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Articles

Comparison of Raltegravir and Elvitegravir on HIV-1 Integrase Catalytic Reactions and on a Series of Drug-Resistant Integrase Mutants[†]

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ABSTRACT: HIV-1 integrase (IN) is the molecular target of the newly approved anti-AIDS drug raltegravir (MK-0518, Isentress) while elvitegravir (GS-9137, JTK-303) is in clinical trials. The aims of the present study were (1) to investigate and compare the effects of raltegravir and elvitegravir on the three IN-mediated reactions, 3′-processing (3′-P), strand transfer (ST), and disintegration, (2) to determine the biochemical activities of seven IN mutants (T66I, L74M, E92Q, F121Y, Q148K, S153Y, and N155H) previously selected from drug-resistant patients and isolates, and (3) to determine the resistance profile for raltegravir and elvitegravir in those IN mutants. Our findings demonstrate that both raltegravir and elvitegravir are potent IN inhibitors and are highly selective for the ST reaction of IN. Elvitegravir was more potent than raltegravir, but neither drug could block disintegration. All resistance mutations were at least partially impaired for ST. Q148K was also markedly impaired for 3′-P. Both drugs exhibited a parallel resistance profile, although resistance was generally greater for elvitegravir. Q148K and T66I conferred the highest resistance to both drugs while S153Y conferred relatively greater resistance to elvitegravir than raltegravir. Drug resistance could not be overcome by preincubating the drugs with IN, consistent with the binding of raltegravir and elvitegravir at the IN−DNA interface. Finally, we found an inverse correlation between resistance and catalytic activity of the IN mutants.

The high mutation rate in the HIV-1 viral genome and the accumulation of virus in persistent reservoirs during HIV-1 infection prevent complete virus eradication in patients treated with current treatment strategies. HAART (highly active antiretroviral therapy) is based on the association of inhibitors targeting the HIV reverse transcriptase and protease viral enzymes, and this cocktail has transformed AIDS from a fatal to a chronic disease. However, prolonged therapy usually leads to drug resistance. One way to overcome such resistances and to reduce therapeutic side effects is to identify novel targets for therapy.

In October 2007, the new pyrimidinone carboxamide raltegravir (MK-0518, Isentress, Merck & Co., Figure 1D) became the first integrase (IN)[1] inhibitor approved by the FDA for the treatment of HIV-1 infection in treatment-experienced adult patients who have evidence of viral replication and HIV-1 strains resistant to multiple antiretroviral agents (http://www.fda.gov/bbs/topics/NEWS/2007/NEW01726.html). Elvitegravir (GS-9137, JTK-303, Figure

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[1] Abbreviations: IN, HIV-1 integrase; CCD, catalytic core domain; 3′-P, 3′-processing; ST, strand transfer.

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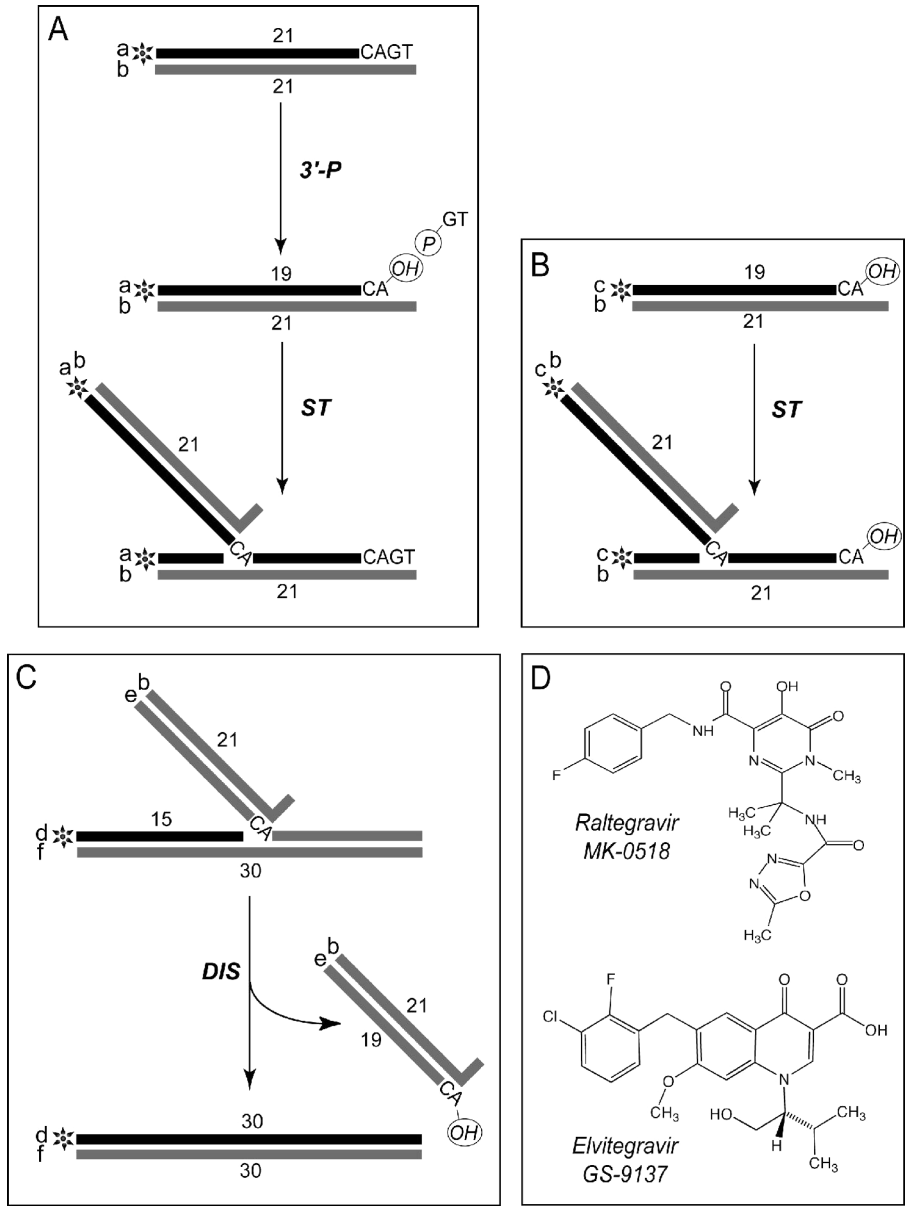

FIGURE 1: Schematic representation of the three integrase reactions and assays used to test raltegravir and elvitegravir. (A) Schematic representation of the 3′-processing (3′-P) and strand transfer (ST) reactions using full-length DNA substrate. (B) Schematic representation of the ST assay using precleaved substrate. (C) Schematic representation of the disintegration reaction (reverse of the ST reaction). Sequences are reported in Table 1, and asterisks indicate the 5′-end labeling with ^{32}P. (D) Structures of raltegravir and elvitegravir.

Table 1: Oligonucleotide Sequences Used in Assays

oligonucleotide	size	sequence
a	21-mer	5′-GTG TGG AAA ATC TCT AGC AGT-3′
b	21-mer	5′-ACT GCT AGA GAT TTT CCA CAC-3′
c	19-mer	5′-GTG TGG AAA ATC TCT AGC A-3′
d	15-mer	5′-GAA AGC GAC CGC GCC-3′
e	34-mer	5′-GTG TGG AAA ATC TCT AGC AGG GGC TAT GGC GTC C-3′
f	30-mer	5′-GGA CGC CAT AGC CCC GGC GCG GTC GCT TTC-3′

1D, Gilead Sciences), a quinolone carboxylic acid, is the other IN inhibitor in advanced stage of human clinical trials (*1*). Both raltegravir and elvitegravir have been reported as ST-selective inhibitors (*2, 3*).

HIV-1 IN, one of the three viral enzymes encoded from the POL gene, catalyzes the insertion of viral cDNA into host chromosomes (*4*−*6*). This reaction consists in two sequential steps: first, following reverse transcription, the 3′-terminal nucleotides (generally a dinucleotide) are removed from both 3′-ends of the HIV-1 DNA genome immediately 3′ from their conserved CA sequence (3′-processing, 3′-P), and second, the 3′-processed DNA ends are integrated into a host genome (strand transfer, ST) (see Figure 1A). It is possible to study each of these reactions *in vitro* using oligonucleotides that mimic the terminal portion of the U5 viral long terminal repeat (LTR) and recombinant IN (*6*−*8*) (Figure 1).

Despite major clinical activity of raltegravir in the treatment of multidrug refractory patients, ongoing studies have already evidenced mutations in the IN gene associ-

ated with therapeutic failure in patients receiving raltegravir [Merck Protocol 005 (*2, 9*)]. Resistance to raltegravir and elvitegravir has been associated with specific IN mutations. For raltegravir the two main mutations are N155H or Q148H/R/K with 10- and 25-fold *in vivo* resistance, respectively (*9*). Additional mutations have been reported (L74M, E92Q, E138K, G140S/A, and G163R) (*9*). For elvitegravir, T66I and E92Q are the two mutations that contribute the most to resistance (37- and 36-fold reduced susceptibility to elvitegravir, respectively) (*10*). Other mutations reported for elvitegravir are H51Y, T66I, Q95K, E138K, Q146P, S147G, and E157Q (*10*).

The aim of the present study was to compare raltegravir and elvitegravir, which represent the most advanced and clinically used inhibitors of IN. We tested both drugs side by side to determine their relative potency and to determine their selectivity for ST versus 3′-P and disintegration against wild-type IN. After expression of selected IN mutants reported to be involved in the resistance of one or both drugs, we also evaluated the impact of these single amino acid substitutions on IN biochemical catalytic activities and studied their potential reduction in sensitivity to raltegravir and elvitegravir. Our study is to our knowledge the first to compare side by side these two novel IN inhibitors and to elucidate their cross-resistance profile against recombinant IN with selected mutations.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis and Drugs. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). All oligonucleotides were purified on denaturing 20% polyacrylamide gels. Single-stranded oligonucleotides were 5′-labeled using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) with [γ-^{32}P]ATP (Perkin-Elmer Life and Analytical Sciences, Boston, MA) according to the manufacturers' instructions. Unincorporated nucleotides were removed by Mini Quick Spin Oligo Columns (Roche). The duplex DNA was annealed by addition of an equal concentration of the complementary strand, heating to 95 °C, and slow cooling to room temperature. Sequence of the oligonucleotides and use are indicated in Table 1 and Figure 1, respectively.

Raltegravir (MK-0518) was purified directly from pharmaceutically available tablet formulation (both extraction and HPLC purification was performed). Elvitegravir (JTK-303) was synthesized in a similar manner to known procedures and purified via preparative HPLC. Purity according to LCMS analysis was >95%. ^{1}H NMR (DMSO-d_6, 400 MHz) (δ) ppm: 0.69 (d, J = 6.8 Hz, 3H), 1.10 (d, J = 6.4 Hz, 3H), 2.25−2.39 (m, 1H), 3.70−3.76 (m, 1H), 3.88−3.97 (m, 1H), 3.99 (s, 3H), 4.05 (s, 2H) 4.78−4.84 (m, 1H), 5.13−5.18 (m, 1H), 7.10−7.20 (m, 2H), 7.38−7.44 (m, 2H), 7.96 (s, 1H), 8.81 (s, 1H). LRMS calculated for $C_{23}H_{23}ClFNO_5$ (M + H), 448.1; found, 448.1.

Mutagenesis. IN mutants were created using the Stratagene QuikChange Site-Directed Mutagenesis Kit (La Jolla, CA), according to the manufacturer's recommendations. The presence of desired mutations and the integrity of the remainder of the IN sequence were verified by DNA sequencing.

Integrase Purification. Recombinant wild-type or mutant IN polypeptides were purified from *Escherichia coli* as described previously (*11*).

Integrase Reactions. IN reactions were carried out by mixing 20 nM DNA with 400 nM IN (unless otherwise indicated) in a buffer containing 20 mM MOPS, pH 7.2, 7.5 mM $MgCl_2$, 14 mM 2-mercaptoethanol, and the drug of interest or 10% DMSO. Reactions were incubated at 37 °C for 1 h and quenched by addition of an equal volume of gel loading dye (formamide containing 1% SDS, 0.25% bromophenol blue, and xylene cyanol). Reaction products were separated in 16% polyacrylamide denaturing sequencing gels. Dried gels were visualized using the Typhoon 8600 (GE Healthcare, Piscataway, NJ). Densitometry analyses were performed using ImageQuant software from GE Healthcare.

RESULTS

Comparison of Effects of Raltegravir and Elvitegravir on ST, 3′-P, and Disintegration Reactions Mediated by Wild-Type Integrase. To investigate the influence of raltegravir and elvitegravir on wild-type IN activity, both drugs were compared for their influence on 3′-P and on ST in a gel-based assay that uses a 21 bp oligonucleotide duplex mimicking the terminal U5 sequence of the HIV-1 LTR (Figure 1A) (*7*) (*6, 8, 12*). Drug inhibition assays were also performed using a precleaved substrate, in which the terminal GT dinucleotide, following the conserved CA dinucleotide, has been removed (Figure 1B). Therefore, the direct effect of the drugs on ST could be monitored without any interference of 3′-P. Finally, the influence of the compounds on the disintegration reaction, a reverse ST reaction catalyzed by IN (*13*), was followed by using a Y-shaped DNA substrate (Figure 1C).

Representative gels in Figure 2 (panels A and C) and average quantitation of independent experiments (panel B) demonstrate concentration-dependent inhibition of IN-mediated ST and 3′-P activities by both drugs. Those data also show more intense interference of elvitegravir compared to raltegravir. IC_{50} determined using drug concentration ranges of 0.002−37 μM are reported in Table 2. The ST IC_{50} values derived using full-length substrate are lower than those obtained with precleaved. The drugs show therefore a greater inhibition effect in presence of full-length substrate. Our data demonstrate also that the inhibitory effect of the drugs on ST is not due to an inhibition of 3′-P. This conclusion is consistent with the fact that the inhibition curves for ST did not overlap with the inhibition curves for 3′-P (Figure 2B). The more than 100-fold ratios between IC_{50}'s for ST and 3′-P indicate the high specificity of both compounds to interfere with ST under conditions where 3′-P remains fully effective. Comparison of the two drugs in both assays [full-length vs precleaved substrates (compare panels A and C in Figure 2 and see summary in Table 2)] consistently showed that elvitegravir was able to inhibit ST activity of IN at a concentration 3-fold lower than raltegravir. Elvitegravir was also slightly more potent as a 3′-P inhibitor (Figure 2B and Table 2). Thus, our data show that elvitegravir is slightly more potent than raltegravir against recombinant IN *in vitro* and demonstrate the high specificity of both compounds for discriminating ST vs 3′-P. Thus, the two catalytic steps (ST and 3′-P) are affected at different concentrations for both raltegravir and elvitegravir, which is consistent with prior reports concluding that both raltegravir and elvitegravir are selective ST inhibitors (*14, 15*).

We next determined whether raltegravir and elvitegravir were able to inhibit disintegration. The branched Y-shaped double-stranded oligonucleotide that mimics a half-integration ST product (*13*) is shown in Figure 1C. Figure 3 shows a representative gel demonstrating the inability of raltegravir to inhibit disintegration at concentrations up to 111 μM,

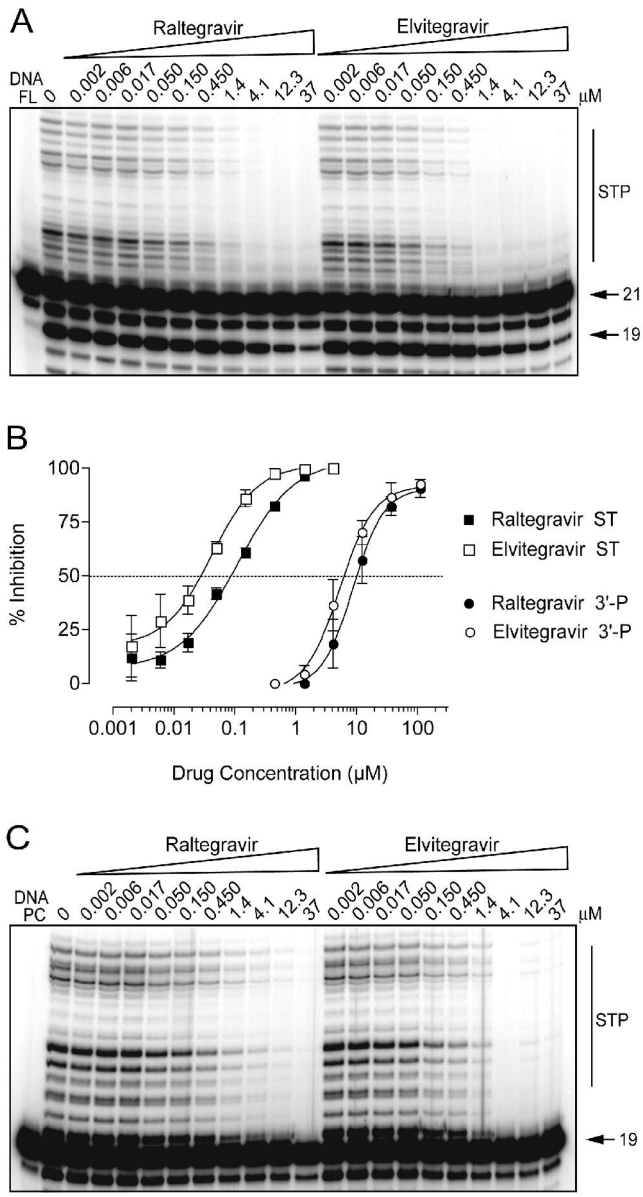

FIGURE 2: Comparative inhibition of wild-type HIV-1 integrase by raltegravir and elvitegravir. (A) Gel image of a typical dose−response experiment using full-length DNA substrate (FL) (see Figure 1A). Bands labeled 21, 19, and STP correspond to DNA substrate, 3′-processing, and strand transfer products, respectively. (B) Quantitation from densitometry analyses of gels performed as in panel A. Error bars indicate SD for at least four independent experiments. (C) Gel image of a typical dose−response experiment using precleaved DNA substrate (PC) (see Figure 1B). Quantitations from at least four independent experiments are summarized in Table 2.

Table 2: Raltegravir and Elvitegravir IC_{50} Value (μM) against IN Wild-Type Activities[a]

	raltegravir	elvitegravir
3′-P	12.79 ± 6	8.06 ± 4.27
ST (FL)	0.087 ± 0.008	0.028 ± 0.006
ST (PC)	0.190 ± 0.056	0.054 ± 0.013
disintegration	>111	>37

[a] Abbreviations: FL, full-length substrate; PC, precleaved substrate (see panels A and B of Figure 1, respectively).

which are at least 3 orders of magnitude above the ST IC_{50} values. Similarly, elvitegravir was ineffective in disintegration reactions (minor inhibition was detectable above 37 μM).

Thus, we conclude that both raltegravir and elvitegravir

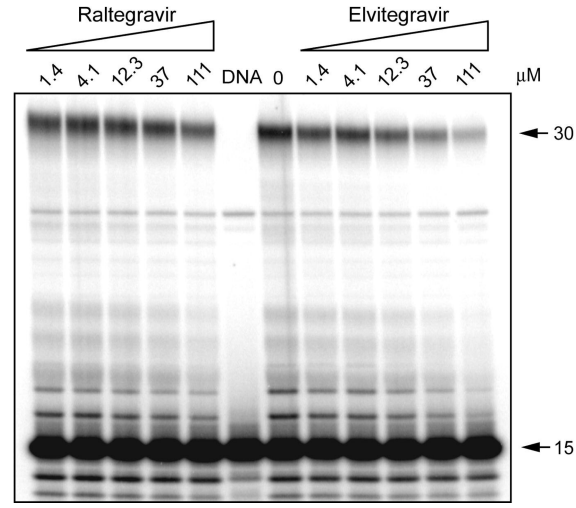

FIGURE 3: Weak or lack of inhibition of wild-type HIV-1 integrase-mediated disintegration by raltegravir and elvitegravir. Bands labeled 15 and 30 correspond to disintegration substrate and product, respectively (see Figure 1C).

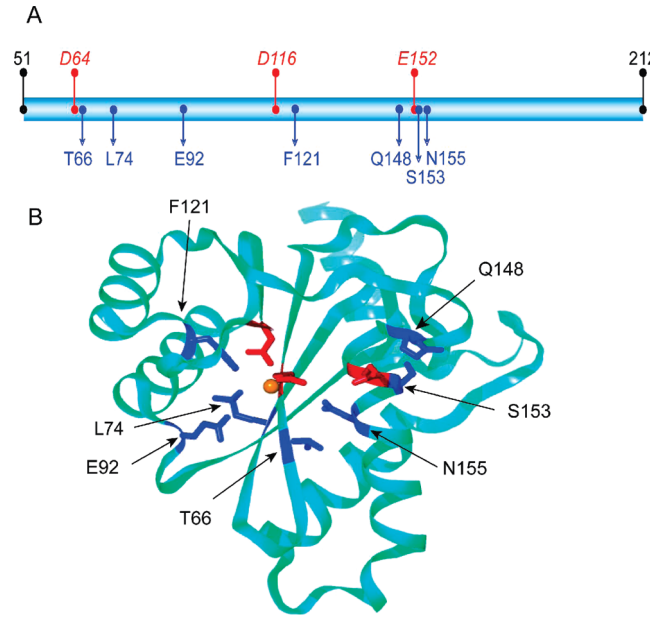

FIGURE 4: HIV-1 integrase core domain residues associated with resistance to raltegravir or elvitegravir. (A) Linear representation of the HIV-1 integrase catalytic core domain (CCD). Catalytic acidic residues (DDE motif) are shown in red. The seven residues mutated in drug-resistant viruses and selected for comparison with wild-type HIV-1 integrase are shown in dark blue. (B) Three-dimensional representation of the HIV-1 integrase CCD. The catalytic residues (red) and the seven selected mutations (dark blue) are shown as stick representation in the crystal structure of the CCD (PDB code 1BI4) (*29*).

are highly specific ST inhibitors. The ranking order for specificity is ST ≫ 3′-P ≫ disintegration (with disintegration being practically immune to the drugs). Moreover, elvitegravir is 2−3-fold more potent than raltegravir in the *in vitro* reactions with recombinant wild-type IN.

Enzymatic Activities of Selected Integrase Drug-Resistant Mutants. We next expressed and analyzed the activity of integrase mutants reported in literature as drug-resistant: T66I, L74M, E92Q, F121Y, Q148K, S153Y, and N155H (*9, 10, 16*−*20*). Figure 4A shows the schematic distribution of the chosen mutations along the catalytic core domain (CCD) of integrase. Examination of the spatial arrangement

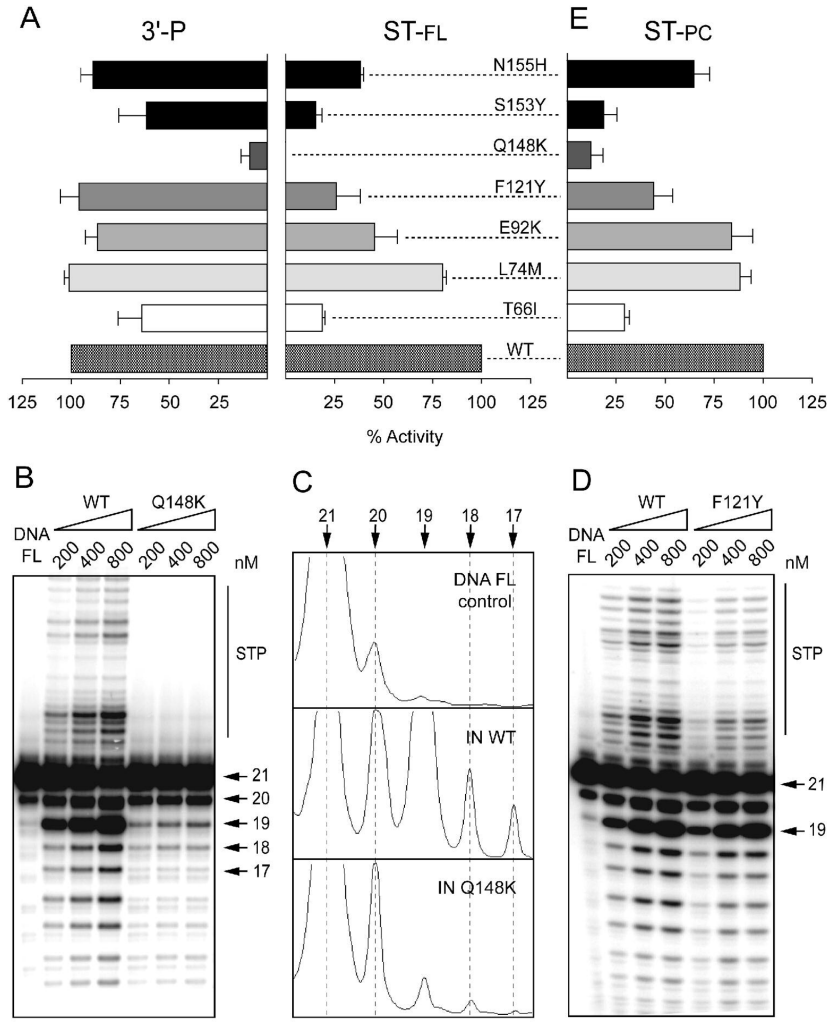

FIGURE 5: Defective catalytic activities of the drug-resistant HIV-1 integrase mutants compared to wild-type HIV-1 integrase (WT). (A) Comparative activities in assays performed with the full-length blunt-ended substrate (FL) (see Figure 1A). Left and right panels: 3′-P and ST activities, respectively. (B) Gel image of typical 3′-P and ST reactions using three different concentrations of Q148K and showing defective ST and 3′-P activities of the Q148K mutant HIV-1 integrase. Increasing enzyme concentrations are indicated above the gel. (C) Defective 3′-P of the Q148K mutant shown as densitometry analysis of the gel presented in panel B (lanes 1, 4, and 7). DNA bands are indicated on top and on dotted lines and correspond to annotation in panel B. (D) Gel image of a typical reaction and showing selective defect for ST vs 3′-P for the F121Y mutant HIV-1 integrase. (E) Strand transfer defect of the integrase mutants (normalized to WT integrase) using the precleaved DNA substrate (PC) (see Figure 1B for assay description).

of those selected mutations in the crystal structure of the CCD of IN (Figure 4B) shows their distribution around and their relative proximity to the triad of catalytic acidic residues of IN (DDE motif shown in red with divalent metal in orange, Figure 4B).

We first compared the enzymatic activities of the IN mutants using the dual ST and 3′-P assay (Figure 5A; see schematic description of the assay in Figure 1A). Plotting all the data after quantification analyses and normalization to WT IN (Figure 5A) shows that the first step catalyzed by IN (3′-P) was only marginally impaired in most mutants. The only mutation that markedly impacted 3′-P was Q148K. The Q148K mutant IN was almost completely unable to catalyze 3′-P. Conversely, the second integration step (ST) was defective for most of the mutant enzymes (Figure 5A, right panel). The ranking order for the most defective mutants for ST in the dual 3′-P and ST assay was Q148K, S153Y, T66I, F121Y, N155H, E92Q. The L74M mutant was the only one whose ST and 3′-P activities were close to WT IN.

Representative 3′-P products for the defective Q148K mutant are shown in Figure 5B,C. A noticeable defect of this mutant was the prevalence of a 20-mer product corresponding to 3′-terminal mononucleotide cleavage. Such product is also produced by wild-type IN, although to a lesser extent than the canonical 19-mer 3′-P processing product. Densitometry tracing for the Q148K mutant (Figure 5C) shows this nonprocessive reaction product (which does not lead to ST) resulting from excision of the last base at the 3′-end of the DNA. This 3′-mononucleotide cleavage reaction was also seen with wild-type IN (compare middle and top panels in Figure 5C and gel images in Figure 5B,D) and the other mutants (example in Figure 5D). Additional experiments with a topoisomerase I DNA substrate that does not bear sequence similarity with the HIV-U5-LTR showed lack of 3′-cleavage for the topoisomerase I substrate (data not shown), indicating that the 3′-mononucleotide cleavage reaction is dependent on the HIV-LTR sequence.

Among all IN mutants, the F121Y mutant was the most deficient for ST, while retaining normal 3′-P (see Figure 5A). However, the integration pattern remained unaltered (Figure 5D). Thus, the mutations leading to ST deficiency appear to affect the efficiency of ST but have no detectable impact on ST integration patterns. Further analyses of ST deficiencies for the mutants were performed with the precleaved substrate

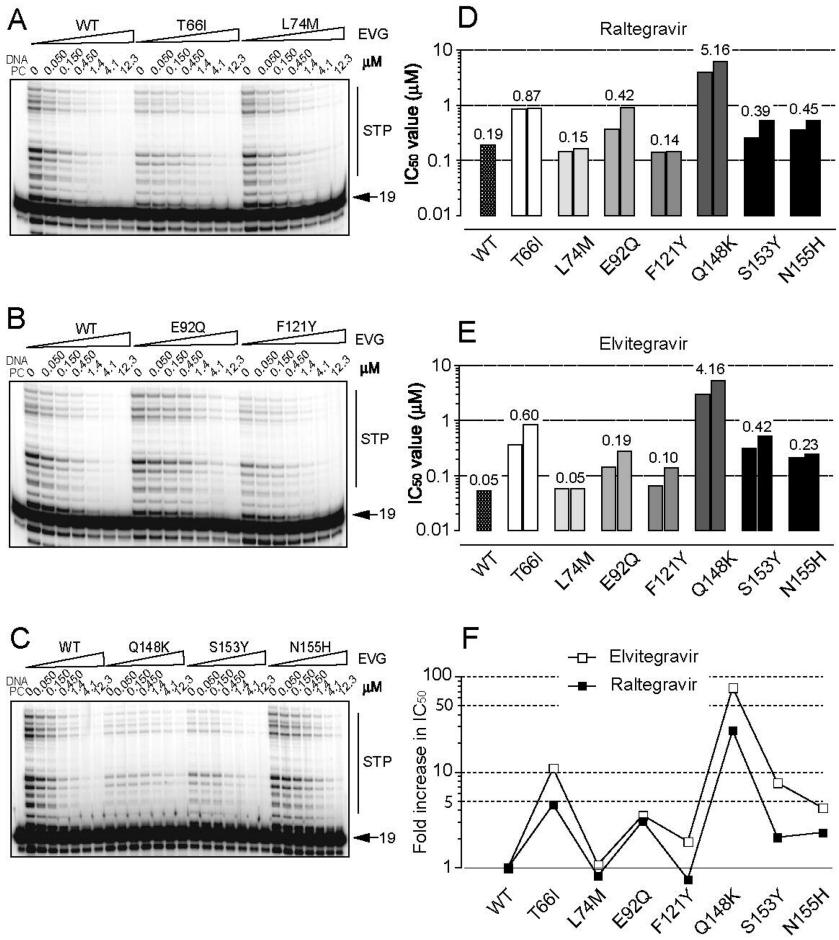

FIGURE 6: Comparative impact of the HIV-1 integrase mutations on integrase strand transfer inhibition by raltegravir and elvitegravir. (A−C) Representative gel images of elvitegravir (EVG) concentration−response experiments using precleaved DNA substrate (PC) (see Figure 1B). Wild-type (WT) HIV-1 integrase and the mutants are indicated above the gel pictures. (D, E) Comparison of drug IC_{50} value derived from gels performed as in panels A−C. Values for WT enzyme were derived from the mean of at least 10 experiments. Values for mutant HIV-1 integrase are reported directly on the graph for two independent experiments. IC_{50} values (μM; mean of at least two independent experiments) are indicated above bars. (F) Graphical representation of the patterns of cross-resistance for raltegravir and elvitegravir. Values were derived from experiments performed as described in panels A−E.

to avoid interference from 3′-P defects (see Figure 1B). This was particularly relevant for the Q148K mutant whose defective 3′-P activity precluded ST determination with the full-length substrate. Figure 5E shows comparable ST deficiency for the mutants when assays were performed with the precleaved and full-length substrates (compare panels A and E of Figure 5).

ST was also generally less efficient using the full-length substrate (Figure 1A) compared to the precleaved substrate (Figure 1B), even when 3′-P was not affected. Clear example is the E92Q mutant, which has a ST activity that reached level similar to WT with precleaved substrate, whereas it was only around 50% with full-length substrate (compare panels E and A in Figure 5).

Together, these experiments demonstrate that only one of the mutants, Q148K, is severely defective for both 3′-P and ST. Two mutants (T66I and S153Y) are partially defective for both 3′-P and ST. Three mutants are selectively defective for ST (F121Y, E92Q, and N155H). Only one mutant (L74M) appeared normal for both 3′-P and ST.

Cross-Resistance of the Integrase Mutants to Raltegravir and Elvitegravir. To examine drug resistance of the IN mutants, we tested raltegravir and elvitegravir on all eight recombinant enzymes. The analysis was performed using the precleaved substrate since both drugs are selective ST inhibitors (see Figures 2 and 3).

Gels of Figure 6A−C show three representative experiments comparing the activity of elvitegravir on ST in the seven mutants and the wild-type IN enzymes. Only two of the mutants, L74M and F121Y, preserved the same sensibility to the compound as wild-type IN. The other mutants showed various degrees of resistance. Similar experiments were performed for raltegravir (data not shown).

Quantitations by densitometry analysis are summarized in Figure 6D−F. Panels D and E show independent duplicate ST IC_{50} values for raltegravir (Figure 6D) and elvitegravir (Figure 6E). Figure 6F reports the fold increase in ST IC_{50} values normalized to WT integrase and shows comparable resistance profiles for the two drugs and a greater resistance of all mutants to elvitegravir. T66I, Q148K, S153Y, and N155H were at least 2-fold more sensitive to raltegravir than to elvitegravir (see numbers above bars in panels D and E). Integrase Q148K displayed the most resistant profile, demonstrating a 77-fold increase for elvitegravir and 27-fold for raltegravir. The next most resistant integrase was T66I with 11- and 4.6-fold resistance for elvitegravir and raltegravir, respectively. Mutations L74M and F121Y did not contribute to integrase resistance to the drug.

The E92Q Mutation Enhances 3′-P Inhibition by Raltegravir and Elvitegravir. To evaluate the drug effects on 3′-P in the seven IN mutants, we performed the same drug

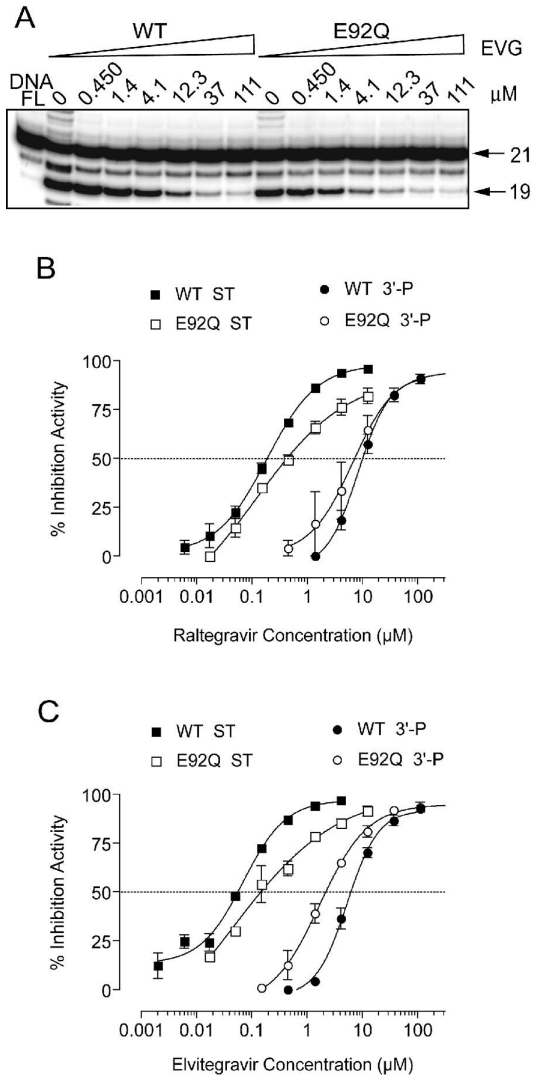

FIGURE 7: Differential effect of the E92Q integrase mutation on inhibition of 3′-processing vs strand transfer by raltegravir and elvitegravir. (A) Gel image of a representative dose−response experiment using full-length DNA substrate (FL) (see Figure 1A) in the presence of elvitegravir. (B, C) Quantitation by densitometry analyses of experiments performed as in panel A for raltegravir (B) and elvitegravir (C).

concentration−response reactions using the full-length substrate (see Figure 1A). For all the mutant enzymes except E92Q both drugs gave similarly weak inhibition as for the WT IN. Figure 7A shows a representative experiment demonstrating increased inhibitory effect of elvitegravir on 3′-P mediated by the E92Q mutant. Similar experiments were performed with raltegravir, and results for both elvitegravir and raltegravir are summarized in Figure 7B,C. These results demonstrate that the E92Q mutation renders IN more resistant to both raltegravir and elvitegravir with regard to ST but sensitize the enzyme to both drugs with respect to 3′-P.

Preincubation of Raltegravir and Elvitegravir with Integrase Does Not Overcome Drug Resistance. Because the previous results had been obtained by adding the drugs to IN followed by the immediate addition of the DNA substrate, we next wished to determine whether preincubation of the drugs with IN or addition of the drugs to the preassembled IN−DNA complexes would affect resistance. To that effect, we designed two experimental protocols (Figure 8A). In protocol 1, IN−DNA complexes were preassembled in the absence of drug for 15 min on ice to prevent catalysis. After this preincubation period, the drugs were added and reactions were initiated by placing the samples at 37 °C. Reactions were carried out for 1 h. In protocol 2, the drugs were mixed with IN for 15 min. Then the DNA was added and reactions were continued for an additional hour. Figure 8B shows a representative experiment performed with the E92Q mutant. It demonstrates the absence of detectable difference for strand transfer IC_{50} values determined in the two different conditions for raltegravir. Similar results were obtained with the T66I mutant and wild-type IN and with elvitegravir. Thus, our results demonstrate that both drugs inhibit IN independently of the order of addition and that preincubating the drugs with IN does not affect IN resistance.

DISCUSSION

The present study compares the inhibitory activities of raltegravir, which has been approved by the FDA as Isentress at the end of 2007, and elvitegravir, which is undergoing clinical trials. Prior to the present study, limited information was available on the molecular pharmacology of those two drugs against the specific biochemical activities of IN (*10, 21*), and there was no published report comparing the two drugs side by side. Our data confirm that both compounds are potent and selective ST inhibitors at low nanomolar concentration (*10, 21*). The drug concentrations required to inhibit 3′-P are at least 2 orders of magnitude higher than those required to inhibit ST, and full inhibition of ST takes place at drug concentrations devoid of effect on 3′-P (see Figure 2 and Table 2). We also find that elvitegravir is approximately 2−3-fold more potent than raltegravir against IN-mediated ST (see Figure 2 and Table 2).

Comparison of the effects of the two drugs against the different reactions mediated by IN *in vitro* demonstrates that neither drug shows significant activity against disintegration (see Figure 3 and Table 2), although disintegration is considered as a reverse reaction compared to ST (see Figure 1) (*13*). Moreover, both drugs inhibited ST more efficiently (by approximately 2-fold) when reactions were performed with the full-length vs the precleaved substrate (see Figure 1 and Table 2). Together, these findings suggest that both raltegravir and elvitegravir are highly specific for an intermediate step in the integrase catalytic cycle, which immediately follows 3′-P. Such selectivity is remarkable considering that all three reactions (3′-P, ST, and disintegration) utilize the same catalytic site with the three catalytic acidic residues of IN (see Figure 4). The specificity for the ST step indicates that the drugs can selectively recognize a specific configuration of the IN−DNA complex and that they probably bind at the interface of the IN−viral DNA processed complex by forming a quaternary complex with the catalytic metal in the enzyme active site together with the 3′-processed donor (viral) DNA (*5, 22, 23*). Our finding that preincubation of the drugs with IN does not increase the drug inhibitory activities (see Figure 8) is also consistent with the binding of raltegravir and elvitegravir at the IN−DNA interface (*5*) rather than to IN alone. Thus, raltegravir and elvitegravir are candidate interfacial inhibitors (*24*).

Comparison of the effects of raltegravir and elvitegravir on a series of seven resistant mutants previously identified from viral isolates selected upon drug exposure (*9, 10, 21*) enabled us to compare the resistance profiles for both drugs (see Figures 6 and 9). Our data extend recent publications for raltegravir showing resistance for the E92Q and the

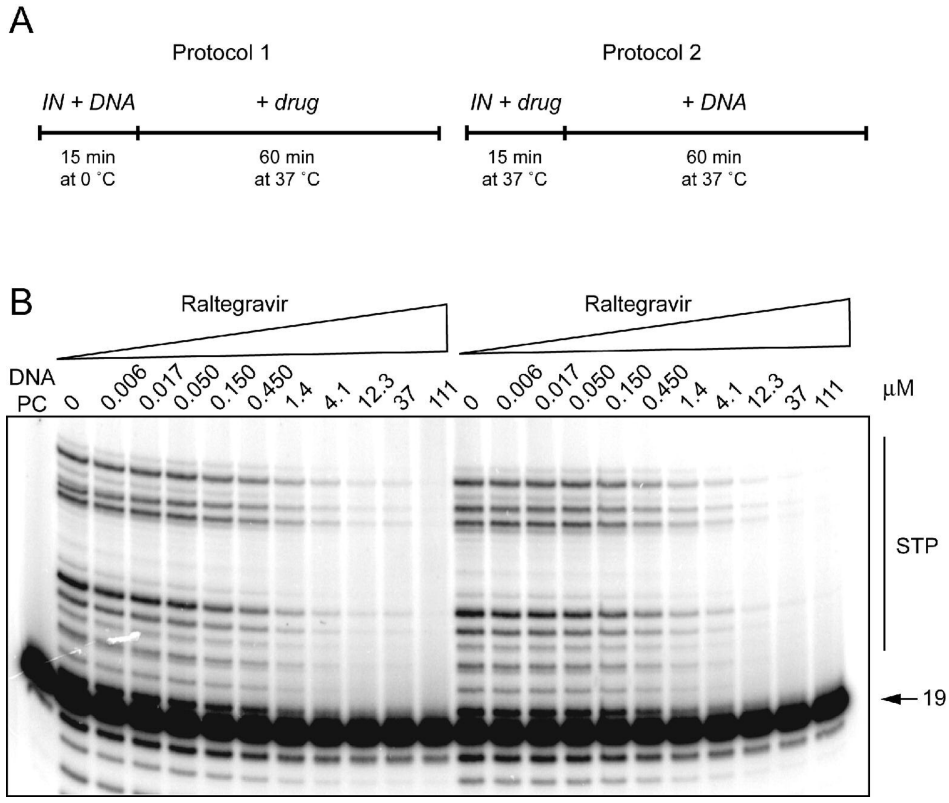

FIGURE 8: Comparison of the inhibition of HIV-1 integrase by raltegravir and elvitegravir using different orders of addition. (A) Schematic representation of the two protocols used. (B) Gel image of the inhibition of HIV-1 integrase E92Q by raltegravir using both protocols.

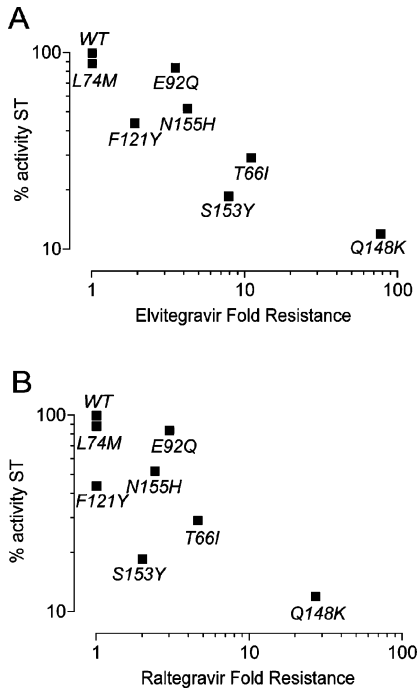

FIGURE 9: Correlation between drug resistance and defective catalytic activity for the HIV-1 integrase mutants. (A) Elvitegravir. (B) Raltegravir.

N155H mutants (*21*). The quantitative differences in resistance levels between the Malet publication (*21*) and our study (7−8-fold vs 3-fold for the E92Q mutant and 14-fold vs 2.4-fold for the N155H mutant, respectively) might be due to differences in assay conditions and enzyme preparations. Our data also extend the analyses for elvitegravir and are in agreement with the recent publication of Shimura et al. (*10*), who reported a 4.3-fold resistance level for the E92Q mutant (we found 3.5-fold; see Figure 6E). Our data also show that overall resistance to elvitegravir is greater than to raltegravir (see Figure 6F). The qualitative similarity in drug resistance profile across the seven mutants for raltegravir and elvitegravir is consistent with the similarities of the two drugs with respect to ST selectivity vs 3′-P and disintegration (see above and Table 2). Thus, these results suggest that raltegravir and elvitegravir interact in a similar way with IN and that they bind to a common region in the IN catalytic site. That region is probably outlined by the drug resistance mutations (see Figure 4B).

Although all the IN tested were more sensitive to elvitegravir than raltegravir, detailed analyses of the resistance profiles to both drugs (see Figure 6) suggest that the mutant enzymes can be divided in three groups (see Figure 6F). The first includes mutations that confer no selectivity to either drug: L74M and E92Q. The second group includes mutations that confer approximately 2-fold less resistance to raltegravir than elvitegravir (T66I, F121Y, Q148K, and N155H). The third group comprises the S153Y mutation, which appears somewhat more selectively resistant to elvitegravir than raltegravir. Nevertheless, based on these results with recombinant IN enzymes bearing resistance mutations, it seems unlikely that elvitegravir will be a rationale choice to overcome resistance to raltegravir.

The concept that drug resistance mutations lead to loss of viral fitness was first proposed by Hazuda and co-workers upon selection of resistance mutations to the diketo acid ST inhibitors (*25*). The fact that the seven mutations analyzed in the present study conferred a range of activity deficiency (see Figure 5) and drug resistance (see Figure 6) enabled us to examine the relationship between catalytic activity and drug resistance. This relationship is represented graphically

in Figure 9. Panel A shows an overall linear correlation between loss of ST activity of the mutants and resistance to elvitegravir. The three most resistant enzymes (Q148K, T66I, and S153Y) were also most defective in ST activity. A similar trend was observed for raltegravir (Figure 9B). The correlation between resistance mutations and catalytic defects is consistent with the specific interaction of the drugs with the IN catalytic site.

During antiretroviral therapy, the selection of drug-resistant mutants reflects a complex interaction between the effect of a mutation on drug activity and the effect of that mutation on the viral replication potential ("fitness") (*26*). There seems to exist two phases in the evolution of viral fitness: the first characterized by the selection of viruses with reduced drug susceptibility but which also display an impaired replication potential and the second with the generation and selection of additional compensatory mutations that restore replication (*27, 28*). All enzymes considered in this study have at least a partially impaired activity. Natural polymorphisms of the HIV-1 IN gene can also have important implications for the onset of resistance. IN S153Y and N155H are among the natural polymorphisms analyzed in the present study. Unexpectedly, we find that the S153Y IN mutant is markedly deficient in ST activity. Thus, it is likely that such mutations do not appear alone but in association with secondary mutations and/or changes in cofactors (viral or cellular cofactors associated with the integration complexes) that allow recovery of levels of catalytic activity similar to wild-type IN.

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