Bifunctional Anti-Human Immunodeficiency Virus Type 1 Small Molecules with Two Novel Mechanisms of Action

Li Huang, Xiong Yuan, Christopher Aiken, and Chin Ho Chen

Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710, and Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

A class of betulinic acid derivatives was synthesized to target two critical steps in the human immunodeficiency virus type 1 (HIV-1) replication cycle, entry and maturation. Each mechanism of HIV-1 inhibition is distinct from clinically available anti-HIV therapeutics. The viral determinants of the antientry and antimaturation activities are the bridging sheet of HIV-1 gp120 and the P24/p2 cleavage site, respectively.

Betulinic acid derivatives are a class of small molecules that exhibit anti-human immunodeficiency virus type 1 (anti-HIV-1) activity (6, 7, 11). The targets of betulinic acid derivatives are varied, depending primarily on the side chain structures of the compounds (6) (Fig. 1). For example, betulnilyl aminoctanoyl-leucine (LH15) and N-(3-O-(3',3'-dimethylsucinyl)-lup-20(29)-en-28-oyl)leucine (LH55) are two of these compounds with potent anti-HIV activity.

Synthesis and anti-HIV-1 activity of bifunctional small molecules LH15 and LH55. The protocol used to synthesize LH15 and LH55 was modified from previously described procedures (4, 12). The antientry functional groups, leucine and aminoundecanoic acid methyl esters, were introduced into the backbone of betulinic acid at position 28. The resulting intermediates were refluxed with 2,2-dimethylsuccinic anhydride in the presence of pyridine and dimethylaminopyridine to introduce the antimaturation side chains at position 3. The final products were purified with high-performance liquid chromatography to yield LH15 and LH55. 1H nuclear magnetic resonance with signal assignment and mass spectrometry were performed to verify the structures.

The anti-HIV activity of these compounds was evaluated with an HIV-1 infectivity assay described previously (5). A diluted HIV-1 stock at a multiplicity of infection of 0.001 50% tissue culture infective dose per cell was used to infect MT4 cells in the presence of various concentrations of the compounds. The compounds IC9564 and DSB (4, 5) inhibit various HIV-1 isolates at submicromolar concentrations but not the protease inhibitor-resistant strain PI-R, which is less sensitive to DSB (Table 1). LH15 and LH55, in general, are at least a log more potent than either IC9564 or DSB. The results shown in Table 1 also demonstrate that LH15 and LH55 are potent inhibitors of the multiple-protease-inhibitor-resistant strain PI-R (2) and the multiple-reverse transcriptase (RT)-inhibitor-resistant strain RTI-R (10).

Inhibition of HIV-1 entry. The side chain at position 28 is critical for the antifusion activity of the betulinic acid derivatives. Betulinic acid derivatives with the same side chains as LH15 and LH55 at position 28 but without a functional side chain at position 3 exhibit antifusion activity (12). Figure 2a shows that the potency of LH55 against NL4-3 envelope-induced membrane fusion is similar to that of IC9564 and T20. DSB, lacking a side chain at position 28, does not significantly affect the envelope-mediated membrane fusion. It has previously been demonstrated that the key determinant for IC9564 sensitivity is gp120 (5). However, the detailed mechanism of fusion inhibition remains unclear. IC9564 does not affect CD4-gp120 interaction. The binding of gp120 to CD4 and the subsequent interaction with chemokine receptors are two critical fusion events for HIV-1 entry. Thus, it is likely that the chemokine receptor interactive site on gp120 is a key determinant for the antifusion activity of IC9564.

To test this hypothesis, we chose a pair of HIV-1 strains, NLDH120 and M2-NLDH, that exhibit significant differences in the accessibility of their chemokine receptor interactive sites (15). The two viruses differ in one amino acid at position 198 in the bridging sheet of gp120 (15). The envelope sequences of these two viruses were cloned into an expression vector, pSRHS, and used in an envelope-mediated membrane fusion assay (5). The fusion mediated by the M2-NLDH envelope, M2-pSW120, is approximately 10-fold more sensitive to IC9564 than that of the NLDH120 envelope, pSW120 (Fig. 2b). Since the bridging sheet is a critical structural motif in-
involved in HIV-1 entry (9), the results strongly support the notion that the bridging sheet is involved in IC9564 sensitivity.

**Interference with the processing of p25 resulted in an inhibition of HIV-1 maturation.** Based on our previous observation that DSB does not affect the production of HIV-1 viral particles (4), we speculated that DSB treatment might lead to the production of immature viral particles that have lost infectivity. In order to test this hypothesis, HIV-1 particles produced in the presence of LH55 were lysed and analyzed using Western blots. There was an accumulation of p25 in the virus produced in the presence of LH55 (Fig. 3). Accumulation of p25 was also observed in the virus particles produced in the presence of DSB (data not shown). The processing of p25 to p24 and p2 is the last step in sequential protease cleavage of Gag precursor into mature Gag proteins, which is critical for viral infectivity (3, 8, 13). The unique mode of action of DSB or LH55 is that these compounds affect the processing of only p25. The processing of other Gag proteins, such as p17, is not affected by these compounds (Fig. 3).

The key structural feature that enables LH55 and its analogs to possess the dual mode of action is the presence of both side chains at position 3 and position 28. The unique biological activity of these compounds is that they not only inhibit the viruses resistant to HIV-1 RT and protease inhibitors but also inhibit viruses that are resistant to compounds which bear the side chains only at position 3 or position 28 (data not shown). The bridging sheet of gp120 and the maturation of p24 are the critical determinants for drug sensitivity to this class of compounds. The antifusion activity of LH55 or LH15 allows the

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**TABLE 1.** Anti-HIV activity of betulinic acid derivatives

<table>
<thead>
<tr>
<th>Virus</th>
<th>IC₅₀ of indicated compound (μM)</th>
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<tbody>
<tr>
<td></td>
<td>DSB</td>
</tr>
<tr>
<td>NL4-3</td>
<td>0.096 ± 0.012</td>
</tr>
<tr>
<td>PI-R</td>
<td>1.71 ± 0.15</td>
</tr>
<tr>
<td>RTI-R</td>
<td>0.085 ± 0.008</td>
</tr>
</tbody>
</table>

*PI-R is an HIV-1 strain, HIV-1M46I/L63P/V82P/I84V, resistant to multiple protease inhibitors (2). RTI-R is an HIV-1 strain, HIV-1RTMDR1/MT2 (RT 74V, 41L, 106A, 215Y), resistant to multiple HIV-1 reverse transcriptase inhibitors (10). PI-R and RTI-R were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

*IC₅₀ is the drug concentration required to inhibit 50% of HIV-1 replication. The highest concentration tested for these compounds was 5 μM; there was no observable cytotoxicity at this concentration. The numbers in the table represent the means ± the standard deviations of three independent experiments done in duplicate.
FIG. 3. LH55 inhibits HIV-1 maturation. HIV-1 viral particles were produced from ACH-2 cells in the absence (A) or presence (B) of LH55. The virus particles were purified by ultracentrifugation using a sucrose gradient (14). The collected viral samples were lysed and analyzed using a sodium dodecyl sulfate–12.5% polyacrylamide gel followed by a Western blot using an HIV-1-positive human serum. kd, molecular mass standards in kilodaltons.

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