Supporting Information

Incorporation of Privileged Structures into Bevirimat Can Improve Activity against Wild-type and Bevirimat-resistant HIV-1

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Figure S1. All designed compounds showed improved activity against NL4-3 and NL4-3/V370A virus as compared with 2. A) The fold change in IC$_{50}$ (NL4-3) value of compound relative to 2 is the IC$_{50}$ (NL4-3) of compound / IC$_{50}$ (NL4-3) of 2; B) The fold change in IC$_{50}$ (NL4-3/V370A) value of compound relative to 2 is the IC$_{50}$ (NL4-3/V370A) of compound / IC$_{50}$ (NL4-3/V370A) of 2; C) The fold change in RFC of compound relative to 2 is the RFC of compound / RFC of 2.
Table S1. Statistical analysis of activity of each compound compared to that of 2a

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 against NL4-3 (µM)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>18a</td>
<td>0.029±0.0093</td>
<td>0.0421</td>
</tr>
<tr>
<td>18b</td>
<td>0.050±0.021</td>
<td>0.411</td>
</tr>
<tr>
<td>18c</td>
<td>0.019±0.0054</td>
<td>0.0157</td>
</tr>
<tr>
<td>19</td>
<td>0.028±0.010</td>
<td>0.0405</td>
</tr>
<tr>
<td>23</td>
<td>0.012±0.0041</td>
<td>0.0092</td>
</tr>
<tr>
<td>20</td>
<td>0.49±0.12</td>
<td>-</td>
</tr>
<tr>
<td>14c</td>
<td>&gt; 90</td>
<td>-</td>
</tr>
<tr>
<td>12c</td>
<td>&gt; 90</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.065±0.019</td>
<td></td>
</tr>
</tbody>
</table>

aData are presented as the mean ± standard deviation (SD), and determined in three separate experiments; NS means no selective anti-HIV activity (CC50/IC50 < 5); Analysis of anti-HIV-1 activity of each compound compared to that of compound 2 was performed using a two-sided Student t test (GraphPad, http://www.graphpad.com/quickcalcs/ttest2/). A P-value of < 0.05 denotes a significant increase/difference in antiviral potency. The p-value reported are not adjustments for multiple comparisons.

Table S2. In vitro microsomal stability and in-silico drug-like profile profiles of 18c and 2.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>HLMa</th>
<th>CLint (mL/min/mg)</th>
<th>Solubilityc</th>
<th>PSAd</th>
<th>Hepatotoxicitye</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18c</td>
<td>128</td>
<td>0.054</td>
<td>2</td>
<td>102.463</td>
<td>-12.9317</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>0.128</td>
<td>0</td>
<td>123.513</td>
<td>-11.1763</td>
</tr>
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</table>

aData Human liver microsomal incubation assay, data from two experiments in parallel. Predicted properties were calculated by ADMET module of Discovery Studio 2.5. a aqueous solubility level in water at 25 ºC. 0 (extremely low), 1 (very low), 2 (low), 3 (good), 4 (optimal). d PSA : Fast polar surface area (compounds with 10 or fewer rotatable bonds and PSA below 140 Å would have a higher chance of being orally bioavailable. e hepatotoxicity (<0.4095: Nontoxic; >0.4095: Toxic).

General procedures for synthesis of compounds 13a-c.

The appropriate acids 12a-c were prepared using previously described methods1 or purchased directly from Sigma-Aldrich or other commercial sources. N-Boc piperazine (1.0 equiv), 12a-c (1.5 equiv), and Et3N (1.1 eq) were dissolved in anhydrous CH2Cl2. To the solution, EDCI (2 equiv) and HOBt (1.1 equiv) were added. The mixture was stirred at r.t. overnight until all starting material was consumed. The solution was then diluted with DCM and washed twice with brine. The organic layer was dried over Na2SO4 and concentrated in vacuum. The residue was chromatographed using a silica gel column to give the pure compounds.

Compound 13a. 1.0 g (99%), starting from 500 mg of N-Boc piperazine; colorless oil. 1H NMR (400 MHz, CDCl3): δ 7.65 (1H, d, J = 15.2 Hz, CH=), 7.11 (1H, dd, J = 1.6, 8.4
Hz, 6-Ph), 7.03 (1H, d, J = 1.6 Hz, 2-Ph), 6.87 (1H, d, J = 8.0 Hz, 5-Ph), 6.71 (1H, d, J = 15.2 Hz, CH=), 3.94 and 3.92 (3H each, s, 2×OCH$_3$), 3.66 and 3.47 (4H each, m, CON(CH$_2$CH$_2$)$_2$-N-), 1.48 (9H, s, COO(CH$_3$)$_3$).

Compound 13b. 1.1 g (94%), starting from 500 mg of NVBoc piperazine; white colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.61 (1H, d, J = 15.6 Hz, CH=), 7.34 (1H, s, 2VPh), 7.15 (2H, s, 5,6VPh), 6.71 (1H, d, J = 15.6 Hz, CH=), 5.26 (4H, s, 2×OVCH$_2$VO), 3.66 and 3.48 (4H each, m, CON(CH$_2$CH$_2$)$_2$-N-), 3.54 and 3.52 (3H each, s, 2×OCH$_3$), 1.48 (9H, s, COO(CH$_3$)$_3$).

Compound 13c. 962 mg (99%), starting from 500 mg of NVBoc piperazine; white amorphous powder. Mp 155-156 °C. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.61 (1H, d, J = 15.6 Hz, CH=), 7.04 (1H, d, J = 1.6 Hz, 2-Ph), 7.01 (1H, dd, J = 1.6, 8.4 Hz, 6-Ph), 6.81 (1H, d, J = 8.4 Hz, 5VPh), 6.68 (1H, d, J = 15.2 Hz, CH=), 6.00 (2H, s, O-CH$_2$-O), 3.66 and 3.49 (4H each, br s, CON(CH$_2$CH$_2$)$_2$-N-). HRMS (ESI, m/z) Calcd for C$_{14}$H$_{17}$N$_2$O$_3$, 261.1239 [M+H$^+$]; found, 261.1239.

Synthesis of compound 14c. To a solution of 13c (150 mg, 0.42 mmol) in DCM (7 mL), TFA (0.48 mL, 6.25 mmol) was added. The reaction was stirred for 2 h at r.t. until all starting material was consumed. The solvent was removed under vacuum. The residue was chromatographed using a silica gel column to give the pure compound 14c (58.3 mg, 54%); colorless oil. $^1$H NMR (400 MHz, CD$_3$OD): δ 7.49 (1H, d, J = 15.2 Hz, CH=), 7.21 (1H, d, J = 1.6 Hz, 2VPh), 7.05 (1H, dd, J = 1.6, 8.4 Hz, 6-Ph), 6.95 (1H, d, J = 15.2 Hz, CH=), 6.81 (1H, d, J = 8.4 Hz, 5VPh), 5.98 (2H, s, O-CH$_2$-O), 3.67 and 2.84 (4H each, br s, CON(CH$_2$CH$_2$)$_2$-N-). HRMS (ESI, m/z) Calcd for C$_{14}$H$_{17}$N$_2$O$_3$, 261.1239 [M+H$^+$]; found, 261.1239.

General procedures for synthesis of compounds 16a-c.

To a solution of 13a or 13c (1 mmol) in CH$_2$Cl$_2$ (17.23 mL), TFA (1.15 mL, 15 mmol) was added. The reaction was stirred for 2 h at r.t. until all starting material was consumed. The solvent was removed under vacuum to give the Boc deprotected product, which was used without further purification.

To a solution of 13b (1 equiv) in DCM (5 mL), ZnBr$_2$ (5 equiv) was added. The mixture was stirred overnight at r.t. treated with water (20 mL) and stirred for 2 h. The organic layer was separated and aqueous layer was extracted twice with DCM. The combined organic solvent was dried over Na$_2$SO$_4$ and concentrated in vacuum to give the Boc deprotected product, which was used without further purification.

Betulinic acid-3-O-acetate (3-OAc-BA) was prepared using previously reported methods. Oxalyl chloride solution (2 M in DCM) was added to 3-OAc-BA (1 equiv) in CH$_2$Cl$_2$. The mixture was stirred for 2 h. After removal of solvent under vacuum, the residue was treated with appropriate Boc deprotected product of 13a-c (1.5 equiv), obtained above, and Et$_3$N (15 equiv) in anhydrous CH$_2$Cl$_2$. The mixture was stirred at r.t. overnight until all starting material was consumed. The solution was then diluted with CH$_2$Cl$_2$ and washed twice with brine. The organic layer was dried over Na$_2$SO$_4$ and concentrated. The residue was chromatographed using a silica gel column to yield the pure compounds.
Compound 16a. 231 mg (61%), starting from 250 mg of 3-OAC-BA; white amorphous powder. Mp 170-171 °C. 1H NMR (400 MHz, CDCl3): δ 7.65 (1H, d, J = 15.2 Hz, CH=), 7.12 (1H, dd, J = 2.0, 8.0 Hz, 6-Ph), 7.03 (1H, d, J = 2.0 Hz, 2-Ph), 6.87 (1H, d, J = 8.0 Hz, 5-Ph), 6.72 (1H, d, J = 15.2 Hz, CH=), 4.74 and 4.60 (1H each, 2s, H-29), 4.47 (1H, t, J = 9.2 Hz, H-3), 3.94 and 3.92 (3H each, s, 2×OCH3), 3.67 (8H, brs, 28-CON(CH2CH2)2-N-), 3.01 (1H, m, H-19), 2.04 (s, 3H, -OAc), 1.69 (3H, s, H-30), 0.97 (3H, s, CH3), 0.94 (3H, s, CH3), 0.85 (3H, s, CH3), 0.84 (3H, s, CH3), 0.83 (3H, s, CH3). HRMS (ESI, m/z) calced for C47H68N2O6Na, 779.4975 [M+Na]+; found, 779.4965.

Compound 16b. 489 mg (53%), starting from 566 mg of 3-OAC-BA; colorless oil. 1H NMR (400 MHz, CDCl3): δ 7.62 (1H, d, J = 15.2 Hz, CH=), 7.34 (1H, s, 2-Ph), 7.15 (2H, s, 5,6-Ph), 6.72 (1H, d, J = 15.2 Hz, CH=), 5.27 (4H, s, 2×O-CH2-O), 4.73 and 4.59 (1H each, 2s, H-29), 4.47 (1H, t, J = 9.2 Hz, H-3), 3.67 (8H, brs, 28-CON(CH2CH2)2-N-), 3.54 and 3.52 (3H each, s, 2×OCH3), 2.99 (1H, m, H-19), 2.04 (s, 3H, -OAc), 1.69 (3H, s, H-30), 0.96 (3H, s, CH3), 0.94 (3H, s, CH3), 0.85 (3H, s, CH3), 0.83 (6H, s, 2×CH3). HRMS (ESI, m/z) calced for C49H73N2O8, 817.5367 [M+H]+; found, 817.5362.

Compound 16c. 436 mg (98%), starting from 300 mg of 3-OAC-BA; white amorphous powder. Mp 172-173 °C. 1H NMR (400 MHz, CDCl3): δ 7.62 (1H, d, J = 15.6 Hz, CH=), 7.04 (1H, d, J = 1.6 Hz, 2-Ph), 7.01 (1H, dd, J = 1.6, 8.0 Hz, 6-Ph), 6.81 (1H, d, J = 8.0 Hz, 5-Ph), 6.68 (1H, d, J = 15.2 Hz, CH=), 6.00 (2H, s, O-CH2-O), 4.73 and 4.59 (1H each, 2s, H-29), 4.47 (1H, t, J = 7.2 Hz, H-3), 3.66 (8H, brs, 28-CON(CH2CH2)2-N-), 2.98 (1H, m, H-19), 2.04 (s, 3H, -OAc), 1.69 (3H, s, H-30), 0.96 (3H, s, CH3), 0.94 (3H, s, CH3), 0.85 (3H, s, CH3), 0.84 (3H, s, CH3), 0.83 (3H, s, CH3). HRMS (ESI, m/z) calced for C46H65N2O6, 741.4842 [M+H]+; found, 741.4828.

1HNMR and HRMS data for compounds 17a-c.

Compound 17a. 186 mg (86%), starting from 230 mg of 16a; white amorphous powder. Mp 155-156 °C. 1H NMR (400 MHz, CDCl3): δ 7.66 (1H, d, J = 15.6 Hz, CH=), 7.12 (1H, dd, J = 1.6, 8.0 Hz, 6-Ph), 7.03 (1H, d, J = 1.6 Hz, 2-Ph), 6.88 (1H, d, J = 8.0 Hz, 5-Ph), 6.73 (1H, d, J = 15.6 Hz, CH=), 4.74 and 4.60 (1H each, 2s, H-29), 3.94 and 3.92 (3H each, s, 2×OCH3), 3.67 (8H, brs, 28-CON(CH2CH2)2-N-), 3.17 (1H, dd, J = 5.6, 10.8 Hz, H-3), 3.01 (1H, m, H-19), 2.04 (s, 3H, -OAc), 1.69 (3H, s, H-30), 0.97 (3H, s, CH3), 0.94 (3H, s, CH3), 0.85 (3H, s, CH3), 0.84 (3H, s, CH3), 0.83 (3H, s, CH3). HRMS (ESI, m/z) calced for C45H67N2O7, 715.5050 [M+H]+; found, 715.5015.

Compound 17b. 97.6 mg (84%), starting from 123 mg of 16b; white amorphous powder. Mp 155-156 °C. 1H NMR (400 MHz, CDCl3): δ 7.62 (1H, d, J = 15.6 Hz, CH=), 7.34 (1H, d, J = 1.2 Hz, 2-Ph), 7.15 (2H, s, 5,6-Ph), 6.72 (1H, d, J = 15.6 Hz, CH=), 5.27 and 5.26 (2H each, 2s, 2×O-CH2-O), 4.74 and 4.59 (1H each, 2s, H-29), 3.66 (8H, brs, 28-CON(CH2CH2)2-N-), 3.54 and 3.52 (3H each, 2s, 2×OCH3), 3.18 (1H, dd, J = 5.2, 11.2 Hz, H-3), 2.99 (1H, m, H-19), 1.69 (3H, s, H-30), 0.97 (3H, s, CH3), 0.96 (3H, s, CH3), 0.94 (3H, s, CH3), 0.83 (3H, s, CH3), 0.76 (3H, s, CH3). HRMS (ESI, m/z) calced for C45H67N2O7, 775.5261 [M+H]+; found, 775.5211.

Compound 17c. 182.5 mg (82%), starting from 235.8 mg of 16c; white amorphous powder. Mp 171-172 °C. 1H NMR (400 MHz, CDCl3): δ 7.62 (1H, d, J = 15.2 Hz, CH=), 7.04 (1H, s, 2-Ph), 7.01 (1H, d, J = 8.4 Hz, 6-Ph), 6.81 (1H, d, J = 8.4 Hz, 5-Ph), 6.69
(1H, d, J = 15.2 Hz, CH=), 6.01 (2H, s, O-CH₂-O), 4.74 and 4.59 (1H each, 2s, H-29), 3.66 (8H, brs, 28-CON(CH₂CH₂)₂-N-), 3.18 (1H, dd, J = 5.2, 11.2 Hz, H-3), 2.99 (1H, m, H-19), 1.69 (3H, s, H-30), 0.97 (3H, s, CH₃), 0.96 (3H, s, CH₃), 0.94 (3H, s, CH₃), 0.83 (3H, s, CH₃), 0.76 (3H, s, CH₃). HRMS (ESI, m/z) calcd for C₄₄H₆₂N₂O₅Na, 721.4557 [M+Na⁺]; found, 721.4546.

Synthesis of compound S-1. Betulinic acid-3-O-acetate (3-OAc-BA,) was prepared using previously reported methods. Oxalyl chloride solution (2.04 mL, 24.10 mmol) was added to 3-OAc-BA (600 mg, 1.20 mmol) in 12 mL CH₂Cl₂. The mixture was stirred for 2 h. After removal of solvent under vacuum, the residue was treated with 1-Boc-piperazine (336 mg, 1.81 mmol), obtained above, and Et₃N (0.25 mL, 1.81 mmol) in anhydrous CH₂Cl₂. The mixture was stirred at r.t. overnight until all starting material was consumed. The solution was then diluted with CH₂Cl₂ and washed twice with brine. The organic layer was dried over Na₂SO₄ and concentrated. The residue was chromatographed using a silica gel column to yield the pure compound S-1 (657.2 mg, 82% yield); white amorphous powder. Mp 169-170 °C. ¹H NMR (400 MHz, CDCl₃): δ 4.74 and 4.60 (1H each, 2s, H-29), 4.48 (1H, dd, J = 6.0, 9.0 Hz, H-3), 3.58 and 3.41 (8H, brs, 28-CON(CH₂CH₂)₂-N-), 3.00 (1H, m, H-19), 2.05 (s, 3H, -OAc), 1.70 (3H, s, H-30), 1.49 (9H, s, COO(CH₃)₃), 0.97 (3H, s, CH₃), 0.95 (3H, s, CH₃), 0.86 (3H, s, CH₃), 0.85 (3H, s, CH₃), 0.85 (3H, s, CH₃). HRMS (ESI, m/z) calcd for C₄₁H₆₇N₂O₅, 667.5050 [M+H⁺]; found, 667.5017.

Synthesis of compound S-2. To a solution of S-1 (600 mg, 0.90 mmol) in MeOH (1 mL) and THF (2 mL) was added 4 N NaOH (1 mL). The reaction was stirred overnight until all starting material was consumed. The mixture was then neutralized with 1 N HCl and extracted with CH₂Cl₂ three times. The combined organic layer was dried over Na₂SO₄ and concentrated under vacuum. The residue was chromatographed using a silica gel column to yield the pure compound S-2 (523.6 mg, 93% yield); white amorphous powder. ¹H NMR (400 MHz, CDCl₃): δ 4.73 and 4.58 (1H each, 2s, H-29), 3.57 and 3.39 (4H
each, brs, 28-CON(CH₂CH₂)₂-N), 3.18 (1H, d, J = 4.8, 11.0 Hz, H-3), 2.98 (1H, m, H-19), 1.68 (3H, s, H-30), 1.47 (9H, s, COO(CH₃)₃), 0.96 (6H, s, 2×CH₃), 0.93 (3H, s, CH₃), 0.83 (3H, s, CH₃), 0.76 (3H, s, CH₃). HRMS (ESI, m/z) calcd for C₃₀H₆₅N₂O₄, 625.4944 [M+H⁺]; found, 625.4903.

**Synthesis of compound 20.** Compound S-2 (120.0 mg, 0.19 mmol), 2,2-dimethylsuccinic anhydride (73.9 mg, 0.58 mmol), and DMAP (11.7 mg, 0.1 mmol) were dissolved in anhydrous pyridine. The mixture was stirred at 150 °C for 2 h in a microwave oven (Biotage). The reaction mixture was diluted with EtOAc and neutralized with HCl (1N) and then extracted twice with EtOAc. The combined organic layer was washed with brine, dried over Na₂SO₄ and concentrated in vacuum to afford the crude product, which was chromatographed using a silica gel column to give the pure compound 20 (46.3 mg, 37% yield); colorless oil. ¹H NMR (400 MHz, CDCl₃): δ 4.73 and 4.60 (1H each, 2s, HV₂₉), 4.48 (1H, dd, J = 6.8, 10.8 Hz, H-3), 3.71 and 2.99 (4H each, br s, 28-CON(CH₂CH₂)₂-NV), 2.97 (1H, m, HV₁₉), 2.66 and 2.60 (1H each, d, J = 15.2 Hz, H-2'), 1.69 (3H, s, H-30), 1.31 and 1.28 (3 H each, s, 2×CH₃V₃'), 0.97 (3H, s, CH₃), 0.93 (3H, s, CH₃), 0.84 (3H, s, CH₃), 0.83 (3H, s, CH₃), 0.81 (3H, s, CH₃). HRMS (ESI, m/z) Calcd for C₄₀H₆₅N₂O₅, 653.4893 [M+H⁺]; found, 653.4867.

**Synthesis of compound 22.** Compound 21 was prepared using previously described methods.² To a solution of compound 13c (350 mg, 0.97 mmol) in CH₂Cl₂ (16 mL), TFA (1.12 mL, 14.6 mmol) was added. The reaction was stirred for 2 h at r.t. until all starting material was consumed. The solvent was removed under vacuum to give the Boc deprotected product, which was dissolved in anhydrous CH₂Cl₂. To the solution, compound 21 (405.9 mg, 0.65 mmol), Et₃N (1.35 mL, 9.72 mmol), EDCI (372.6 mg, 1.94 mmol) and HOBt (144.4 mg, 1.07 mmol) were added. The mixture was stirred at r.t. overnight until all starting material was consumed. The solution was then diluted with CH₂Cl₂ and washed with brine two times. The organic layer was dried over Na₂SO₄ and concentrated in vacuum. The residue was chromatographed using a silica gel column to provide compound 22 (343.6 mg, 61%); colorless oil. ¹H NMR (400 MHz, CDC1₃): δ 7.63 (1H, d, J = 15.2 Hz, CH=), 7.04 to 7.00 (2H, overlap, 2,6VPh), 6.81 (1H, d, J = 7.6 Hz, 5-Ph), 6.68 (1H, d, J = 15.2 Hz, CH=), 6.01 (2H, s, O-CH₂-O), 4.72 and 4.57 (1H each, 2s, H-29), 3.74 to 3.52 (12 H, overlap, 28-CON(CH₂CH₂)₂-N and -CON(CH₂)₂), 3.18 (1H, dd, J = 4.8, 11.2 Hz, H-3), 2.98 (1H, m, H-19), 2.39 (6H, br s, -(CH₂)₂-N and N-CH₂), 1.68 (3H, s, H-30), 0.96 (6H, s, 2×CH₃), 0.94 (3H, s, CH₃), 0.82 (3H, s, CH₃), 0.75 (3H, s, CH₃). HRMS (ESI, m/z) calcd for C₅₀H₇₉N₄O₆, 867.5999 [M+H⁺]; found, 867.5957.

**HIV-1/NL4-3 and NL4-3/V370 A Replication Inhibition Assay in MT-4 Lymphocytes.** BVM-R variants were constructed as previously reported.³ A previously described HIV-1 infectivity assay was used.⁴ Briefly, a 96-well microtiter plate was used to set up the HIV-1/NL4-3 and NL4-3/V370A replication screening assay. NL4-3 or NL4-3/V370A variants at a multiplicity of infection (MOI) of 0.01 were used to infect MT4 cells. Culture supernatants were collected on day 4 PI for the p24 antigen capture using an ELISA kit from ZeptoMetrix Corporation (Buffalo, NY). The 50% inhibition concentration (IC₅₀) was defined as the concentration that inhibits HIV-1/NL4-3 replication by 50%.
**Cytotoxicity Assay.** A CytoTox-Glo cytotoxicity assay (Promega) was used to determine the cytotoxicity of the synthesized derivatives. Mock-infected MT-4 cells were cultured in the presence of various concentrations of the compounds for 2 days. Percent of viable cells was determined by following the protocol provided by the manufacturer. The 50% cytotoxic concentration (CC$_{50}$) was defined as the concentration that caused a 50% reduction of cell viability.

**TZM-bl Assay.** Anti-HIV-1 activity was measured as reduction in Luc reporter gene expression after a single round of virus infection of TZM-bl cells. HIV-1 at 200 TCID$_{50}$ and test samples were mixed in a total volume of 100 mL of growth medium in 96-well black solid plates (Corning-Costar). After 48 h of incubation, culture medium was removed from each well and 100 mL of Bright Glo luciferase reagent was added to each culture well. The luciferase activity in the assay wells was measured using a Victor 2 luminometer. The 50% inhibitory dose (IC$_{50}$) was defined as the sample concentration that caused a 50% reduction in relative luminescence units (RLU) compared to virus control wells after subtraction of background RLU.

**Fusion Assay.** The fusion assay used in this study was described previously. The fusion assay was performed by transfecting monkey kidney cells (COS) with the expression vector that contained HIV-1 Env and tat genes. COS cells (1 × 106 cells/mL) were mixed with 5 µg of the Env-expressing vector and incubated on ice for 10 min. Electroporation was performed using a gene pulsar (Bio-Rad, Hercules, CA) with capacitance set at 950 µF and voltage at 150 V. After the transfected COS cells were cultured for one day, they were mixed with TZM-bl cells. TZM-bl cells were incubated with the Env-expressing COS cells in the presence of inhibitors in 96-well flat-bottom plates (Costar) overnight. Fusion was measured by quantifying luciferase activity in the fused cells using a Bright-Glow luciferase assay kit (Promega, Luis Obispo, CA). Inhibition of the Env-mediated membrane fusion was expressed as a percentage of the control (Env-mediated membrane fusion in the absence of inhibitors).

**Microsomal Stability Assay.** Stock solutions of 18c, 2, and reference compound propranolol (0.5 mM) were prepared by dissolving the pure compound in DMSO and were stored at 4 °C. Before the assay, the stock solution was diluted with acetonitrile (ACN) to 0.1 mM. For measurement of metabolic stability, all compounds were brought to a final concentration of 1 µM with 0.1 M potassium phosphate buffer at pH 7.4, which contained 0.1 mg/mL human liver microsomes and 5 mM MgCl$_2$. The incubation volumes were 300 µL, and reaction temperature was 37 °C. Reactions were started by adding 60 µL of NADPH (final concentration of 1.0 mM) and quenched by adding 600 µL of ice-cold ACN to stop the reaction at 5, 15, 30, and 60 min time points. Samples at 0 min time point were prepared by adding 600 µL of ice-cold ACN first, followed by 60 µL of NADPH. Incubations of all samples were conducted in duplicate. After quenching, all samples were centrifuged at 12 000 rpm for 7 min at 4 °C. The supernatant was collected, and 30 µL of the supernatant were injected directly onto a Shimadzu LC-MS-IT-TOF system by autosampler. The LC/MS/MS analysis was carried out with an electrospray ionization source (ESI). An Alltima C18 column (3 µm, 20 mm × 2.1 mm) was used for HPLC with a gradient elution at a flow rate of 0.3 mL/min. The elution conditions for 18c were ACN (B) in water (A) at 65% for 0–2 min, 95% for 2–8 min, and 65% for 8–12 min. The CDL temperature was 250 °C, and heat block temperature was
200 °C. The neutralizing gas flow was 1.5 L/min. The peak heights of test compounds at different time points were converted to the percentage remaining, and the peak height values at initial time (0 min) served as 100%. The slope of the linear regression from log of the percentage remaining versus incubation time relationships (\(-k\)) was used to calculate in vitro half-life (\(t_{1/2}\)) by the formula of in vitro \(t_{1/2} = \frac{0.693}{k}\), regarded as first-order kinetics. Conversion to in vitro clearance [CL\(_{\text{int}}\) in units of (mL/min)/mg protein] was calculated by the formula: \(\text{CL}_{\text{int}} = \frac{6.93}{(\text{in vitro} \ t_{1/2}) \ [(\text{mL incubation})/(\text{mg of microsomes})]}\). Moderate metabolizing reference compounds propranolol have \(t_{1/2}\) of 43.8 min in these assay conditions.

References


