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Differential Sensitivities of Retroviruses to Integrate Strand Transfer Inhibitors

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Integrase inhibitors are emerging anti-human immunodeficiency virus (HIV) drugs, and multiple retroviruses and transposable elements were evaluated here for susceptibilities to raltegravir (RAL) and elvitegravir (EVG). All viruses, including primate and nonprimate lentiviruses, a Betaretrovirus, a Gammaretrovirus, and the Alpharetrovirus Rous sarcoma virus (RSV), were susceptible to inhibition by RAL. EVG potently inhibited all lentiviruses and intermittently inhibited Betaretrovirus and Gammaretrovirus infections yet was basically ineffective against RSV. Substitutions based on HIV type 1 (HIV-1) resistance changes revealed that integrase residue Ser150 contributed significantly to the resistance of RSV. The drugs intermittently inhibited intracisternal A-particle retrotransposition but were inactive against Sleeping Beauty transposition and long interspersed nucleotide element 1 (LINE-1) retrotransposition.

Reverse transcription of retroviral RNA yields linear viral DNA (vDNA) containing a copy of the long terminal repeat (LTR) at each end. Integrase (IN) is an essential retroviral enzyme that catalyzes two reactions to insert the vDNA into cellular chromosomal DNA. IN prepares the LTR ends by hydrolyzing phosphodiester bonds adjacent to invariant CA dinucleotides, yielding reactive 3′ deoxyadenylate (dAOH) termini. In the nucleus, IN catalyzes DNA strand transfer by using the 3′ OHs to cut the chromosome in a staggered fashion, concomitantly joining the vDNA ends to 5′ phosphates. Host-mediated repair of the resulting DNA recombination intermediate completes the integration process. See reference 8 for an overview of retroviral reverse transcription and integration.

IN belongs to the polynucleotidyl transferase superfamily of nucleic acid-metabolizing enzymes (7). Conserved amino acid residues (typically Asp and Glu [32]) arranged commonly on an RNase H structural fold comprise active sites that coordinate divalent metal ions for in-line nucleophilic attack of phosphodiester bonds. Due to its critical role in replication, human immunodeficiency virus type 1 (HIV-1) IN has long been targeted for drug development, and the first-in-class inhibitor raltegravir (RAL) was licensed in 2007 (45). Because RAL and related compounds preferentially inhibit DNA strand transfer activity, the drugs are primarily used to target IN strand transfer inhibitors (INSTIs) (24). Elvitegravir (EVG) is another well-studied INSTI (41). Recently determined X-ray crystal structures revealed that the drugs work by ejecting the 3′ dA and its associated OH nucleophile from the IN active site (10, 11). Drug resistance occurs through mutations in the downstream region of the pol gene, in the region that encodes IN (reviewed in references 24 and 27).

Drugs discovered through their abilities to adversely affect HIV-1 replication show divergent activities against other retroviruses. Nonnucleoside reverse transcriptase (RT) inhibitors such as nevirapine are highly selective for HIV-1 (50), whereas the nucleoside RT inhibitor (NRTI) azidothymidine (AZT) inhibits infection by a variety of viruses, including the primate lentiviruses HIV-2 (35) and simian immunodeficiency virus (SIV) (47), the nonprimate lentiviruses bovine immunodeficiency virus (BIV) (46) and feline immunodeficiency virus (FIV) (31), gammaretroviruses (34, 39, 42, 43), and Spumavirus (29). Protease inhibitors harbor an intermediate phenotype which is highly active against HIV-2/SIV (18) but ineffective against the Gammaretrovirus xenotropic murine leukemia virus-related virus (42). RAL and EVG were previously shown to be effective against HIV-2/SIV (21, 38, 40), gammaretroviruses (1, 34, 40, 42, 43), and the Spumavirus prototype foam virus (PFV) (48), suggesting that they, like NRTIs, might harbor pantoic antiretroviral activities. To comprehensively address this question, we determined RAL and EVG concentrations required to inhibit infection by vectors derived from five different lentiviruses, the Betaretrovirus Mason-Pfizer monkey virus (MPMV), the Alpharetrovirus Rous sarcoma virus (RSV), and the Gammaretrovirus Moloney murine leukemia virus (MLV). Moreover, we extended the analysis to nonviral elements that transpose intracellularly.

Experimental system. Resistance to RAL arises through one of three genetic pathways, Y143H/R/C, Q148H/R/K, or N155H (5), and substitutions at Gln148 confer significant cross-resistance to EVG (16). To start, an ex vivo infection assay (17) was calibrated to wide-ranging INSTI sensitivities by...
determining the 50% effective concentrations (EC50s) and 
EC95 doses of RAL and EVG using the wild-type (WT) IN and 
the Q148H, G140S, and Q148H/G140S mutants constructed in 
HIV-1NLX.Luc.R/H11002, a single-round strain that expresses firefly 
luciferase from the HIV-1 NL4-3 nef position (22). Viruses pseudo-
typed with vesicular stomatitis virus G (VSV-G) glycoprotein 
by cotransfecting 293T cells were measured by exogenous RT 
assay, and equal counts per minute (cpm) were applied in 
duplicate to HeLa-T4 cells (17). Two days after being infected, 
cells were lysed and resulting luciferase activities were cor-
rected for total protein concentrations. The normalized level 
of infection by the Q148H IN mutant was reduced about 9-fold 
comparably to that of the WT, whereas the G140S mutation 
conferred about a 2-fold defect in virus infectivity (Fig. 1A). 
The double mutant, by contrast, infected cells at a level similar 
to that of the WT (Fig. 1A). The EC50 and EC95 values for 
RAL (AIDS Research and Reference Reagent Program 
[RRRP], Germantown, MD) and EVG (Selleck Chemicals, 
Houston, TX) against WT HIV-1NLX.Luc.R/H11002 were about 8 
and 1.3 nM, respectively (Table 1). The G140S mutation elicited 
relatively small fold changes (FCs) in the EC50 and EC95 values for 
RAL and EVG, approximately 1.5 and 4.3, respectively. About 24- 
and 10-fold-higher concentrations of RAL and EVG, respectively, 
were required to thwart 50% of Q148H infectivity. The double 
mutation conferred significant resistance to both compounds, 
as the EC50s of RAL and EVG were about 1.9 μM and 2.3 μM, 
respectively (FC values, 241 and 1,762, respectively; Table 1). 
These results are fully consistent with those of previous studies 
that concluded the secondary G140S change increased the 
overall level of resistance to RAL conferred by the primary 
Q148H mutation and repaired an inherent IN catalytic defect 
(6, 26). Moreover, they established our ability to detect rela-
tively large FCs in sensitivity to INSTIs (Table 1).

### Inhibition of retroviral infection by INSTIs.

Cells were next 
challenged with VSV-G-pseudotyped single-round reporter 
constructs derived from HIV-2 strain ROD (54), SIV from 
macaques (SIVmac), RSV (51), FIV, BIV, MLV (20), or 
MPMV (30) in the absence or presence of AZT (AIDS 
RRRP), RAL, or EVG. FCs in EC50 and EC95 values for the 
drugs were determined based on the EC50s and EC95s against 
HIV-1NLX.Luc.R/H11002, which was included in parallel infections. 
Accordingly, a virus for which the FC in EC50 was 
5 was 
considered highly susceptible to the challenge compound, that 
for which the FC was 
5 and 
50, moderately drug sensitive, 
and that for which the FC was 
50, relatively insensitive. As 
expected (28, 31, 35, 39, 46, 47), AZT effectively inhibited all 
retroviruses, yielding FC values that ranged from a low of 0.4 
for RSV to a high of 2.4 for FIV (Table 2). In contrast, 
relatively large FC spectra were determined for the INSTIs. As 
previously reported (21, 38, 40), RAL and EVG each potently 
inhibited infection by HIV-2 and SIVmac. RAL potently in-
hibited MLV (1), while EVG acted moderately, with an FC of 
39. RAL also potently inhibited MPMV, whereas EVG was 
particularly effective against all lentiviruses. RAL displayed 
intermediate strength against FIV, BIV, and RSV, while an

![FIG. 1. IN mutant virus infectivities. (A) Normalized levels of 
HIV-1 IN mutant infectivities compared to that of the WT, which was 
set at 100%. (B) Same as panel A, except that RSV was studied.](http://jvi.asm.org/)

### Table 1. Activities of RAL and EVG against wild-type and IN mutant viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>RAL</th>
<th>EVG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (μM)</td>
<td>EC95 (μM)</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.008 ± 0.002</td>
<td>0.089 ± 0.004</td>
</tr>
<tr>
<td>Q148H mutant</td>
<td>0.012 ± 0.002 (1.5)</td>
<td>0.16 ± 0.11 (1.8)</td>
</tr>
<tr>
<td>Q148H/G140S mutant</td>
<td>0.19 ± 0.10 (24)</td>
<td>1.64 ± 0.89 (18)</td>
</tr>
<tr>
<td>Q148H/G140S/G140S mutant</td>
<td>1.93 ± 0.24 (241)</td>
<td>45.8 ± 6.8 (515)</td>
</tr>
</tbody>
</table>

*a* Means ± standard deviations obtained from three independent experiments, each conducted in duplicate.

*b* The FC in drug resistance of the IN mutant relative to that of the wild-type is indicated in parentheses.
intermediate dose of EVG was required to inhibit MPMV. Of all tested virus-drug combinations, one stood out as relatively ineffective: RSV naturally resisted EVG (Table 2).

IN residue Ser150 contributes significantly to RSV resistance to EVG. The majority of RAL and EVG resistance changes occur within the catalytic core domain (CCD) of HIV-1 IN and, moreover, cluster around Glu152 within its D-D-35-E active-site motif (27). To appreciate IN amino acid residues that might confer resistance to RAL or EVG, sequences from the heart of CCDs, corresponding to HIV-1 IN residues 91 to 169, were aligned (Fig. 2). To facilitate inter-comparison, residues that when altered can contribute to HIV-1 resistance were color coded green, and amino acid differences known to confer resistance to RAL or EVG, se-

TABLE 2. Antiviral activities of INSTIs and AZT

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genus</th>
<th>RAL EC50 (µM)</th>
<th>RAL EC95 (µM)</th>
<th>EVG EC50 (µM)</th>
<th>EVG EC95 (µM)</th>
<th>AZT EC50 (µM)</th>
<th>AZT EC95 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>Lentivirus</td>
<td>0.0006 ± 0.0028</td>
<td>0.098 ± 0.0029</td>
<td>0.0019 ± 0.0013</td>
<td>0.035 ± 0.0097</td>
<td>0.071 ± 0.027</td>
<td>0.81 ± 0.10</td>
</tr>
<tr>
<td>HIV-2</td>
<td>Lentivirus</td>
<td>0.020 ± 0.026 (2.4)</td>
<td>0.20 ± 0.04 (2.0)</td>
<td>0.0031 ± 0.0011 (1.6)</td>
<td>0.064 ± 0.018 (1.8)</td>
<td>0.04 ± 0.009 (0.6)</td>
<td>0.87 ± 0.13 (1.1)</td>
</tr>
<tr>
<td>SIVmac</td>
<td>Lentivirus</td>
<td>0.009 ± 0.002 (1.1)</td>
<td>0.11 ± 0.01 (1.1)</td>
<td>0.003 ± 0.0003 (1.6)</td>
<td>0.054 ± 0.017 (1.5)</td>
<td>0.03 ± 0.007 (0.5)</td>
<td>1.1 ± 0.14 (1.4)</td>
</tr>
<tr>
<td>FIV</td>
<td>Lentivirus</td>
<td>0.17 ± 0.05 (20)</td>
<td>3.85 ± 0.1 (39)</td>
<td>0.0056 ± 0.0006 (2.9)</td>
<td>0.095 ± 0.002 (2.7)</td>
<td>0.17 ± 0.08 (2.4)</td>
<td>7.9 ± 8.9 (2.8)</td>
</tr>
<tr>
<td>BIV</td>
<td>Lentivirus</td>
<td>0.016 ± 0.006 (23)</td>
<td>6.9 ± 1.4 (70)</td>
<td>0.0075 ± 0.0011 (3.0)</td>
<td>0.25 ± 0.08 (6.6)</td>
<td>0.12 ± 0.03 (1.7)</td>
<td>8.8 ± 0.2 (11)</td>
</tr>
<tr>
<td>RSV</td>
<td>Alpharetrovirus</td>
<td>0.13 ± 0.005 (15)</td>
<td>5.8 ± 1.8 (59)</td>
<td>7.2 ± 0.8 (3,789)</td>
<td>&gt;10 (&gt;268)</td>
<td>0.03 ± 0.009 (0.4)</td>
<td>0.52 ± 0.10 (0.6)</td>
</tr>
<tr>
<td>MPMV</td>
<td>Betaretrovirus</td>
<td>0.0086 ± 0.0007 (1.0)</td>
<td>0.27 ± 0.03 (2.8)</td>
<td>0.050 ± 0.0007 (26)</td>
<td>0.99 ± 0.02 (28)</td>
<td>0.066 ± 0.016 (0.9)</td>
<td>3.0 ± 0.6 (3.7)</td>
</tr>
<tr>
<td>MLV</td>
<td>Gammaretrovirus</td>
<td>0.0042 ± 0.0010 (1.5)</td>
<td>0.21 ± 0.03 (2.1)</td>
<td>0.0755 ± 0.0011 (39)</td>
<td>1.14 ± 0.31 (33)</td>
<td>0.043 ± 0.005 (0.56)</td>
<td>0.66 ± 0.05 (0.8)</td>
</tr>
<tr>
<td>XMRV</td>
<td>Gammaretrovirus</td>
<td>0.0022 ± 0.0011 (0.3)</td>
<td>ND</td>
<td>0.067 ± 0.029 (46)</td>
<td>ND</td>
<td>0.06 ± 0.02 (0.09)</td>
<td>ND</td>
</tr>
<tr>
<td>PFV</td>
<td>Spumavirus</td>
<td>0.06 (7.1)</td>
<td>ND</td>
<td>0.8 (421)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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a Means ± standard deviations obtained from three independent experiments, each conducted in duplicate.

b The FC in EC50 or EC95 relative to that of HIV-1 is indicated in parentheses. ND, not determined.

c Data are from reference 42.
d Data are from reference 48.

FIG. 2. IN sequence alignment and contribution of potential amino acid residues to resistance to INSTIs. Green indicates residues that when changed can confer resistance to RAL and/or EVG; magenta marks residues known to confer resistance when present at the analogous HIV-1 position; gray indicates residues with unknown effects on potential HIV-1 resistance; red indicates active-site residues; and blue highlights a conserved DNA binding residue (15). Numbers above the alignment indicate HIV-1 amino acid positions; those to the left and right mark positions in the respective IN or transposase protein sequences. Underlining marks the positions of secondary structural elements for HIV-1 (19), SIV (4), RSV (52), and PFV (10) INs and the positions of SB elements from a structure-based alignment with the related Mos1 transposase (37).
WT. Because this yielded an EC$_{50}$ of 0.1 ± 0.02 µM, we concluded that Ser150 contributes significantly to the natural resistance of RSV to EVG.

**Nonviral sensitivities to INSTIs.** The abilities of RAL and EVG to inhibit intracellular transposition of noninfected elements were tested next. Intracisternal A-particles (IAPs) are LTR retroelements that rely on RT and IN activities for retrotransposition (reviewed in reference 2) and are indigenous to mice. Sleeping Beauty (SB) is a member of the Tc1/mariner family of DNA transposable elements that moves from one genomic position to another via the activity of its transposase protein, which harbors a D.D-34-E active-site motif (Fig. 2), in the absence of an RNA intermediate (reviewed in reference 36). Though long interspersed nucleotide element 1 (LINE-1) retrotransposition occurs via an RNA intermediate, chromosomal DNA nicking in this case occurs via a functionally distinct endonuclease that is not a member of the polynucleotidyl transferase superfamily (49), and therefore LINE-1 served as a negative control in these assays.

Transposition was assessed using recombinant elements carrying the reporter gene for green fluorescence protein (GFP). Accordingly, IAP retrotransposition was scored in HeLa cells (30,000 plated the previous day in 48-well plates) cotransfected with 0.1 µg of pDE1 bearing a GFP-intron cassette and 0.1 µg of pQ14CAG (13), which encodes IAP proteins, or 0.1 µg of pUC19 to define the assay background, using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) in the absence or presence of RAL, EVG, or AZT. Seven days thereafter, GFP-positive cells were quantified using a FACSCanto flow cytometer equipped with FACSDiva software (BD, Franklin Lakes, NJ). For SB transposition, cells cotransfected with 0.15 µg of pT2βGFP (53) and 0.075 µg of pSB100X (14, 23) or 0.075 µg of pUC19 were developed similarly by flow cytometry at day 7. Cells transfected with 0.2 µg of LINE-1 bearing L1gp-EGFP(puro) or negative control pL1gpJM111)-EGFP(puro) were also scored after 7 days, with the addition of puromycin (2 µg/ml) at 48 h posttransfection to enhance the selection of de novo events (33).

As expected, neither RAL nor EVG detectably inhibited LINE-1 retrotransposition (Table 4). Similarly, AZT was inactive against SB transposition. IAP retrotransposition was acutely inhibited by AZT, moderately inhibited by RAL, and somewhat less sensitive to EVG. Despite utilizing a DDE active site, SB transposase was not detectably inhibited by RAL or EVG (Table 4).

**Conclusions.** The results of this study reveal that the prototype INSTIs RAL and EVG differentially inhibit the activities of LTR-containing retroviruses and retrotransposons yet are ineffective against the non-LTR LINE-1 retrotransposon and the DNA transposon SB. EVG was more lentiviral specific than RAL, and RSV rather impressively resisted EVG (Table 2), which was in large part attributable to IN residue Ser150 (Table 3). Based on these results, we speculate that element-specific amino acid sequences likely dictate sensitivities to INSTIs. We note that SB transposase, like RSV IN, harbors serine at the position analogous to Pro145 in HIV-1 IN (Fig. 2). Differences at transposase residues analogous to HIV-1 IN positions Tyr143, Gln148, and Asn155 might accordingly account for the RAL resistance of this element. Unlike LTR retroelements that harbor the strand transfer nuclease as part of a recessed 3’ end, or, more rarely, blunt DNA end, transposition of SB occurs via a 3’ overhang (36). Because key INSTI-vDNA contacts occur via the penultimate LTR C/G base pair (10, 11), it seems possible that the lack of pairing bases at the reactive transposon end might also contribute to INSTI resistance.

Based on the relative activities of low-micromolar preclinical compounds, the Tc1/mariner Mos1 transposase has been proposed as a surrogate to identify HIV-1 IN inhibitors (3). Our results indicate potential limitations to utilizing Tc/mariner elements to identify highly efficacious drugs.

The inability to degrade preintegrative retrotransposon

### TABLE 4. RAL and EVG activities against nonviral elements

<table>
<thead>
<tr>
<th>Element</th>
<th>RAL (µM)</th>
<th>EVG (µM)</th>
<th>AZT (µM)</th>
<th>Transposition rate of no drug control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LINE-1</td>
<td>&gt;10 (&gt;1,190)$^c$</td>
<td>&gt;10 (&gt;5,263)</td>
<td>&gt;10$^b$ (&gt;140)</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>IAP</td>
<td>0.37 ± 0.18 (44)</td>
<td>0.33 ± 0.19 (174)</td>
<td>0.020 ± 0.004 (0.3)</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>SB</td>
<td>&gt;10 (&gt;1,190)</td>
<td>&gt;10 (&gt;5,263)</td>
<td>&gt;10 (&gt;140)</td>
<td>22.0 ± 5.1</td>
</tr>
</tbody>
</table>

$^a$ Means ± standard deviations from two to three independent experiments of concentrations required to inhibit 50% of the control transposition rate.

$^b$ Percentage of GFP-positive cells after background correction.

$^c$ The FC in concentration compared to that for HIV-1 (Table 2) is indicated in parentheses.

$^d$ 45% inhibition at 10 µM.
DNA can increase susceptibility to autoimmune dysfunction (44), and inhibition by RAL transiently increases unintegrated LTR circular DNA forms during acute HIV-1 infection (12). The rate of onset of autoimmune disease has, moreover, been observed to increase in susceptible mice treated with RAL (1). As we have demonstrated differential susceptibilities of retroviruses and retrotransposons to inhibition by RAL versus EVG (Tables 2 and 4), novel INSTIs should be evaluated individually for inhibitory activities against potentially medically relevant (1, 25) non-HIV elements.

We are indebted to the following colleagues for their generous donations of reagents: H. Kazazian for L1Pr-EGFP(puro) and pL1Pr(JM1111)-EGFP(puro), J. Takeda for pDE1 and pQ14cAG, P. Mead for pT2sGFPP, Z. Izsvák for pSB100X, W. Johnson and E. Hunter for pSAR-EGFP, and T. Hatziioannou for the HIV-2 reporter virus. RAL and AZT were obtained from the NIH AIDS Research and Reference Reagent Program.

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20. Lu, R., et al., 2004. Class II integrase mutants with changes in putative nuclear localization signals are primarily blocked at a postnuclear step of the viral DNA transcriptional initiation by RAL versus EVG (Tables 2 and 4), novel INSTIs should be evaluated individually for inhibitory activities against potentially medically relevant (1, 25) non-HIV ele-

ments.

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