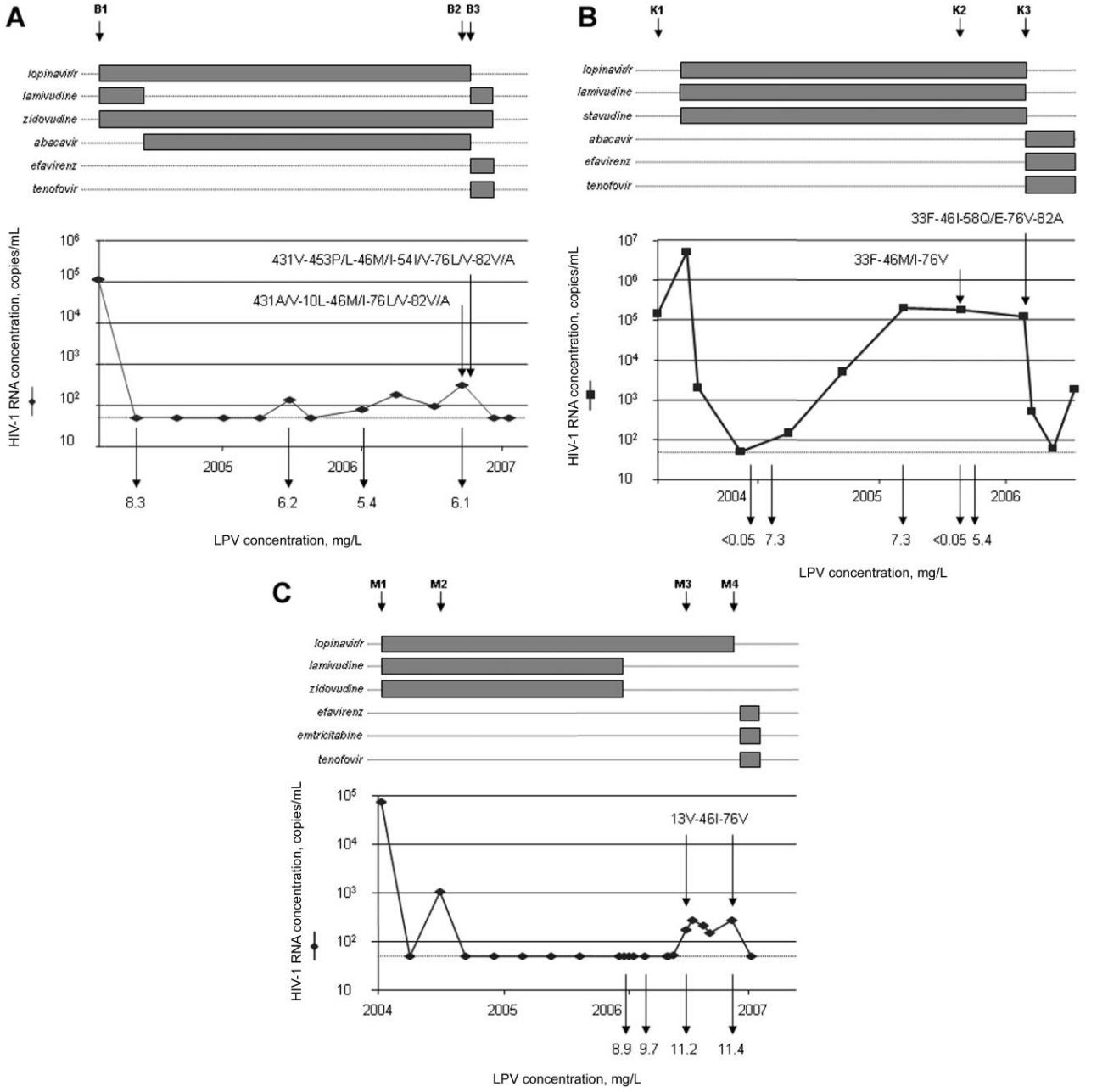
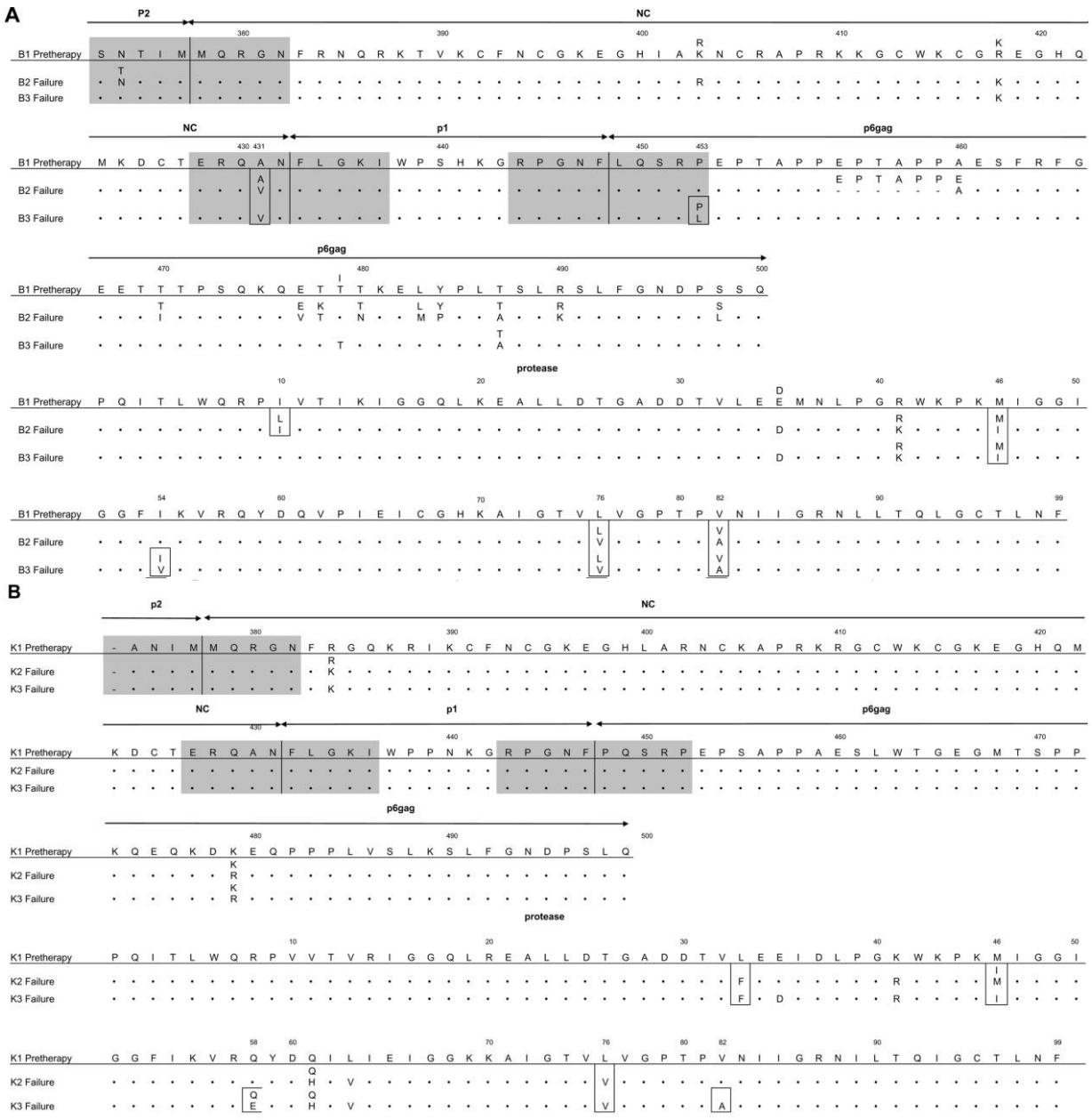


Figure 1. Human immunodeficiency virus (HIV) RNA concentrations in and treatment history of patient B *(A),* patient K *(B),* and patient M *(C).* At several times during therapy, plasma lopinavir (LPV) concentrations were measured. The arrows indicate the sequential times at which plasma samples were used for genotypic resistance analysis (protease resistance–associated mutations [18], compared with the patient's baseline sample, are shown). Resistance-associated protease substitutions and/or polymorphisms [18] were already observed at baseline in patient B (L10I, I62V, and L63P), patient K (L10V, I13V, K20R, M36I, and H69K), and patient M (I62V and L63P).

that was not associated with selection of resistance. Several months after simplification of the drug regimen to lopinavir/r monotherapy, low levels of viral RNA (50–300 copies/mL) were observed, despite sufficient plasma lopinavir levels (figure 1*C*). At the time of virological failure (samples M3 and M4), genotypic analysis indicated infection with HIV-1 subtype B and the selection of M46I and L76V (figures 1*C* and 2*C*). In addition, several other amino acid changes in the C-terminal part of Gag and protease were observed (figure 2*C*). Subsequently, re-initiation of combination therapy (efavirenz, emtricitabine, and tenofovir) rapidly resuppressed the plasma viral load (<50 copies/mL). To investigate the order of appearance of changes in viral protease and Gag in more detail, we performed a clonal sequence analysis of longitudinal samples


A
P2
NC
B1 Pretherapy S N T I M M Q R G N F R N Q R K T V K C F N C G K E G H I A K N C R A P R K K G C W K C G R E G H Q
B2 Failure
B3 Failure
NC
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p6gag
B1 Pretherapy M K D C T E R Q A N F L G K I W P S H K G R P G N F L Q S R P E P T A P P E P T A P P A E S F R F G
B2 Failure
B3 Failure
p6gag
B1 Pretherapy E E T T T P S Q K Q E T T T K E L Y P L T S L R S L F G N D P S S Q
B2 Failure
B3 Failure
protease
B1 Pretherapy P Q I T L W Q R P I V T I K I G G Q L K E A L L D T G A D D T V L E E M N L P G R W K P K M I G G I
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B3 Failure
B1 Pretherapy G G F I K V R Q Y D Q V P I E I C G H K A I G T V L V G P T P V N I I G R N L L T Q L G C T L N F
B2 Failure
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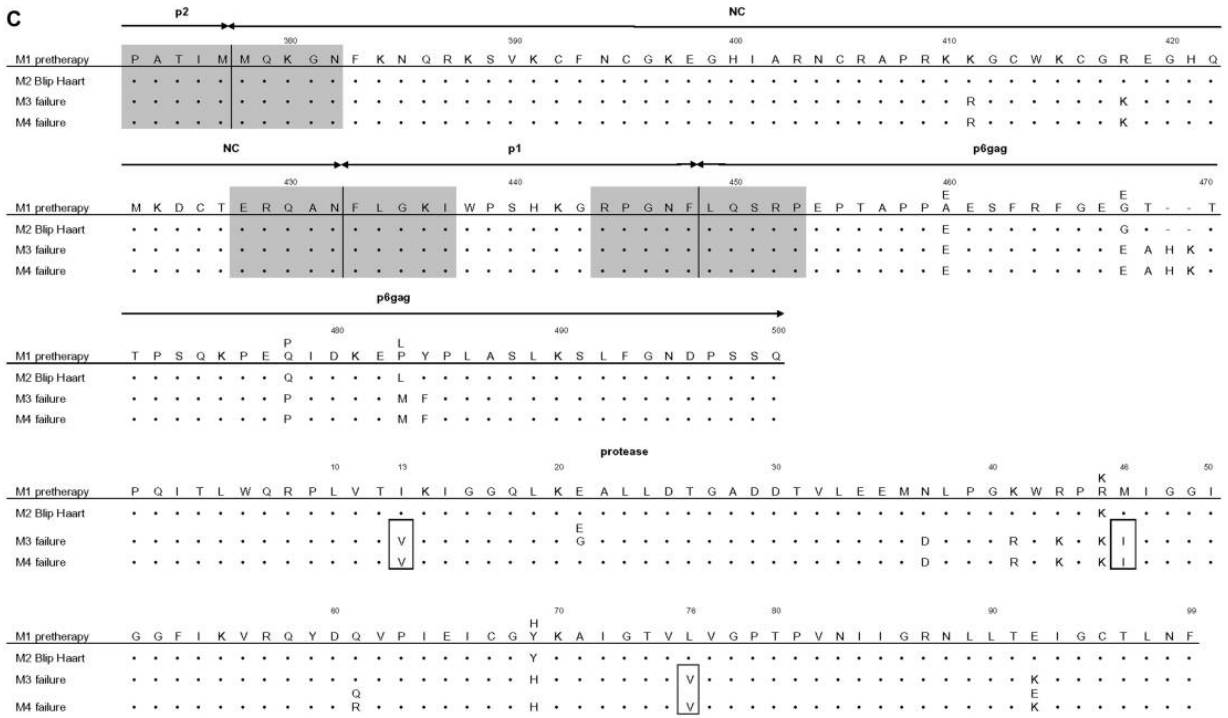

Figure 2. Evolution of the C-terminal part of human immunodeficiency type 1 (HIV-1) Gag (p2-p6gag; amino acid 373–500) and HIV protease during failure of first-line therapy with lopinavir boosted with ritonavir in patient B *(A),* patient K *(B),* and patient M *(C).* Protease cleavage sites, as present in the C-terminal part of Gag, are shaded. Protease resistance–associated mutations [18] are highlighted in open squares.

from patients B and K, who had the most complex drug resistance profiles.

Clonal analysis of the viral population of patient B. Phylogenetic analysis revealed that the viral population before the start of therapy was relatively heterogeneous (figure 3*A*). Remarkably, the NC/p1 Gag CS variant A431V was the predominant single mutant observed during therapy failure. This variant was observed in at least 2 different sequence clusters, and separation of these clusters suggests that they represent independent events, although recombination can never be excluded. The only other single mutant that was observed was the V82A variant. The progressive pattern of evolution continued in just 1 of the clusters, because the A431V was observed together with M46I, M46I+V82A, or M46I+L76V. A clonal analysis of the viral population that was performed a few weeks later revealed that the latter A431V+M46I+L76V became dominant. This resulted in a more homogenous population, as indicated by the presence of just 1 major sequence cluster.

Clonal analysis of the viral population in samples from patient K. Phylogenetic analysis revealed that all pretherapy sequences clustered together (figure 3*B*). Subsequently, during lopinavir/r therapy failure, a new cluster of viruses with just the L76V alone or in combination with M46I was observed. Finally, at the last time point, continued viral evolution generated a homogenous population of M46I+L76V+V82A.

Impact on PI Susceptibility and Replication Capacity

A set of SDMs was generated to investigate the impact on PI susceptibility and RC (figures 4 and 5). All single mutants (A431V, M46I, L76V, and V82A) were generated in the background of HIV-1 subtype B HXB2. Remarkably, the NC/p1 Gag CS change A431V confers a 2.7-fold increase in one-half the maximal inhibitory concentration (IC_{50}) to lopinavir with no obvious replication defect. This may explain the observation of A431V as the initial escape mutant in patient B. Of the other single mutants, V82A and M46I replication was comparable to wild-type replication, but they did not reduce lopinavir susceptibility. The L76V mutation conferred a 3.5-fold increase in IC_{50} to lopinavir but had severely hampered replication (0.1%). Of interest, addition of M46I partly compensated for the poor RC of the L76V variant (5%). Furthermore, the M46I mutation, which alone did not confer any lopinavir resistance, increased the IC_{50} of the double mutant above the lower clinical cutoff level (11.6-fold). Analysis of patient-derived viral protease clones representing wild-type, L76V, and M46I+L76V variants revealed comparable RC data. The wild-type HIV variant from

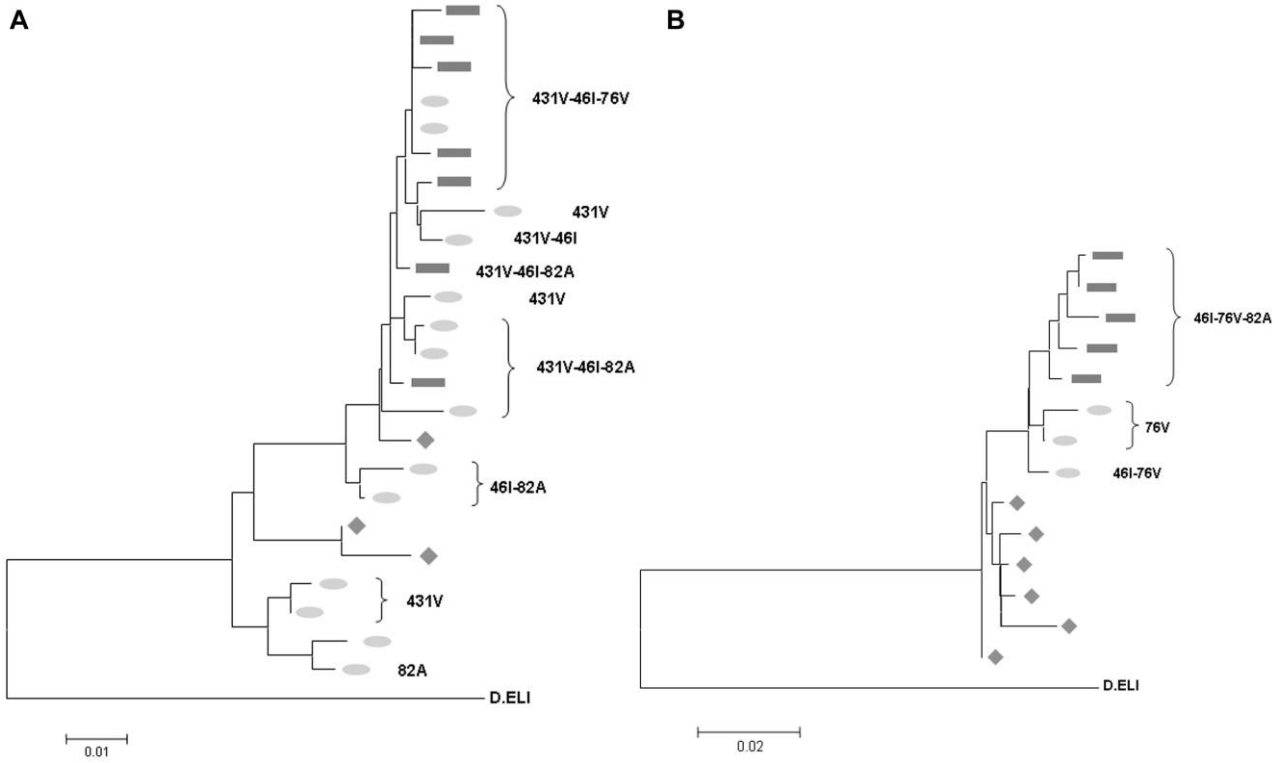

Figure 3. Phylogenetic tree constructed using C-term Gag and protease sequences from plasma samples from patient B *(A)* and patient K *(B)* that were obtained during failure of first-line therapy with lopinavir boosted with ritonavir. For each clone, the sampling moment is indicated by a symbol: baseline *(diamonds),* first therapy failure moment *(oval),* and last therapy failure moment *(rectangle).*

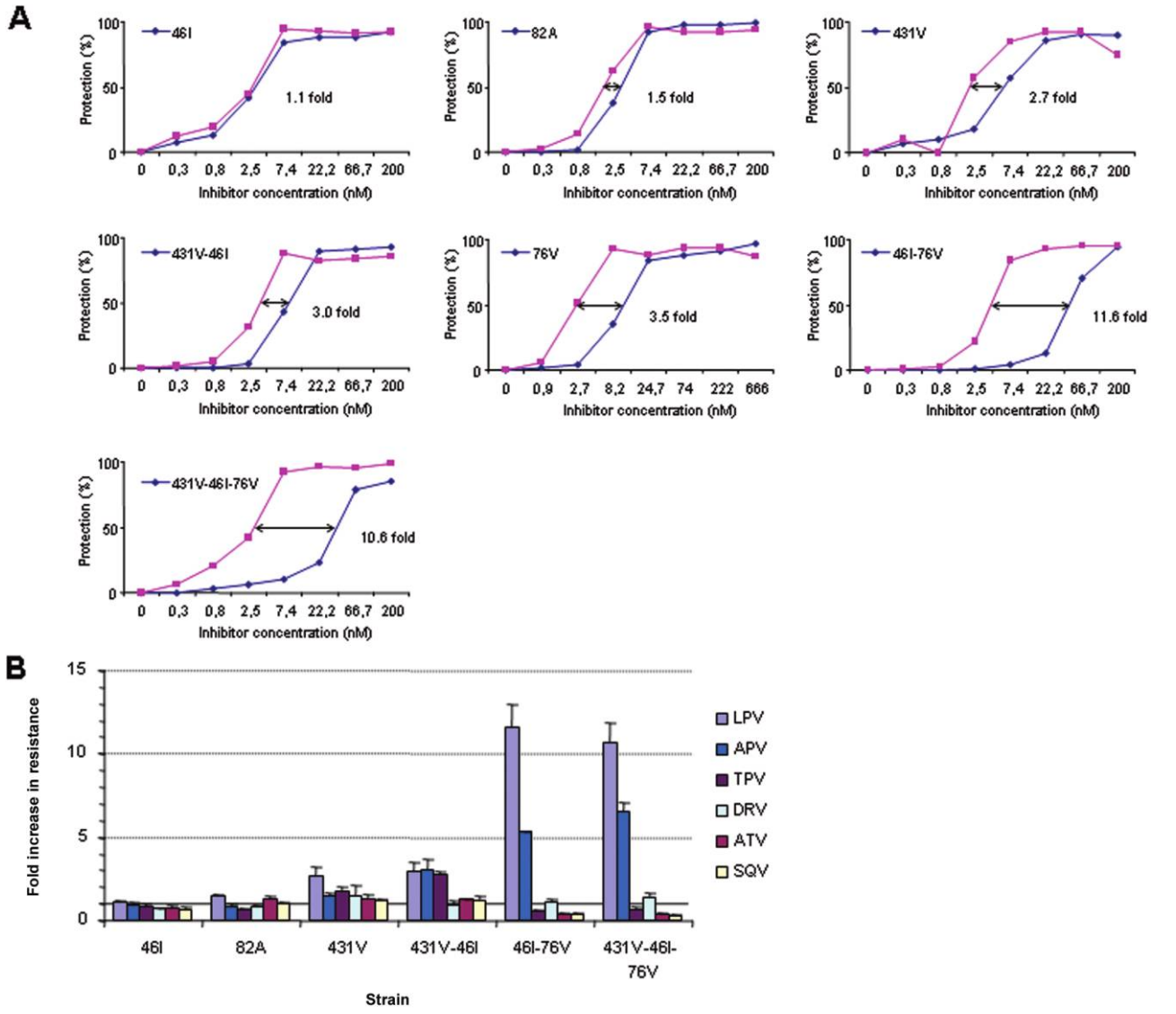

Figure 4. Determination of phenotypic drug susceptibility and replication capacity of a set of site-directed mutants (SDMs) generated in the background of a subtype B reference strain (HXB2). *A,* Investigation of phenotypic drug susceptibility to the protease inhibitor lopinavir (LPV) of the SDMs *(diamonds),* compared with the wild-type reference strain *(square).* Fold increases in half the maximal inhibitory concentration are indicated. *B,* Representation of the fold increases in phenotypic drug resistance of the different SDMs, compared with the wild-type reference strain, to the protease inhibitors LPV, amprenavir (APV), tipranavir (TPV), darunavir (DRV), atazanavir (ATV), and saquinavir (SQV). The L76V variant hardly replicated, and therefore, susceptibility to only LPV was analyzed. Error bars indicate the standard error of the mean.

the patient replicated as well as did our reference strain HXB2. In line with the SDM data, L76V hardly replicated (few syncytia; p24 level not above the cutoff of the assay), and its poor replication capacity was partially compensated in the M46I+L76V variant. The A431V+M46I SDM conferred 3-fold resistance and demonstrated intermediate RC. A variant with a combination of mutations at protease codons M46I+L76V+V82A did not result in infectious virus. The virus with the most prevalent combination of mutations, as observed in patient B (A431V+M46I+L76V), demonstrated 10.6-fold resistance to lopinavir and a further improvement of the RC (10% RC). Cross-resistance analysis demonstrated an IC_{50} increase for amprenavir (6.6-fold), hypersusceptibility to saquinavir (0.3-fold) and atazanavir (0.4-fold), 0.7-fold resistance to tipranavir, and 1.4-fold resistance to darunavir. Finally, a patient-derived viral clone with the same set of mutations conferred an 8-fold increase in lopinavir IC_{50}.

Incidence of L76V and Association with Lopinavir Prescription

The incidence of L76V in a large US clinical database increased significantly during 2000–2007. In 2000, L76V was detected in

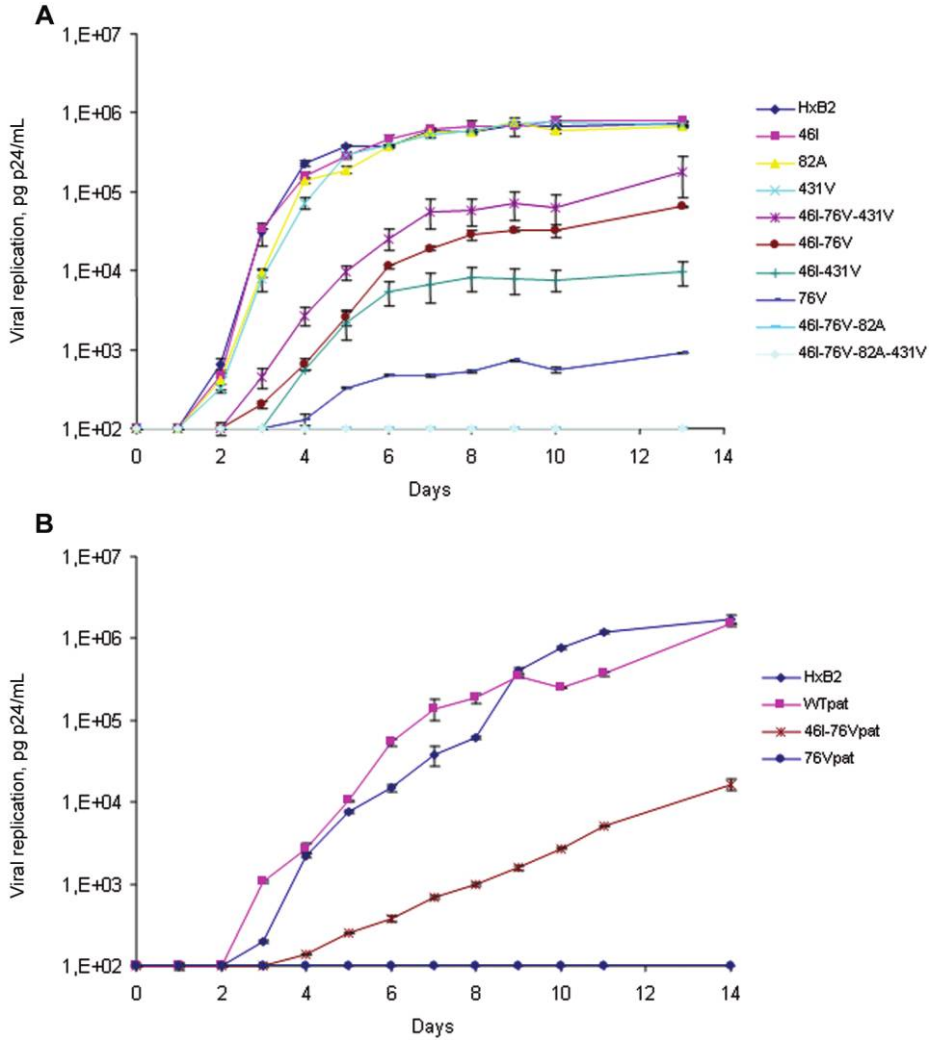

Figure 5. *A,* Viral replication curves of the site-directed mutants (SDMs), compared with the wild-type reference strain. *B,* Viral replication curves of patient-derived viral protease and C-terminal part of Gag, compared with the wild-type reference strain. The virus variants were used to infect SupT1 cells in duplicate, and viral replication was monitored by p24 production. Error bars indicate the standard error of the mean.

only 0.42% of samples with any predicted antiretroviral resistance and in 0.51% of samples with predicted PI resistance. In 2007, the frequency significantly ($P < .001$, by χ^2 test) increased to 138 (1.16%) of 11,914 viruses with any antiretroviral resistance and to 133 (3.39%) of 3920 PI-resistant viruses. To investigate whether the increase in incidence was associated with prescription of a particular PI, we analyzed prescription use in the same period. Prescription use of lopinavir, approved for clinical use in 2000, increased steadily during 2000–2003 and was closely correlated to increasing incidence of L76V (Spearman ρ, 0.93) (figure 6). Use decreased or did not change for other PIs available during 2000–2003; thus, no correlation was seen between use of other PIs and L76V.

The M46I substitution was found in 80.7% of PI-resistant viruses with L76V sequenced during 2003–2006, which was 2.9 times (95% confidence interval, 2.7–3.0 times) more frequently than expected from chance alone; the binomial correlation coefficient phi, a measure of covariation, was 0.19 ($P < .001$; statistically significant after correction for multiple comparisons). An additional analysis of 62 protease amino acid substitutions, including 49 International AIDS Society USA–listed mutations, at 37 protease positions found only 2 additional substitutions with a phi value of 0.1 (I84V and K55R).

DISCUSSION

In the present study, we revealed that HIV protease substitution L76V, in combination with M46I, represents a novel pathway to first-line lopinavir/r therapy failure. In the past, development of PI resistance has severely hampered the treatment of HIV-infected patients. To prevent the rapid onset of PI resistance, ritonavir boosting of PI has been successfully implemented.

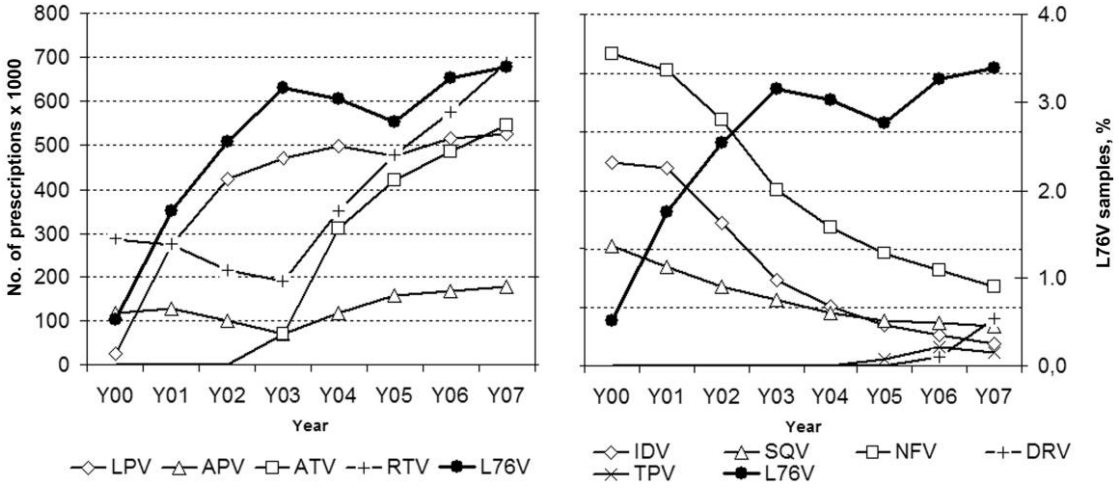

Figure 6. Number of protease inhibitor prescriptions in the United States during 2000–2007 (obtained from Verispan [http://www.verispan.com]). The percentage of clinical samples with L76V in a large US reference laboratory database was determined as the proportion of samples with predicted resistance to at least 1 protease inhibitor (surrogate marker for antiretroviral treatment). APV, amprenavir; ATV, atazanavir; DRV, darunavir; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; TPV, tipranavir.

Ritonavir inhibits the cytochrome P4503A4 enzyme [12], and as a result, the level of PI in the plasma of the patient is elevated. Consequently, an increased number of protease mutations is needed to achieve clinical resistance, thereby increasing the genetic barrier required to escape drug inhibition. In large clinical trials of first-line lopinavir/r-based therapy, virological failure could not be attributed to the selection of previously identified resistance mutations in protease [15, 16]. In addition, in clinical practice, failure of first-line lopinavir/r therapy for viruses resistant to lopinavir is rare, and to date, only 2 cases have been reported [17, 19].

We identified 3 therapy-naive patients who experienced failure of lopinavir/r therapy, and all of them had 2 common mutations in the viral protease (M46I and L76V). A detailed virological analysis of SDM revealed that the L76V variant confers low-level lopinavir resistance but has severely hampered replication. The M46I variant alone does not reduce lopinavir susceptibility. Addition of the M46I to L76V has a dual effect; it not only results in levels of lopinavir resistance above the lower clinical cutoff but also partly compensates for the reduced RC. Nonetheless, the RC of the double mutant is still compromised, compared with wild-type virus. It could be speculated that particular compensatory mutations could be present in these patient isolates to favor the emergence of the L76V mutation. However, analysis of the RC of wild-type, L76V, and M46I+L76V variants from clinical samples demonstrated RC profiles comparable to those observed for the SDM, showing poor RC of the single and double mutants.

Of interest, low-level viremia was observed in 2 of our patients carrying these M46I+L76V mutant viruses. This may be explained by a residual antiviral effect; however, the reduced RC of the double mutant could also play a role.

Our data revealed that just 2 nucleotide changes can be sufficient for viral breakthrough during first-line lopinavir/r therapy. However, the combination of M46I+L76V is not frequently reported, which may be explained by lack of resistance conferred by the M46I and/or the severe reduction in RC of the other intermediate L76V variant. This indicates that a genetic barrier to resistance can not be simply calculated by the sum of mutations. The interaction of mutations and their effect on RC, which are factors that determine whether viral escape can be observed, should be included in the genetic barrier.

Because we identified only 3 patients who experienced failure of first-line lopinavir/r therapy and who had virus with M46I+L76V, we investigated the prevalence in a larger population. An extensive clinical database analysis revealed a significant increase in the prevalence of L76V in a population with predicted PI resistance during 2000–2007 (from 0.5% to 3.4%). This increase is in line with a recent study that reported a prevalence of L76V of 2.7% among patients who experienced failure of a PI regimen [27]. Because current testing guidelines recommend resistance testing before the initiation of therapy, we have observed an increasing number of samples submitted for testing in recent years that have no evidence of any antiretroviral resistance. The majority of such samples more than likely were obtained from treatment-naive patients, and inclusion of these in the denominator when estimating frequencies of resistance-associated mutations would obscure any trends seen for changes in prevalence for mutations such as L76V. Indeed, in 2007, we found 133 L76V-containing sequences

(3.4%) in PI-resistant samples but only 4 (0.023%) in samples with no resistance. Although some primary resistance mutations may occur as natural polymorphisms in the absence of treatment exposure, their prevalence is low.

Furthermore, our analysis showed that the increasing frequency of L76V was closely correlated to lopinavir use. This is in agreement with the observation that the M46I+L76V variant confers reduced susceptibility to only 2 PIs (lopinavir and amprenavir). M46I+L76V conferred hypersusceptibility to 2 other PIs (atazanavir and saquinavir). It was reported that L76V alone confers hypersusceptibility, which could be exploited during subsequent salvage therapy [28, 29].

A detailed analysis of viral isolates from a patient demonstrated that the gag NC/p1 substitution A431V was the predominant single mutant. The frequent selection of this mutant can be explained by the fact that it confers low-level resistance and no obvious replication defects. This is in line with a study that we recently had published that revealed that gag NC/p1 CS changes, including the A431V, can confer low-level PI resistance in the absence of substitutions in the viral protease [11]. The additional role of A431V becomes more evident as viral evolution continues. A431V increases RC of the M46I+L76V. This compensatory effect of A431V was described previously, both in vitro and in vivo [7, 9, 31], and was related to the increased processing in the background of drug resistance mutations in the viral protease (codons 46, 82, 84, and 90) [30].

In a previous case report of first-line lopinavir treatment failure, a patient was infected with subtype C virus [17]. The authors suggested that a protease polymorphism at codon M36I at baseline may have contributed to virological failure. In our study, a patient was infected with subtype K virus and also had virus with the M36I change. In the MONARK trial, which investigated lopinavir/r monotherapy, 3 of 5 patients who experienced therapy failure selected the L76V protease substitution and were infected with HIV-1 CRF_02 [32]. Impact of HIV-1 subtype on selection of PI resistance may become more important, because non-B subtypes are becoming more frequent, and lopinavir/r will be used as part of second-line regimens in developing countries.

In conclusion, the HIV protease substitution L76V, in combination with M46I, confers levels of lopinavir resistance above the lower clinical cutoff and represents a novel resistance pathway to first-line lopinavir/r therapy. However, HIV replication is severely compromised, which may explain why this particular combination of mutations is not observed more frequently during first-line lopinavir/r treatment failure. Additional studies are warranted to investigate whether long-term treatment failure under lopinavir/r pressure may result in the appearance of additional compensatory mutations.

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