Mutations in Human Immunodeficiency Virus Type 1 Integrase Confer Resistance to the Naphthyridine L-870,810 and Cross-Resistance to the Clinical Trial Drug GS-9137

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To gain further insight into the understanding of the antiviral resistance patterns and mechanisms of the integrase strand transfer inhibitor L-870,810, the prototypical naphthyridine analogue, we passaged the human immunodeficiency virus type 1 strain HIV-1(IIIB) in cell culture in the presence of increasing concentrations of L-870,810 (IIIpL-L-870,810). The mutations L74M, E92Q, and S230N were successively selected in the integrase. The L74M and E92Q mutations have both been associated in the past with resistance against the diketo acid (DKA) analogues L-708,906 and S-1360 and the clinical trial drugs MK-0518 and GS-9137. After 20, 40, and 60 passages in the presence of L-870,810, IIIpL-L-870,810 displayed 22-, 34-, and 110-fold reduced susceptibility to L-870,810, respectively. Phenotypic cross-resistance against the DKA analogue CHI-1043 and MK-0518 was modest but that against GS-9137 was pronounced. Recombination of the mutant integrase genes into the wild-type background reproduced the resistance profile of the resistant IIIpL-L-870,810 strains. In addition, resistance against L-870,810 was accompanied by reduced viral replication kinetics and reduced enzymatic activity of integrase. In conclusion, the accumulation of L74M, E92Q, and S230N mutations in the integrase causes resistance to the naphthyridine L-870,810 and cross-resistance to GS-9137. These data may have implications for cross-resistance of different integrase inhibitors in the clinic.

Human immunodeficiency virus (HIV) resistance is a major problem encountered during present day anti-HIV treatment, which comprises mainly inhibitors of the viral enzymes reverse transcriptase (RT) and protease. Among viremic patients, 76% have resistance to one or more antiretroviral drugs (31). Therefore, there is a growing need for drugs active against resistant virus, particularly drugs with a novel mechanism of action. Integrase (IN), the third viral enzyme required for HIV type 1 (HIV-1) replication, catalyzes the insertion of viral DNA into the host cell chromosome through a multistep process that includes two catalytic reactions, namely, 3’ cleavage of the viral DNA ends and strand transfer of the processed viral DNA into the host DNA (35). After integration, the proviral DNA is replicated and genetically transmitted as part of the cellular genome. As such, integration defines a point of no return in the establishment of HIV infection. Since no human counterpart of the enzyme is known, there is substantial interest in developing effective inhibitors of HIV IN (24).

The identification of a series of diketo acids (DKA) that specifically target strand transfer and prevent HIV-1 replication in cell culture provided the first proof of principle for HIV-1 IN inhibitors as antiviral agents (15). L-731,988 is the prototype of these IN strand transfer inhibitors (INSTIs). CHI-1043, a novel DKA analogue and INSTI, was also included in the study (Fig. 1) (L. De Luca, M. L. Barreca, S. Ferro, N. Iraci, Z. Debyser, M. Witvrouw, and A. Chimirri, submitted for publication). Since then, the Merck group characterized a series of metabolically stable heterocyclic compounds, represented by L-870,810 (Fig. 1), containing an 8-hydroxy-[1,6]-naphthyridine-7-carboxamide pharmacophore as a substitute for the 1,3-DKA moiety (39). In HIV-1-infected patients, L-870,810 resulted in a 50-fold reduction in viral load, but clinical studies were halted due to liver and kidney toxicity observed in dogs. The follow-up compound MK-0518 (raltegravir) (Fig. 1) recently received FDA approval for use in the clinic. Although MK-0518 is a hydroxyprimidinone carboxamide and is structurally distinct from DKA and naphthyridines, the compound acts much like all INSTIs (34). MK-0518 reduced viral loads to undetectable levels (below 50 copies/ml) in nearly two-thirds of highly treatment-experienced patients infected with triple-class-resistant HIV and was generally well tolerated (6, 32). Gilead Sciences developed the INSTI GS-9137 (elvitegravir) (Fig. 1) (41). An ongoing phase II clinical trial showed that GS-9137 at its highest dose level was able to significantly reduce HIV loads compared with a protease inhibitor regimen.

The mechanism of action of DKAs has been the subject of intensive study because of the importance of DKAs and DKA-like derivatives as antiviral lead compounds. Research suggests that DKAs selectively bind to a unique conformation of the IN acceptor site for host DNA. This distinct conformation of the IN chromosomal DNA acceptor site may only be present after prior complexing of IN with the viral DNA and subsequent 3’ processing. Together, both events would induce the necessary
structural changes in IN for accommodating the host DNA or, in this case, the DKA ligands. The DKA ligands thereby compete with the host DNA for binding to IN. It has been proposed that binding of DKA ligands involves chelating the critical divalent metal ions in the IN catalytic core, resulting in subsequent sequestration of the metal cofactor, which acts normally as a coordination bridge between the IN DDE motif, the viral cDNA donor, and the host DNA (9, 13).

With MK-0518 in the clinic, antiviral resistance profiling should be pursued. Data on antiviral resistance development against INSTIs are still limited (Table 1). Resistance of HIV to INSTIs is typically observed after 3 to 6 months of in vitro passaging in the presence of the drug (11, 12, 14). HIV-1 strains that were selected in the presence of the DKA L-708,906 carried the mutations T66I, S153Y, and M154I (15) or T66I, L74M, and S230R (12). Nine mutations accumulated in HIV-1 IN of strains grown in the presence of the DKA analogue S-1360. The T66I, L74M, and Q146K mutations were present in the whole virus population, whereas the substitutions E138K, S153A, K160D, V165I, and V201I were detected as a mixture (11). Apparently, mutations that result in resistance to these prototype inhibitors map to the IN active site.

<table>
<thead>
<tr>
<th>Integrase inhibitor (description)</th>
<th>Selection process</th>
<th>Integrase mutation(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-708,906 (DKA strand transfer inhibitor)</td>
<td>In vitro</td>
<td>T66I/M154I</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>T66I/S153Y</td>
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<tr>
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<td>In vitro</td>
<td>N155S</td>
<td>14</td>
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<tr>
<td></td>
<td>In vitro</td>
<td>T66I/L74M/S230R</td>
<td>12</td>
</tr>
<tr>
<td>S-1360 (DKA analogue strand transfer inhibitor)</td>
<td>In vitro</td>
<td>T66I/L74M/A128T/E138K/Q146K/S153A</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>K160D/V165I/V201I</td>
<td>14</td>
</tr>
<tr>
<td>L-870,810 (naphthyridine carboxamide strand transfer inhibitor)</td>
<td>In vitro</td>
<td>V72I/F121Y/T125K/V151I</td>
<td>11</td>
</tr>
<tr>
<td>MK-0518 (hydroxypyrimidinone carboxamide strand transfer inhibitor)</td>
<td>In vitro</td>
<td>G140A/E138A/Q148K</td>
<td>36</td>
</tr>
</tbody>
</table>

Two main pathways

- In patients N155H (+ L74M, E92Q, V151I, T97A, and G163R) | 6 |
- In patients Q148K/R/H (+ G140S/A and E138K) | 6, 16 |
- In patients Y143R/C (+ L74A/l, E92Q, I203M, and S230R) | 6 |

Most common pathway

- In patients Q148K/R/H | 16, 36 |

Other pathway

- In patients Q148K/R/H | 16, 36 |

GS-9137 (dihydroquinoline carboxylic acid strand transfer inhibitor)

- In vitro E92Q (+ H51Y, S147G, and E157Q) | 21, 23 |
- In vitro T66I (+ R263K or + S153Y and F121Y) | 21, 41 |
proximal to residues that coordinate divalent metals (15). HIV-1 strains that were made resistant to the naphthyridine carboxamide L-870,810 in vitro carried the mutations V72I, F121Y, T125K, and V151I (14). Only minor cross-resistance against DKA was reported (14). Although clinical data are still limited, it appears that there are two distinct molecular pathways that confer MK-0518 resistance in patients, through N155H or through Q148K/R/H (6, 16, 36). Additional mutations were frequently observed in both pathways, including N155H plus L74M, E92Q, V151L, T97A, and G163E or Q148K/H/R plus G140S/A and E138K (6). A minor pathway involving Y143C/R, L74A/I, E92Q, I203M, and S230R has been reported as well (6). In vitro resistance profiling of GS-9137 showed that the drug can select two primary resistance patterns, involving either T66I or E92Q mutation in HIV-1 IN (21). In vitro data suggest possible cross-resistance between the IN inhibitors MK-0518 and GS-9137 (21, 36).

To gain further insight into patterns and mechanisms of antiviral resistance to INSTIs, we selected HIV strains resistant to the naphthyridine L-870,810. The mutations L74M, E92Q, and S230N found in the IN were different from those reported before (V72I, F121Y, T125R, and V151I) (14). Phenotypic resistance to L-870,810 was apparent. Cross-resistance to CHI-1043, GS-9137, and MK-0518 was evidenced. In addition, we studied the impact of the selected IN mutations on viral fitness and enzymatic activity and constructed a three-dimensional (3D) model of the mutant IN.

MATERIALS AND METHODS

**Compounds.** AMD3100 was provided by AnorMED (Langley, BC, Canada) and was synthesized as described previously (2). Dextran sulfate (DS) (average molecular weight, 5,000) was purchased from Sigma (Bornem, Belgium). Zidovudine was synthesized according to the method described by Horwitz et al. (19). Dextran sulfate (average molecular weight, 750,000) was a gift from FMC Biopolymer (Bloomfield, NJ). CHI-1043, a novel INSTI, was synthesized by A. Chimirri, Messina, Italy (De Luca et al., submitted). Ritonavir (ABT538) was obtained from J. M. Leonard, Abbott Laboratories (Abbott Park, IL). L-870,810 was synthesized at our institute. MK-0518 and GS-9137 were kindly provided by Tibotec (Mechelen, Belgium). The compounds were dissolved in dimethyl sulfoxide at 10 mg/mL, except for DS, which was dissolved in MilliQ water.

**Cells.** MT-4 cells (27) were grown in a humidified atmosphere with 5% CO2 at 37°C and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1% sodium bicarbonate, and 20 µg/mL of gentamicin.

**Virus strains.** The origin of HIV-1(IIIb) has been described previously (29).

**Plasmids.** The HIV-1 plasmid pNL4.3 (1) is a molecular clone obtained from Malcolm Martin through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD). The bacterial expression plasmid pRPl102 (R. H. A. Plasterk, Dutch Cancer Institute, Amsterdam, The Netherlands), encoding HIV-1 IN, was used to generate the double E92Q S230N mutant. Site-directed mutagenesis was performed using the Kirsch and Joly method (22). The presence of the expected mutations was confirmed by DNA sequencing of the entire IN coding region.

**Selection of antiviral resistance.** The resistance selection of HIV-1(IIIb) against L-870,810 was initiated at a low multiplicity of infection (MOI; 0.01) in MT-4 cells and a drug concentration equal to the 50% effective concentration (IC50; 0.005 µM), as determined in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Every 3 to 4 days, cell culture was monitored for the appearance of HIV-induced cytopathic effect (CPE). When CPE was observed, the cell-free culture supernatant was used to infect fresh MT-4 cells in the presence of an equal or higher concentration of the compound. When no virus breakthrough was observed, the infected cell culture was subcultivated in the presence of the same concentration of the compound.

**PCR amplification and sequencing of the coding regions for integrase, reverse transcriptase, and gp160.** Proviral DNA extraction of MT-4 cells infected with different passages of HIV-1(IIIb) selected in the presence of L-870,810 was performed using a QIAamp blood kit (Qiagen, Hilden, Germany). PCR amplification and sequencing of the IN, RT, and gp160 coding sequences were done as described previously (18). Mutations present in >25% of the global virus population can be detected as a mixture with the wild-type (WT) amino acid by means of population sequencing.

**Chimeric virus recombination assay.** Integrate chimeric virus technology was done as described earlier (12). The IN sequences after recombination were verified to be identical to those of the respective WT or in vitro-selected parental IIIbL810 strains (data not shown). In addition, we verified the proportion of each mutation in the recombined strains by sequencing; the recombined strains showed similar proportions of each mutation to those in the parental strains (data not shown).

**Drug susceptibility assay.** The inhibitory effect of antiviral drugs on the HIV-induced CPE in MT-4 cell culture was determined by MTT assay (28). This assay is based on the reduction of the yellow compound MTT by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective doses of the HIV strains were determined by titration of the virus stocks, using MT-4 cells. For drug susceptibility assays, MT-4 cells were infected with 100 to 300 50% cell culture infective doses of the HIV strains in the presence of fivefold serial dilutions of the antiviral drugs. The concentration of the compound achieving 50% protection against the CPE of HIV, defined as the IC50, was determined.

**Production and purification of recombinant integrase.** The purification of WT and mutant HIV-1 IN was done as described elsewhere (3).

**Overall integration assay.** Enzymatic integration reactions were carried out as described previously (5, 7), with minor modifications (12). Overall integration activities of the different enzymes were determined in this assay by measuring the respective amounts of strand transfer products, using ImageJ (http://rsb.info.nih.gov/ij).

**Determination of replication fitness of HIV-1 strains selected in the presence of L-870,810.** Inocula of various HIV-1 strains containing equal amounts of HIV-1 p24 antigen (10, 5, and 2.5 pg/mL) were added to MT-4 cells (5 × 104/mL). Starting at 3 days postinfection, cells were examined daily for the appearance of HIV-1-induced cytopathicity. In addition, aliquots of cell-free supernatants were taken for determination of virion p24 levels.

**Construction of 3D model of mutant HIV-1 integrase.** On the basis of the published partial X-ray IN structures 1EX4 (37) and 1K0Y (4), a 3D IN model was assembled by using the Brugel package. The two Mg2+ ions and the viral DNA were added according to the crystal structure of the structurally homologous Tn5 transposase crystal structure 1MM8 (33). Host DNA was added to the structure based on available contacts with the viral DNA-IN complex.

RESULTS

**Selection of HIV-1 strains resistant to L-870,810.** L-870,810-resistant HIV-1 strains were selected by serial passage of HIV-1(IIIb) in the presence of increasing concentrations of L-870,810 (Fig. 2). After 20 passages, the selected strain was able to grow at a concentration of 0.13 µM, which is 25-fold higher than the IC50 for WT HIV-1(IIIb) (0.005 µM). After 40 passages, the selected strain was able to grow at a compound concentration of 0.62 µM (125-fold higher than the IC50), while after 50 passages this concentration was 1.1 µM. After 60 passages, the selected strain was able to grow at a compound concentration of 1.8 µM (360-fold higher than the IC50).

**Progressive accumulation of mutations in the integrase genes of selected HIV-1 strains.** The IN-encoding regions of the HIV-1(IIIb) strains selected in the presence of L-870,810 (IIIbL870,810) were sequenced. Several mutations were detected in comparison with the DNA sequence of the WT HIV-1(IIIb) strain (Fig. 2). After 20 passages, the E92Q mutation was present in the whole virus population, whereas the S230N mutation was present as a mixture with the WT residue in 90% of the virus population. Selection for 40 passages resulted in a virus strain containing the L74 (25%)-M74 (75%) mixture in
addition to the E92Q and S230N mutations in the IN. After 50 passages, all three mutations were present in the complete virus population. No additional mutations were selected during selection for up to 60 passages in the presence of L-870,810. The virus harboring the triple mutation is probably fit enough to replicate at the higher concentration of L-870,810 at passage 60. No mutations were found in the genes coding for gp160 and RT.

Evaluation of phenotypic (cross-)resistance of different selected HIV-1 strains by use of MT-4 cell–MTT assay. To corroborate that the HIV strains selected to grow in the presence of L-870,810 were indeed drug resistant, we determined the antiviral activity of L-870,810 against the selected strains in MT-4 cells. HIV-1(IIIB) strains selected during 20, 40, and 60 passages in the presence of L-810,810 are referred to as IIIb/L-870,810(#20), IIIb/L-870,810(#40), and IIIb/L-870,810(#60), respectively. In parallel, sensitivities to two other HIV INSTIs, namely, the DKA analogue CHI-1043 and the clinical trial drug GS-9137, were determined. The inhibitory effects of the RT inhibitors zidovudine and nevirapine, the protease inhibitor ritonavir, and the entry inhibitors AMD3100 and DS were evaluated as well. HIV-1(IIIb) selected for 20 passages in the presence of L-870,810 [IIIb/L-870,810(#20)] displayed 23-fold resistance against L-870,810. The occurrence of the L74M mutation in the virus population at passage 40 resulted in 34-fold resistance against L-870,810, while IIIb/L-870,810(#60) showed 110-fold resistance against L-870,810. Only low-level cross-resistance was observed against the novel IN strand transfer inhibitor CHI-1043. The triple mutant was ninefold resistant against this DKA analogue. Interestingly, high-level (33-fold) cross-resistance to the clinical trial drug GS-9137 was observed with the E92Q S230N double mutant strain [IIIb/L-870,810(#20)]. Addition of the L74M mutation increased cross-resistance to GS-9137 even further (77-fold) [IIIb/L-870,810(#60)]. The compounds AMD3100, DS, zidovudine, nevirapine, and ritonavir remained equally active against the IIIb/L-870,810 strains (Table 2).

Notably, the concentrations of L-870,810 used to select the resistant strains were higher than the IC_{50}s measured for the recovered mutant viruses (Fig. 2). This may be due to differences in MOI (average number of infectious HIV-1 particles per cell). The MOI during the selection process was 100-fold higher than the MOI in the MT-4 cell–MTT test. A higher MOI (i.e., larger number of infectious HIV-1 particles/cell) requires a higher concentration of compound to protect the cells.

Evaluation of IN-recombined HIV-1 strains by use of MT-4 cell–MTT assay. To evaluate the importance of the described mutations in IN for the observed phenotype, we recombined a PCR product of the IN gene of the selected HIV-1 population in a WT backbone, using chimeric virus technology (12). IN recombination was performed with the following strains: HIV-1(IIIb) and the HIV-1(IIIB) strains selected during 20, 40, and 60 passages in the presence of L-810,810 [IIIb/L-870,810(#20), IIIb/L-870,810(#40), and IIIb/L-870,810(#60), respectively]. The recombinant strains are referred to as RIN/IIIb, RIN/L-870,810(#20), RIN/L-870,810(#40), and RIN/L-870,810(#60), respectively. These recombinative virus populations have various frequencies of the identified IN mutations, depending on the frequencies of the IN mutations in the selected virus populations. We verified the proportion of each mutation in the recombinated strains by DNA sequencing; the recombinated strains showed similar proportions of each mutation to those of the parental strains (data not shown). In addition, we constructed the molecular clones NL4.3 L74M, NL4.3 E92Q, and NL4.3 S230N, carry-
HIV-1 resistance to integrase inhibitors

HIV-1(IIIB) wild-type strain recombined with the integrase gene of HIV-1(IIIB).

**TABLE 2. Susceptibilities of selected and recombinant HIV-1 strains to various antiviral compounds as evaluated in MT-4 cells**

| Viral strain | Entry | RT | Neopterin | L-870,810 | CHI-1043 | AMD3100 | Zidovudine | Nevirapine | L-870,810,810 CHI-1043 MK-0518 GS-9137 |
|-------------|-------|----|-----------|----------|----------|---------|------------|------------|-------------------------------------|---------------------|
| NL4.3       | ND    | ND | ND        | ND       | ND       | ND      | ND         | ND         | ND                   | ND                  |
| NL4.3 S230N  | ND    | ND | ND        | ND       | ND       | ND      | ND         | ND         | ND                   | ND                  |
| NL4.3 E92Q   | ND    | ND | ND        | ND       | ND       | ND      | ND         | ND         | ND                   | ND                  |
| IIIB/L-870,810(#60) | 0.006  | 0.001 | 0.002 | 0.002 | 0.002  | 0.001  | 0.005 | 0.006 | 0.001  | 0.006  |
| IIIB/L-870,810(#40) | ND    | ND | ND        | ND       | ND       | ND      | ND         | ND         | ND                   | ND                  |
| IIIB/L-870,810(#20) | ND    | ND | ND        | ND       | ND       | ND      | ND         | ND         | ND                   | ND                  |

**Antiviral susceptibilities of the IN-recombined strains and the single mutant strains were determined by the MT-4 cell MTT assay (Table 2).** The INSTI MK-0518 was included in this setup. The increase in resistance of the IN-recombined strains RIN/L-870,810(#20), RIN/L-870,810(#40), and RIN/L-870,810(#60) with respect to that of the WT recombined strain RIN/IIIB mirrored the increased resistance of the corresponding parental selected strains compared to HIV-1(IIIB) against all antiviral compounds evaluated. More specifically, RIN/L-870,810(#20), RIN/L-870,810(#40), and RIN/L-870,810(#60) showed 4.5-, 16-, and 47.5-fold resistance, respectively, against L-870,810. These data indicate that the observed mutations in IN are responsible for the observed phenotypic resistance to L-870,810 and the cross-resistance to CHI-1043 and GS-9317. In addition, the triple mutant recombinant strain [RIN/L-870,810(#60)] showed low-level cross-resistance to the clinical trial drug MK-0518 (11-fold). By evaluating the single mutant viral clones, we determined the importance of each individual mutation for the phenotypic profile. The E92Q IN mutation appeared to be the most important for phenotypic (cross-)resistance, conferring 18.3-fold, 3.0-fold, and 17.1-fold resistance against L-870,810, MK-0518, and GS-9137, respectively (Table 2). The L74M and S230N IN mutations by themselves did not impact the antiviral susceptibility of HIV-1 NL4.3.

**Resistance to L-870,810 is accompanied by reduced viral replication kinetics and reduced enzymatic activity of integrase.** To investigate whether the selected IN mutations affect viral replication kinetics, the HIV-1 strain selected in the presence of L-870,810 for 60 passages [IIIB/L-870,810(#60)] and its respective recombinant strain [RIN/L-870,810(#60)] were examined for the ability to replicate in MT-4 cells in comparison with parental HIV-1(IIIB) and RIN/IIIB, respectively (Fig. 3A). The replication kinetics of IIIB/L-870,810(#60) and RIN/L-870,810(#60) were reduced in comparison with those of parental HIV-1(IIIB) and RIN/IIIB. In addition, we evaluated the molecular clones NL4.3 L74M, NL4.3 E92Q, and NL4.3 S230N, carrying single IN mutations. Both the E92Q and S230N mutant strains showed comparable replication kinetics to those of WT NL4.3, whereas NL4.3 L74M showed a slight reduction in its replication capacity (Fig. 3B). This relative order was observed in at least two independent experiments.

To characterize the reduced fitness of the virus carrying the triple substitution in integrase in more detail, we studied the impact of the different IN mutations on the enzymatic level, using an oligonucleotide-based integration assay (Fig. 4A and B). The WT enzyme was most active at 1 μM. Both mutants tested, i.e., IN-E92Q/S230N and the triple mutant IN-L74M/E92Q/S230N, showed optimum activity at a concentration of 500 nM. Still, their enzymatic activities were twofold to fourfold lower than that of WT IN (500 nM and 1 μM, respectively), as quantified by measuring strand transfer products with ImageJ (Fig. 4B). The reduced activity of IN at higher concentrations was possibly due to aggregation.

**3D mapping of mutant HIV-1 IN.** A 3D model illustrating the described mutations has been built (Fig. 5A). Positions 74 and 92 are situated in close proximity to each other and to the active site, while position 230 is located in the DNA-binding...
For comparison, 3D mapping of the previously in vitro-selected resistance mutations to L-870,810 (14), the clinical resistance mutations to MK-0518, and the in vitro-selected resistance mutations to GS-9137 was done as well (Fig. 5B to D). Apparently, mutations that confer resistance to current INSTIs generally cluster in the same region in integrase, i.e., at the catalytic site defined by the DDE residues and the residues that are involved in DNA binding (Fig. 5E and F).

**DISCUSSION**

The development of new antiretroviral drugs directed against novel targets with a high genetic barrier to resistance and with improved safety and tolerability is urgently needed for both treatment-naive and treatment-experienced patients, especially those with multidrug-resistant HIV. Next to reverse transcriptase and protease, the two viral enzymes targeted by current antiretroviral therapy, HIV carries a third enzyme, integrase. DKAs were the first compounds reported to interfere with HIV replication through specific inhibition of the strand transfer step of integration (15). At present, MK-0518 has received FDA approval for use in the clinic and GS-9137 is in late-phase clinical trials. Clinical data clearly show that these INSTIs strongly reduce viral load. Still, early clinical data point to the emergence of strains resistant against these INSTIs (6, 16, 32, 36). Accordingly, detailed study of antiviral (cross-) resistance development toward these inhibitors in cell culture is warranted.
Previously, Hazuda and coworkers selected HIV-1 strains in the presence of the naphthyridine carboxamide L-870,810 (14). L-870,810-resistant strains carried the mutations V72I, F121Y, T125K, and V151I in the integrase gene. Only minor cross-resistance against DKAs was observed (14). We have again selected HIV-1(IIIB) strains in the presence of L-870,810 and analyzed the strains (IIIB/L-870,810) genotypically and phenotypically at different stages throughout the selection process. Three mutations, L74M, E92Q, and S230N, emerged successively in integrase in comparison with the WT HIV-1(IIIB) sequence. A 3D model mapping the three mutations associated with resistance to L-870,810 is shown in Fig. 5A. The S230N mutation is located in the C-terminal domain of integrase, at a distance from the active site in the IN monomer.

FIG. 5. HIV-1 integrase mutations leading to resistance to current INSTIs. Integrase models indicate the positions of the mutations associated with resistance toward specific INSTIs. The models were constructed based on the published partial X-ray integrase structures 1EX4 (4) and 1K6Y (37). Active-site residues (D64, D116, and E152) are highlighted in red, and metal ions are shown in gray. (A) Resistance mutations against the naphthyridine L-870,810, selected by us. (B) Resistance mutations to L-870,810 selected by Hazuda et al. (14). (C) Two distinct pathways of resistance mutations selected in vivo during phase II clinical trials with MK-0518 (green and violet) (6, 32, 36). Dark-colored mutations define major mutations, and light-colored mutations define additional minor mutations. (D) Resistance mutations selected in vitro toward the clinical trial drug GS-9137 (21). The two major pathways are colored again in green and violet, and major mutations are presented in dark colors, with minor mutations in light colors. (E) IN complexed with viral DNA, in analogy with the Tn5 transposase-DNA cocrystal (33). Amino acid residues implicated in viral DNA binding are shown in yellow, and active-site residues are highlighted in red. (F) Hypothetical binding of viral (green) and host (red) DNA to the integrase.
the context of the active tetramer, this position is close to the active site (30). The C-terminal domain (residues 213 to 288) has been reported to be important for binding of viral and possibly host DNA (10, 17, 20). As such, this mutation might influence the binding of the compound indirectly, through alteration of the IN–viral cDNA complex. The L74M and E92Q mutations are located in the catalytic core domain, proximal to the active-site residues that coordinate divalent metals (D64, D116, and E152). A water-mediated hydrogen bond has been proposed to occur between E92 and D116 (8). We believe that substituting glutamate for glutamine at position 92 will not affect this bond, since one oxygen molecule in glutamine remains available to make this bond. On the other hand, based on the modeling data, we suggest that this substitution can directly influence the L-870,810 pocket and, as such, the interaction of the compound with IN. The L74M mutation, which lies very close to the active-site residues, may shift the active-site residues, thereby altering the affinity or position of the metals. The selection of these mutations at key positions in IN corroborates the proposed mechanism of action of the DKA and naphthyridine analogues, i.e., inhibiting integrase at its interface with divalent metals and DNA (14). The mutations observed are distinct from those detected by Hazuda and co-workers (V72I, F121Y, T125K, and V151I) (14) but map within the same region of integrase (compare Fig. 5A and B). This may reflect different mechanisms through which resistance to IN strand transfer inhibitors is manifested. Indeed, based on knowledge of the mode of action of current INSTIs and the 3D mapping data (Fig. 5), we propose three main mechanisms through which resistance to current INSTIs may occur, as follows: (i) IN mutations may directly interrupt the binding of the inhibitor to the host DNA acceptor site in IN (e.g., E92Q), (ii) IN mutations may influence the positions of the Mg\(^{2+}\) ions and thereby interfere with the chelating function of the inhibitor (e.g., T66I, L74M, and F121Y), and (iii) IN mutations may indirectly interrupt the binding of the INSTI by altering IN-viral DNA complex formation (e.g., G140S, S147G, Q148K/R/H, V151I, S153Y, and S230N).

The S230N mutation has not yet been associated with in vitro resistance to INSTIs, but the related mutation S230R has been associated with in vitro resistance to L-807,906 (12) and in vivo resistance to MK-0518 (6, 32, 36) (Table 1). The L74M mutation has already been associated with in vitro resistance to the DKA analogues L-708,906 (12) and S-1360 (11) and with resistance to the clinical trial drug MK-0518 in patients (6, 16, 32, 36). The E92Q mutation has also been associated with in vitro resistance to GS-9137 (21) and with resistance to MK-0518 in patients (6, 16, 32, 36) (Table 1). Apparently, some of the selected mutations (i.e., L74M and E92Q) were detected in individuals enrolled in clinical trials with the INSTI MK-0518 who experienced viral breakthrough (6, 16, 32, 36). This supports the clinical relevance of our in vitro selection experiment with the INSTI L-870,810. Furthermore, our findings indicate that these mutations can be induced by the different classes of strand transfer inhibitors developed to date. Possibly, this is due to similar binding modes in the active site of the integrase. Indeed, 3D mapping of the mutations associated with resistance to L-870,810, MK-0518, and GS-9137 (Fig. 5A to F) shows that most mutations are clustered around the catalytic triad that coordinates the divalent metals and the residues involved in DNA binding, supporting the idea of at least partly overlapping binding modes. Although it was reported that mutations conferring resistance to the DKAs and naphthyridines map to distinct locations in the active site (14), the L74M mutation, already reported to be associated with DKA resistance (11, 12), suggests otherwise.

Our genotypic data point to cross-resistance among the different classes of strand transfer inhibitors developed to date, and we corroborated this concept at the phenotypic level. The triple mutant virus displayed high-level in vitro phenotypic resistance against L-870,810 (110-fold). Only low-level cross-resistance against the novel DKA analogue CHI-1043 and MK-0518 was observed, but high-level (77-fold) cross-resistance against the clinical trial drug GS-9137 was detected. By constructing recombinant strains and single mutant clones, we investigated the significance of each mutation for the phenotypic resistance profile. The E92Q IN mutation appears to be the most important for phenotypic (cross-)resistance, conferring 18.3-fold, 3.0-fold, and 17.1-fold resistance against L-870,810, MK-0518, and GS-9137, respectively (Table 2). This is in agreement with the observation that the E92Q mutation is also selected in vitro in the presence of GS-9137 (21, 23) and in patients who fail raltegravir therapy (16). Although the single L74M or S230N IN mutation did not impact the antiviral susceptibility of HIV-1 NL4.3 against various INSTIs, the addition of the mutation at position 74 increased resistance (Table 2).

Next, we investigated the impact of the drug-induced mutations on viral replication capacity and enzymatic activity. The selected and recombined strains were examined for the ability to replicate in MT-4 cells in comparison with their respective WT strains. All triple mutant strains showed a reduced replication capacity in comparison with their respective WT strains (Fig. 3A). Although the single E92Q or S230N IN mutation did not impact viral replication capacity, the mutation at position 74 did reduce viral replication capacity to some extent (Fig. 3B). Previously, it was reported that the single mutant enzymes IN-L74M (12), IN-E92Q (8), and IN-S230N (18) maintain nearly WT levels of enzymatic activities. These data are in good correlation with our virological data. We generated recombinant enzymes containing the double or triple mutation in order to characterize the additive effect of the different mutations on enzymatic activity in an oligonucleotide-based assay. Overall enzymatic activities of both the double mutant and triple mutant enzymes were reduced twofold in comparison to that of WT integrase (Fig. 4A and B). A comparable attenuation of viral growth and IN enzymatic activity has been reported previously for IN mutations associated with antiviral resistance against DKA (11, 12, 15).

In conclusion, the accumulation of L74M, E92Q, and S230N mutations in IN coincided with resistance against L-870,810, the prototype of the naphthyridine analogues. Interestingly, recent studies investigating natural variation of HIV-1 group M integrases showed that both L74 and E92 are nonpolymorphic (26, 40) and S230 is polymorphic (25, 38). Apparently, amino acid polymorphisms, which may confer resistance to current integrase inhibitors, do occur in infected patients, although major mutations within the catalytic domain and extended active sites that are associated with INSTI resistance are infrequent (25). Still, studies evaluating whether genetic
background affects the efficacy of future antiretroviral treatment are warranted. From a clinical point of view, we predict that cross-resistance may readily occur between the different INSTIs. Whether the reduction in viral replication capacity associated with the emergence of the observed mutations will jeopardize viral resistance in vivo will have to be determined. Careful monitoring of the emergence of integrase mutations and antiviral resistance in clinical trials, expanded-access programs, and treatment regimens with INSTIs is recommended. Our data add to the understanding of INSTIs and antiviral (cross)-resistance development against INSTIs and should be helpful in the development of new IN inhibitors with an increased genetic barrier to resistance.

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