Characterization of a replication-competent, integrase-defective human immunodeficiency virus (HIV)/simian virus 40 chimera as a powerful tool for the discovery and validation of HIV integrase inhibitors

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Integrate is actively studied as an antiviral target, but many inhibitors selected from biochemical screens fail to inhibit human immunodeficiency virus (HIV) replication or primarily affect off-site targets. Here we develop and validate a replication-competent, simian virus 40 (SV40)-HIV integrase mutant chimera as a novel tool to classify the mechanism of action of potential integrase inhibitors. Whereas the mutant was more susceptible than wild-type to entry, reverse transcriptase, and protease inhibitors, it specifically resisted the action of integrase inhibitor L-870,810. We furthermore demonstrate inhibition of integration by GS-9137 and GS-9160, and off-site targeting by the 6-aminoquinolone antibiotic WM-5.

Integration of reverse transcribed DNA is essential for productive human immunodeficiency virus type 1 (HIV-1) replication (14, 21), defining integrase (IN) as a target for antiretroviral therapy. Intense effort has been dedicated to developing IN inhibitors, and a variety of chemical classes have been described. Many of these however fail to function in cell culture, or predominantly inhibit off-site targets (26). Zintevir, for example, potently inhibits HIV-1 replication and IN activity in vitro (22), yet primarily blocks virus entry (5, 8). L-chicoric acid and derivatives also counteract IN activity, but virus entry can again be blocked (25, 28). Diketo compounds in contrast specifically inhibit IN activity and HIV-1 integration in cell culture (9-11). Two selective strand transfer inhibitors, S-1360 (Shionogi & Co) (2) and L-870,810 (Merck & Company, Inc) (9) (Fig. 1A), entered into clinical trials, though their development ceased due to unfavorable pharmacokinetics (29). Compounds GS-9137 (Gilead Sciences, Inc) and MK-0518 (Merck & Company, Inc) are in active development (7, 16).

GS-9137, a quinolone antibiotic (Fig. 1B), potently inhibits IN activity and HIV-1 replication (31), though the step in the viral lifecycle inhibited by GS-9137 remains to be
determined. It is important to note that the related 6-aminoquinolone antibiotic WM-5 (Fig. 1D), which likewise inhibits IN \textit{in vitro}, can inhibit Tat-dependent transcription from the HIV-1 promoter (4, 23).

A time-of-addition experiment was performed to examine the replication step(s) affected by GS-9137, WM-5, as well as the novel diketo compound GS-9160 (Fig. 1C). This design determines how long the addition of a compound can be postponed before losing its antiviral function. Replication was determined by p24 content in MT-4 cell supernatants at 31 h following infection with HIV-1\textsubscript{IIIB} (provided by R.C. Gallo and M. Popovic) (27) at a multiplicity of infection (m.o.i.) of 0.5 (method adapted from ref. 24). Control compounds included the entry inhibitor dextran sulfate (DS5000) (Sigma, Bornem, Belgium) (1, 18), reverse transcriptase (RT) inhibitors 3'-azido-3'-deoxythymidine (AZT) (12, 19) and nevirapine (17) (obtained from Boehringer Ingelheim, Ridgefield, CT), IN inhibitor L-870,810 (Fig. 1) (a kind gift from D. Hazuda, Merck & Co., West Point, PA), and protease inhibitor (PI) ritonavir (13) (obtained from Abbott Laboratories, Abbott Park, IL). As expected, DS5000 required its addition along with the virus; a delay of even 1 h counteracted antiviral activity because virus adsorption had already occurred (Fig. 2). PI addition in contrast could be delayed for more than 12 h. RT inhibitor addition could be delayed for 4 to 5 h, whereas L-870,810 could be delayed an additional 2 h. The addition of GS-9137 or GS-9160 (kindly provided by Gilead Sciences, Foster City, CA) could also be postponed for 7 h from the start of the experiment before potency losses were observed (Fig. 2). These results are indistinguishable from that obtained with the naphthyridine carboxamide IN inhibitor (9), consistent with the notion that GS-9137 or GS-9160 each inhibit integration in cell culture. WM-5 (kindly supplied by O. Tabarrini and A. Fravolini, Perugia, Italy) addition in contrast could be delayed for 9 h, which is consistent with the potential to inhibit post-integration transcription (32).
To address the important issue of IN inhibitor specificity, we have developed a simple and straightforward counterscreen based on the unique infectivity profile of a simian virus (SV) 40/HIV-1 chimera (N/N.Tag.oriT) with catalytically inactive IN (15) to validate if compounds inhibit integration in cell culture. The chimera virus utilizes the SV40 origin of DNA replication oriT and trans-acting T antigen protein to drive the replication of class I HIV-1 IN mutant carrying amino acid substitutions D64N and D116N in the enzyme active site (21). To validate this strategy, C8166 T-cells (30) infected with N/N.Tag.oriT in the presence of various concentrations of known inhibitors were monitored for p24 production in culture supernatants at 5 d post-infection. The CXCR4 co-receptor antagonist AMD3100 (6) (kindly provided by AnorMed, Langley, British Columbia), nevirapine, and ritonavir each elicited a strong reduction in viral replication as compared to untreated controls, demonstrating that the co-receptor usage, reverse transcription, and protease functions of N/N.Tag.oriT are intact (Fig. 3A and Table 1). Furthermore, the calculated EC$_{50}$s for these compounds were approximately 5 to 10 times lower as compared to the values obtained with wild-type HIV-1$_{NL4-3}$ (Table 1). These results were not unexpected, considering that the chimera displayed a diminished capacity for replicative spread when compared alongside wild-type HIV-1$_{NL4-3}$ (15). The validated IN inhibitor L-870,810 in stark contrast failed to inhibit N/N.Tag.oriT at concentrations up to 0.23 µM, while the EC$_{50}$ for parental HIV-1$_{NL4-3}$ was 0.013 ± 0.011 µM (Fig. 3B and Table 1). GS-9137 and GS-9160 also failed to inhibit N/N.Tag.oriT at concentrations up to 2.23 and 0.24 µM, respectively, while their EC$_{50}$s for wild-type HIV-1$_{NL4-3}$ were 0.00725 ± 0.00591 and 0.00849 ± 0.00534 µM, respectively (Fig. 3D, 3E, and Table 1). Significantly, WM-5 inhibited N/N.Tag.oriT replication at a lower dose than required for HIV-1$_{NL4-3}$ (Table 1). We therefore conclude that GS-9137 and GS-9160 specifically target the integration step of the virus lifecycle, whereas the main target of WM-5 action lies elsewhere.
We have utilized the unique replication profile of N/N.Tag.oriT to establish a cell-based assay to validate the antiviral target of newly designed IN inhibitors. During the course of this project, a separate study based on the relatively low level (~0.2%) of transcription from IN active site mutant vectors was presented as a method to distinguish the mode of action of potential IN inhibitors in cell culture (3). Due to its naturally-weakened ability to replicate through multiple cycles of HIV-1 growth, an additional advantage of our assay is that N/N.Tag.oriT is innately more sensitive than wild-type HIV-1_{NL4-3} to drugs that target steps in the lifecycle other than integration. On the flip side, the chimera was ~18 to >308 times less susceptible to IN inhibitors (Table 1). The inversion of inhibitor susceptibility phenotype can significantly help to classify unknown inhibitors as to whether they target the integration step. Our method is simpler and thus more amenable to scale-up than previously described molecular techniques like real-time nested Alu-PCR to show that a potential inhibitor specifically interferes with integration in cell culture. The method is applicable to different classes of inhibitors that may differentially inhibit IN 3'-processing versus DNA strand transfer activity and will also be useful to screen inhibitors of potential IN-cell co-factor interactions. We conclude that the method is a valuable counterscreen to detect and validate inhibitors of IN catalytic function and HIV-1 integration in cell culture.
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REFERENCES


**Figure legends**

FIG. 1. Structures of (A) L-870,810, (B) GS-9137, (C) GS-9160, and (D) WM-5.

FIG. 2. Time-of-addition experiment. Compounds (concentrations in µM) were added at the indicated time points post infection.

FIG. 3. Concentration-dependent anti-HIV-1 effects of (A) nevirapine, (B) L-870,810, (C) WM-5, (D) GS-9137, and (E) GS-9160. C8166 T-cells infected with HIV-1NL4-3 or N/N.Tag.oriT were treated with the indicated compound concentrations. One hundred percent was defined as the level of p24 attained in control, non-drug treated samples.
TABLE 1. Antiretroviral activity and cytotoxicity of HIV-1 inhibitors against wild-type HIV-1

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NL4-3</td>
<td>N/N.Tag.oriT</td>
</tr>
<tr>
<td>AMD3100</td>
<td>0.328 ± 0.254</td>
<td>0.049 ± 0.025</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>0.337 ± 0.277</td>
<td>0.075 ± 0.037*</td>
</tr>
<tr>
<td>L-870,810</td>
<td>0.013 ± 0.011</td>
<td>&gt; 0.23*</td>
</tr>
<tr>
<td>GS-9137</td>
<td>0.00725 ± 0.00591</td>
<td>&gt; 2.23*</td>
</tr>
<tr>
<td>GS-9160</td>
<td>0.00849 ± 0.00534</td>
<td>&gt; 0.24*</td>
</tr>
<tr>
<td>WM-5</td>
<td>2.09 ± 1.02</td>
<td>0.88 ± 1.12</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>0.294 ± 0.080</td>
<td>0.059 ± 0.052</td>
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</table>

<sup>a</sup> Mean values and standard deviations, from a minimum of two separate experiments, required to inhibit p24 production by 50%. *, P < 0.05 as compared to data obtained with wild-type HIV-1<sub>NL4-3</sub>. AMD3100, WM5, and ritonavir data were derived from two total experiments, precluding significance analyses in these cases.

<sup>b</sup> Concentration that reduced cell viability by 50% as determined by the MTT assay (20).
Figure 1

A. L-870,810

B. GS-9137

C. GS-9160

D. WM-5
Figure 2

The figure shows the time course of HIV-1 replication in the presence of various drugs. The y-axis represents the Log p24 levels, and the x-axis represents time in hours. Different lines and markers indicate the control and treatments with DS5000, AZT, nevirapine, ritonavir, L-870,810, GS-9137, GS-9160, and WM-5, each with their respective IC50 values (20, 1.8, 7.5, 2.7, 0.23, 0.22, 0.24, 1.3).
Figure 3

A. Nevirapine (µM) vs. Virion-associated p24 (% of control)

B. L870,810 (µM) vs. Virion-associated p24 (% of control)

C. WM-5 (µM) vs. Virion-associated p24 (% of control)
Figure 3 (continued)

D

Virion-associated p24 (% of control)

GS-9137 (µM)

N L4.3

E

Virion-associated p24 (% of control)

GS-9160 (µM)

N L4.3 ~ N NTag, oriT

D

E