
Jimmy P. Xu, Drexel University
Ashwanth Francis, Emory University
Megan E. Meuser, Drexel University
Marie Mankowski, Emory University
Roger G. Ptak, Emory University
Adel A. Rashad, Drexel University
Gregory Melikian, Emory University
Simon Cocklin, Drexel University

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Exploring Modifications of an HIV-1 Capsid Inhibitor: Design, Synthesis, and Mechanism of Action

Jimmy P. Xu, Ashwanth C. Francis, Megan E. Meuser, Marie Mankowski, Roger G. Ptak, Adel A. Rashad, Gregory B. Melikyan, and Simon Cocklin*

1Department of Biochemistry & Molecular Biology, Drexel University College of Medicine, USA
2Department of Pediatrics, Infectious Diseases, Emory University, USA
3Department of Infectious Disease Research, Southern Research Institute, USA

Abstract

Recent efforts by both academic and pharmaceutical researchers have focused on the HIV-1 capsid (CA) protein as a new therapeutic target. An inter-protomer pocket within the hexamer configuration of the CA, which is also a binding site for key host dependency factors, is the target of the most widely studied CA inhibitor compound PF-3450074 (PF-74). Despite its popularity, PF-74 suffers from properties that limit its usefulness as a lead, most notably it’s extremely poor metabolic stability. To minimize unfavorable qualities, we investigated bioisosteric modification of the PF-74 scaffold as a first step in redeveloping this compound. Using a field-based bioisostere identification method, coupled with biochemical and biological assessment, we have created four new compounds that inhibit HIV-1 infection and that bind to the assembled CA hexamer. Detailed mechanism of action studies indicates that the modifications alter the manner in which these new compounds affect HIV-1 capsid core stability, as compared to the parental compound. Further investigations are underway to redevelop these compounds to optimize potency and drug-like characteristics and to deeply define the mechanism of action.

INTRODUCTION

Drug-resistance and toxicity to antiretroviral drugs is a significant problem that drives the search for new inhibitors of HIV. Therefore, identifying new targets and developing therapeutic compounds remains a continuing research priority. Recent efforts by both academic and pharmaceutical researchers have focused on the HIV-1 capsid (CA) protein [1] as a new therapeutic target. An inter-protomer pocket within the hexamer configuration of the CA, which is also a binding site for key host dependency factors [2-4], is the target of the very high potency compound GS-CA1 [5] and the most widely studied CA inhibitor compound PF-3450074 (PF-74). Despite its popularity, PF-74 suffers from properties that limit its usefulness as a lead, most notably its extremely poor metabolic stability. To minimize unfavorable qualities, we investigated bioisosteric modification of the PF-74 scaffold as a first step in redeveloping this compound. Using a field-based bioisostere identification method, coupled with biochemical and biological assessment, we have created four new compounds that inhibit HIV-1 infection and that bind to the assembled CA hexamer. Detailed mechanism of action studies indicates that the modifications alter the manner in which these new compounds affect HIV-1 capsid core stability, as compared to the parental compound. Further investigations are underway to redevelop these compounds to optimize potency and drug-like characteristics and to deeply define the mechanism of action.

MATERIALS AND METHODS

Chemical compounds

PF-3450074 (PF-74) was purchased from MilliporeSigma (Burlington, MA). The CX compounds were synthesized de novo according to the schemes and details that follow.
Table 1: Potency of the CX compounds and PF-74 against isolates from subtypes A, B, and C. The therapeutic index for each compound was calculated using the median IC\textsubscript{50} across all three isolates tested. Chemical structures were drawn using ChemAxon software (Budapest, Hungary).

<table>
<thead>
<tr>
<th>Compound</th>
<th>2D similarity to PF-74</th>
<th>IC\textsubscript{50} HIV-1 92RW025 (µM) Clade A</th>
<th>IC\textsubscript{50} HIV-1 JR-CSF (µM) Clade B</th>
<th>IC\textsubscript{50} HIV-1 93MW965 (µM) Clade C</th>
<th>Median IC\textsubscript{50} (µM)</th>
<th>CC\textsubscript{50} (µM)</th>
<th>Therapeutic Index (CC\textsubscript{50}/IC\textsubscript{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-74</td>
<td>1</td>
<td>1.5 ± 0.9</td>
<td>0.6 ± 0.20</td>
<td>0.6 ± 0.10</td>
<td>9.0 ± 0.5</td>
<td>90.5 ± 5.9</td>
<td>93.9</td>
</tr>
<tr>
<td>CX03</td>
<td>0.489</td>
<td>6.5 ± 2.2</td>
<td>4.9 ± 1.4</td>
<td>6.4 ± 2.1</td>
<td>5.9 ± 0.9</td>
<td>&gt;100</td>
<td>&gt;16.9</td>
</tr>
<tr>
<td>CX04</td>
<td>0.482</td>
<td>18.7 ± 3.9</td>
<td>18.7 ± 2.9</td>
<td>14.0 ± 5.7</td>
<td>17.1 ± 2.7</td>
<td>&gt;100</td>
<td>&gt;5.84</td>
</tr>
<tr>
<td>CX05</td>
<td>0.498</td>
<td>23.1 ± 11.1</td>
<td>16.2 ± 6.5</td>
<td>14.5 ± 3.7</td>
<td>18.0 ± 4.6</td>
<td>&gt;100</td>
<td>&gt;5.58</td>
</tr>
<tr>
<td>CX06</td>
<td>0.494</td>
<td>6.4 ± 0.4</td>
<td>6.5 ± 0.7</td>
<td>4.7 ± 2.1</td>
<td>5.9 ± 1.0</td>
<td>&gt;100</td>
<td>&gt;17.0</td>
</tr>
</tbody>
</table>
Synthetic scheme of CX03:

\[
\begin{align*}
&\text{O} \quad \text{H} \quad \text{O} \\
&\text{NH\textsubscript{2}} \quad \text{OH} \\
\end{align*}
\]

3-1

\[
\begin{align*}
&\text{N} \quad \text{O} \quad \text{NH} \\
&\text{O} \quad \text{N} \\
\end{align*}
\]

3-2

\[
\begin{align*}
&\text{N} \quad \text{O} \quad \text{NH} \\
&\text{O} \\
\end{align*}
\]

3-3

Synthetic scheme of CX04:

\[
\begin{align*}
&\text{O} \quad \text{NH} \\
&\text{Cl} \quad \text{O} \\
&\text{K\textsubscript{2}CO\textsubscript{3}, DMF} \\
&\text{52\%} \\
\end{align*}
\]

4-2

\[
\begin{align*}
&\text{N} \quad \text{O} \quad \text{NH} \\
&\text{O} \\
\end{align*}
\]

4-3

Synthetic scheme of CX05:

\[
\begin{align*}
&\text{O} \quad \text{N} \\
&\text{LiAlH\textsubscript{4}, THF, r.t.} \\
&\text{90\%} \\
\end{align*}
\]

5-2

\[
\begin{align*}
&\text{N} \quad \text{O} \\
&\text{O} \\
\end{align*}
\]

5-4
Preparation of CX03

**Preparation of 2-isopropyl-4,5,6,7-tetrahydro-1H-benzo[d]imidazole (3-2):**

To a solution of 3-1 (1.0 g, 8.9 mmol) in EtOH (20 mL) were added NH₄OAc (4.1 g, 53.4 mmol) and aldehyde (634 mg, 8.9 mmol), the resulting mixture was heated to 80 °C for 4 hours. After cooled down to room temperature, the reaction was poured into water (30 mL) and then extracted with EAc (25 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash chromatography (silica gel, ethyl acetate (10%) in petroleum ether) to provide 3-2 (992 mg, 68%).

**Preparation of ethyl 2-(2-isopropyl-4,5,6,7-tetrahydro-1H-benzo[d]imidaz -ol -1-yl)acetate (3-3):**

To a solution of 3-2 (1 g, 6.0 mmol) in DMF (10 mL) was added NaH (264 mg, 6.6 mmol), the resulting mixture was stirred at 60 °C for 4 hours. After cooled down to room temperature, the reaction was poured into water (30 mL) and then extracted with EAc (25 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over Na₂SO₄, filtered and concentrated. The residue was used in the next step without further purification (65%).
Preparation of 2-(2-isopropyl-4,5,6,7-tetrahydro-1H-benzo[d]imidazol-1-y1)acetic acid (3-4):

To a solution of crude 3-3 in EtOH (10 mL) and H2O (5 mL) was added NaOH, the resulting mixture was stirred at 60 °C for 8 hours. After cooled down to room temperature, the reaction was poured into water (30 mL) and then extracted with EA (25 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over NaSO4, filtered and concentrated. The residue was used in the next step without further purification (76%).

Preparation of 2-(2-(2-isopropyl-4,5,6,7-tetrahydro-1H-benzo[d]imidazol-1-yl)acetamido)-N-methyl-N,3-diphenylpropanamide (CX03):

At room temperature, to a solution of 3-4 (223 mg, 1 mmol) in DCM (5 mL) were added amine (280 mg, 1.1 mmol), T3P (1.2373 g, 2.0 mmol) and DIPEA (516 mg, 4.0 mmol), the resulting mixture was stirred at room temperature overnight. Then it was washed with water and 5% HCl aqueous solution and concentrated to give crude product which was purified by Pre-HPLC to afford CX03 (27 mg, 6%) as white solid. LC-MS (ESI): m/z 459.55 (M+1). H NMR (400 MHz, CDCl3) δ 7.44 – 7.25 (m, 3H), 7.16 – 7.05 (m, 3H), 7.02 – 6.90 (m, 2H), 6.77 – 6.59 (m, 3H), 4.74 (td, J = 8.4, 6.1 Hz, 1H), 4.37 (s, 2H), 3.74 – 3.54 (m, 4H), 3.30 – 3.11 (m, 3H), 2.90 – 2.74 (m, 2H), 2.64 – 2.43 (m, 3H), 2.33 – 2.04 (m, 2H), 1.31 – 1.10 (m, 6H).

Preparation of CX04

Preparation of ethyl 1-(difluoromethyl)-1H-pyrazole-4-carboxylate (4-2):

To a solution of 4-1 (100 mg, 0.351 mmol) in DMF (1.2 mL) were added 2-chloro-2,2-difluoroacetate (136 mg, 0.428 ml) and K2CO3 (197 mg, 0.714 mmol), the resulting mixture was stirred at 60°C for 5 hours. The reaction was quenched with water, and extracted with EA. The combined organic phases were washed with brine (15 mL), dried over NaSO4, and filtered. The filtrate was concentrated, and the residue was purified by flash chromatography (silica gel, MeOH (6%) in DCM) to provide 4-2 (69 mg, 52%).

Preparation of (1-(difluoromethyl)-1H-pyrazol-4-yl) methanol (4-3):

To a solution of 4-1 (100 mg, 0.351 mmol) in DMF (1.2 mL) were added 2-chloro-2,2-difluoroacetate (136 mg, 0.428 ml) and K2CO3 (197 mg, 0.714 mmol), the resulting mixture was stirred at 60°C for 5 hours. The reaction was quenched with water, and extracted with EA. The combined organic phases were washed with brine (15 mL), dried over NaSO4, and filtered. The filtrate was concentrated, and the residue was purified by flash chromatography (silica gel, MeOH (6%) in DCM) to provide 4-2 (69 mg, 52%).
To a solution of 4-2 (40 mg, 0.21 mmol) in THF (1 mL) was added LAH (40 mg, 1.05 mmol), the resulting mixture was stirred at room temperature for 1 hour. The reaction was quenched with water, and extracted with EA. The combined organic phases were washed with brine (15 mL), dried over NaSO$_4$, and filtered. The filtrate was concentrated, and the residue was used in next step without purification (81%).

**Preparation of 4-(bromomethyl)-1-(difluoromethyl)-1H-pyrazole (4-4):**

![Structure of 4-3](image1)

![Structure of 4-4](image2)

58%

A solution of the 4-3 (600 mg, 4.02 mmol) in DCM was added CBr$_4$ (2.0 g, 6 mmol) and PPh$_3$ (1.6 g, 6 mmol). After stirred at r.t. for 12h, the reaction mixture was filtered and concentrated to give the crude product which was purified by flash chromatography (silica gel, 10%-50% EA in PE) to afford the product 4-4 (500 mg). Yield: 58%. LC-MS (ESI): m/z M + 2 = 211.10 / 213.01

**Preparation of 2-(1-(difluoromethyl)-1H-pyrazol-4-yl) acetonitrile (4-5):**

![Structure of 4-4](image3)

![Structure of 4-5](image4)

86%

A solution of 4-4 (500 mg, 2.3 mmol) in DMSO was added KCN (184 mg, 2.8 mmol). After stirred at r.t. for 15 min, the reaction mixture was concentrated to give the crude product which was purified by flash chromatography (silica gel, 10%-50% EA in PE) to afford the product 4-5 (320 mg). Yield: 86%. LC-MS (ESI): m/z M + 1 = 159.32

**Preparation of 2-(1-(difluoromethyl)-1H-pyrazol-4-yl) acetic acid (4-6):**

![Structure of 4-5](image5)

![Structure of 4-6](image6)

100%

A solution of 4-5 (320 mg, 2.03 mmol) in EtOH was added 1N NaOH (2.03 mL). After stirred at 60°C for 24h, the reaction mixture concentrated to give the crude product (200 mg) without further purification. LC-MS (ESI): m/z M + 1 = 177.21

**Preparation of 2-(2-(1-(difluoromethyl)-1H-pyrazol-4-yl) acetamido)-N-methyl-N,3-diphenylpropanamide (CX04):**

![Structure of 4-6](image7)

![Structure of CX04](image8)

21%

A solution of 4-6 (100 mg, 0.57 mmol) in DCM was added amine (160 mg, 0.62 mmol), T$_3$P (361 mg, 1.14 mmol) and DIPEA (300 mg, 2.28 mmol). After stirred at r.t. for 1h, the reaction mixture was concentrated to give the crude product which was purified by Gilson (C18, 20%-100% MeCN in H$_2$O with 0.1% HCOOH) to afford the product (40 mg). Yield: 21%. LC-MS (ESI): m/z M + 1 = 413.53, 1H NMR (400 MHz, CDCl$_3$) δ 7.58 (s, 1H), 7.47 – 7.36 (m, 1H), 7.34 – 7.25 (m, 3H), 7.17 – 7.08 (m, 3H), 6.94 – 6.83 (m, 2H), 6.77 (dd, J = 7.2, 2.0 Hz, 2H), 6.43 (d, J = 8.3 Hz, 1H), 4.77 (dd, J = 15.3, 7.4 Hz, 1H), 3.30 (s, 3H), 3.16 (s, 3H), 2.83 (dd, J = 13.4, 6.8 Hz, 1H), 2.62 (dd, J = 13.4, 7.5 Hz, 1H).
Preparation of CX05

Preparation of (1-methyl-5-(trifluoromethyl)-1H-pyrazol-4-yl) methanol (5-2):

\[
\begin{align*}
\text{(5-1)} & \xrightarrow{\text{LiAlH}_4, \text{THF}, \text{r.t.}} \text{(5-2)} \\
\end{align*}
\]

At 0°C, to a solution of 5-1 (200 mg, 0.9 mmol) in THF (4 mL) was added LiAlH\(_4\) (171 mg, 4.5 mmol), the resulting mixture was stirred at room temperature for 3 hours. The reaction was quenched with water, and extracted with Et\(_2\)O. The combined organic phases were washed with brine (15 mL), dried over Na\(_2\)SO\(_4\), and filtered. The filtrate was concentrated, and the residue was used for the next step directly (190 mg, 100%).

Preparation of 4-(bromomethyl)-1-methyl-5-(trifluoromethyl)-1H-pyrazole (5-3):

\[
\begin{align*}
\text{(5-2)} & \xrightarrow{\text{CBr}_4, \text{PPh}_3, \text{MeCN}, \text{r.t.}} \text{(5-3)} \\
\end{align*}
\]

To a solution of 5-2 (500 mg, 2.8 mmol) in anhydrous MeCN (15 mL) were added PPh\(_3\) (1.5 g, 5.72 mmol) and CBr\(_4\) (1.88 g, 5.67 mmol) at 0°C. The resulting mixture was stirred at room temperature overnight. The reaction was concentrated and purified by flash chromatography (silica gel, 30% ethyl acetate in petroleum ether) to provide 5-3 (469 mg, 69%).

Preparation of 2-(1-methyl-5-(trifluoromethyl)-1H-pyrazol-4-yl) acetonitrile (5-4):

\[
\begin{align*}
\text{(5-3)} & \xrightarrow{\text{NaCN, DMF, 100 °C}} \text{(5-4)} \\
\end{align*}
\]

A solution of 5-3 (420 mg, 2.2 mmol) in DMF was added KCN (286 mg, 4.4 mmol). After stirred at r.t. for 4 hours, the reaction mixture was concentrated to give the crude product which was purified by flash chromatography (silica gel, 10%~50% EA in PE) to afford the product 5-4 (357 mg, 86%).

Preparation of 2-(1-methyl-5-(trifluoromethyl)-1H-pyrazol-4-yl) acetic acid (5-5):

\[
\begin{align*}
\text{(5-4)} & \xrightarrow{\text{NaOH(1N), EtOH, 60 °C}} \text{(5-5)} \\
\end{align*}
\]

To a solution of 5-4 (150 mg, 0.7 mmol) in EtOH (8 mL), KOH (18 mg, 4 mmol) and water (2 mL) were added. The resulting mixture was stirred at 60°C for 8 hours. Then the solution was concentrated to give the crude product which was used for the next step without further purification (104 mg, 72%).

Preparation of N-methyl-2-(2-(1-methyl-5-(trifluoromethyl)-1H-pyrazol-4-yl) acetamido)-N,3-diphenylpropanamide (CX05)
To a solution of 5-5 (140 mg, 0.673 mmol) in DCM (3 mL) were added amine (188 mg, 0.74 mmol), T₃P (860 mg, 2.7 mmol) and DIPEA (350 mg, 2.69 mmol), the resulting mixture was stirred at room temperature for 12 hours. Then it was washed with water and 5% HCl aqueous solution and concentrated to give crude product which was purified by Pre-HPLC to afford CX05 (25 mg, 7%) as white solid. LC-MS (ESI): m/z 445.42 [M+1], 1H NMR (400 MHz, CDCl₃) δ 7.36 – 7.22 (m, 4H), 7.16 – 7.06 (m, 3H), 6.84 – 6.66 (m, 4H), 6.03 (d, J = 8.2 Hz, 1H), 4.76 (dd, J = 15.1, 7.6 Hz, 1H), 3.91 (d, J = 0.8 Hz, 3H), 3.38 (d, J = 1.0 Hz, 2H), 3.13 (s, 3H), 2.80 (dd, J = 13.3, 7.6 Hz, 1H), 2.62 (dd, J = 13.2, 6.7 Hz, 1H).

Preparation of CX06

Preparation of ethyl 1-ethyl-1H-pyrazole-4-carboxylate (6-2):

A solution of 6-1 (500 mg, 3.57 mmol) in DMF (5 mL) was added NaH (314 mg, 3.93 mmol) at 0°C. After 20 mins, iodoethane was added and the mixture was stirred at room temperature for 16 hours. The reaction was quenched with water (30 mL), and extracted with EA (30 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over Na₂SO₄, and filtered. The filtrate was concentrated, and the residue was used for the next step directly (57%).

Preparation of ethyl 1-ethyl-5-formyl-1H-pyrazole-4-carboxylate (6-3):

At -78°C, to a solution of LDA (127 mg, 1.2 mmol) in THF (10 mL) was added 6-2 (100 mg, 0.6 mmol). The resulting mixture was stirred at -78°C for 5 mins. Then DMF (346 mg, 0.8 mmol) was added, and the mixture was warmed to room temperature for another 1 hour. The resulting solution was diluted with water (10 mL) and extracted with EA (15 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash chromatography (silica gel, 30% ethyl acetate in petroleum ether) to provide 6-3 (36 mg, 31%).

Preparation of ethyl 5-(difluoromethyl)-1-ethyl-1H-pyrazole-4-carboxylate (6-4):

At -78°C, to a solution of LDA (127 mg, 1.2 mmol) in THF (10 mL) was added 6-2 (100 mg, 0.6 mmol). The resulting mixture was stirred at -78°C for 5 mins. Then DMF (346 mg, 0.8 mmol) was added, and the mixture was warmed to room temperature for another 1 hour. The resulting solution was diluted with water (10 mL) and extracted with EA (15 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over Na₂SO₄, filtered and concentrated. The residue was purified by flash chromatography (silica gel, 30% ethyl acetate in petroleum ether) to provide 6-3 (36 mg, 31%).
To a solution of 6-3 (50 mg, 0.255 mmol) in DCM (1 mL) was added DAST (102.8 mg, 0.638 mmol) at 0°C. The resulting mixture was stirred at room temperature for 16 hours. Then the solution was diluted with water (10 mL) and extracted with EA (15 mL×3). The combined organic phases were washed with brine (25 mL×3), dried over NaSO$_4$, filtered and concentrated. The residue was used in the next step without purification (66%).

**Preparation of (5-(difluoromethyl)-1-ethyl-1H-pyrazol-4-yl) methanol(6-5):**

![6-4](image1) \rightarrow LiAlH$_4$ \rightarrow THF, r.t. \rightarrow 100% \rightarrow ![6-5](image2)

To a solution of 6-4 (370 mg, 1.697 mmol) in anhydrous THF (5 mL) was added LiAlH$_4$ (322 mg, 8.486 mmol) at 0°C. The resulting mixture was stirred at room temperature for 30 mins. Then the reaction was quenched with water and extracted with Et$_2$O; the combined organic phases were dried over NaSO$_4$, filtered and concentrated to give the crude product which was used for the next step without further purification (100%).

**Preparation of 4-(bromomethyl)-5-(difluoromethyl)-1-ethyl-1H-pyrazole (6-6):**

![6-5](image3) \rightarrow CBr$_4$, PPh$_3$ \rightarrow MeCN, r.t. \rightarrow 43% \rightarrow ![6-6](image4)

To a solution of 6-5 (150 mg, 0.852 mmol) in anhydrous MeCN (15 mL) were added PPh$_3$ (565 mg, 1.704 mmol) and CBr$_4$ (447 mg, 1.704 mmol) at 0°C. The resulting mixture was stirred at room temperature overnight. The reaction was concentrated and purified by flash chromatography (silica gel, 20% ethyl acetate in petroleum ether) to provide 6-6 (87 mg, 43%).

**Preparation of 2-(5-(difluoromethyl)-1-ethyl-1H-pyrazol-4-yl)acetonitrile (6-7):**

![6-6](image5) \rightarrow KCN, 100 °C \rightarrow DMF \rightarrow 100% \rightarrow ![6-7](image6)

A solution of 6-6 (100 mg, 0.418 mmol) in DMF was added KCN (30 mg, 0.418 mmol). After stirred at r.t. for 15 min, the reaction mixture was concentrated to give the crude product which was purified by flash chromatography (silica gel, 10%~50% EA in PE) to afford the product 6-7 (77 mg, 100%).

**Preparation of 2-(2-(5-(difluoromethyl)-1-ethyl-1H-pyrazol-4-yl) acetamido)-N-methyl-N,3-diphenylpropanamide (CX06)**

![6-7](image7) \rightarrow KOH, 60 °C \rightarrow H$_2$O \rightarrow 62% \rightarrow ![6-8](image8)

To a solution of 6-7 (80 mg, 0.4 mmol) in EtOH (4 mL), NaOH (120 mg) and water (2 ml) were added. The resulting mixture was stirred at 60°C overnight. Then the solution was concentrated to give the crude product which was used for the next step without further purification (62%).

**Preparation of 2-(2-(5-(difluoromethyl)-1-ethyl-1H-pyrazol-4-yl) acetamido)-N-methyl-N,3-diphenylpropanamide (CX06)**
To a solution of 6-8 (55 mg, 0.127 mmol) DCM (5 mL) were added amine (70 mg, 0.27 mmol), T3P (250 mg, 0.54 mmol) and DIPEA (140 mg, 1.08 mmol), the resulting mixture was stirred at room temperature for 12 hours. Then it was washed with water and 5% HCl aqueous solution and concentrated to give crude product which was purified by Pre-HPLC to afford CX06 (25 mg, 21%) as white solid. LC-MS (ESI): m/z 441.42 [M+1], 1H NMR (400 MHz, CDCl3) δ 7.38 – 7.23 (m, 4H), 7.18 – 7.03 (m, 3H), 6.90 – 6.73 (m, 4H), 6.68 (s, 1H), 6.15 (d, J = 8.2 Hz, 1H), 4.74 (dd, J = 15.4, 7.1 Hz, 1H), 4.21 (q, J = 7.2 Hz, 2H), 3.32 (s, 2H), 3.18 – 3.03 (m, 3H), 2.79 (dd, J = 13.3, 7.3 Hz, 1H), 2.60 (dd, J = 13.3, 6.9 Hz, 1H), 1.45 – 1.35 (m, 3H).

**Cells**

Human embryonic kidney 293T cells (a gift from Dr. Irwin Chaiken, Drexel University, Philadelphia, PA) and TZM-bl reporter cell lines (obtained through the NIH AIDS Reagent Program from Dr. John C. Kappes, Dr. Xiao-yun Wu and Tranzyme Inc.) [12] were cultured in Dulbecco’s Modified Eagles Medium (DMEM), 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. Human astroglia U87 cells stably expressing CD4/CCR5 or CD4/CXCR4 were obtained from Professor Hongkui Deng, Peking University, and Prof. Dan Littman, New York University, USA, through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NI [13,14]. U87 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, 300 µg/ml G418 (Thermo Scientific, Waltham, MA) and 1 µg/ml Puromycin (Thermo Scientific). All cells were incubated continuously, unless otherwise stated, at 37°C in a humidified chamber containing 5% CO2 air environment.

**Antibodies and peptides**

Antibodies were obtained from the following sources: Mouse anti-p24 monoclonal antibody and rabbit anti-p24 polyclonal antibody from Abcam, Cambridge, United Kingdom (Cat numbers: ab9071 and ab63913, respectively); HRP-conjugated goat anti-rabbit antibody from Life Technologies, Carlsbad, CA (Cat numbers: 31460). Antibodies were used at a concentration of 0.5 µg/ml unless otherwise indicated. N-terminal biotinylated CPSF6 (308-327) peptide (DRPPPPVLFPGQPFGQPPLG) and non-biotinylated CPSF6 (313-327) peptide (PVLFPGQPFGQPPLG) were synthesized by GenScript Corp. (Piscataway, NJ).

**Elisa-based quantification of capsid (p24) content**

An ELISA plate was coated with 50 ng of mouse anti-p24 (Abcam, ab9071) per well for 2 hours at room temperature, blocked with 3% BSA for 2 hours at room temperature and washed with PBST buffer (0.1% Tween-20 in PBS). Pseudovirals stocks were lysed with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 hour and added to the plate overnight at 4°C. Simultaneously, p24 protein (produced and purified as described below) was added for the generation of a standard curve. Following the overnight incubation, the plate was washed with PBST buffer and 1:5000 dilution of rabbit anti-p24 (Abcam, ab63913) was added for 2 hours at room temperature. After washing the unbound rabbit anti-p24 off the plate with PBST buffer, goat anti-rabbit-HRP at a 1:5000 dilution were added for 2 hours at room temperature. The plate was then extensively washed with PBST buffer. Subsequently, a solution of 0.4 mg/ml o-phenylenediamine in a phosphate-citrate buffer with sodium perborate (Sigma-Aldrich) was added and incubated in the dark for 10 minutes. Optical densities were then obtained at 450 nm in a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific).

**Recombinant capsid overproduction, purification, and mutagenesis**

The overproduction and purification of p24 (HIV-1 CA) were performed as previously described [15]. Briefly, the procedure is also described here: plasmid containing C-terminally His-tagged HIV-1NL4-3 CA (a gift from Dr. Eric Barklis, Oregon Health and Science University, Portland, OR) was transformed into BL21 Codon Plus (DE3)-RIL Competent Cells (Agilent Technologies, Wilmington, DE) and grown up in auto-induction medium ZYP-5052 overnight with shaking (225 rpm) at 30°C [16]. Bacterial cultures were spun down at 5000 rpm (J2-HC centrifuge, Beckman), and the supernatant was discarded. Cell pellets were resuspended in PBS and lysed via sonication. The resultant supernatant was ultracentrifuged at 40,000 rpm (Optima LE-80K ultracentrifuge, Beckman) for 1 hour and immediately applied to a Talon cobalt resin affinity column (Clontech Laboratories, Mountain View, CA) and eluted with 0.2 M imidazole PBS buffer. The eluted protein was dialyzed overnight in 20 mM Tris-HCl pH 8.0 at 4°C, concentrated and applied to a Q-sepharose column (HiTrap Q HP, GE 71-7149-00 AP, GE), then washed with 20mM Tris-HCl pH 8.0 and eluted with 0.3M NaCl in 20 mM Tris-HCl pH 8.0. Purified capsid protein was dialyzed into 20 mM Tris-HCl pH 8.0 at 4°C, concentrated to 120 µM, flash frozen.
in liquid nitrogen, aliquoted and stored at -80°C. Capsid hexamer was generated by introducing mutations at following sites: A14C, E45C, W184A, and M185A. Capsid mutagenesis was done by site-directed mutagenesis (Stratagene).

Production of pseudotyped viruses

Single-round infectious specific envelope-pseudotyped luciferase-reporter viruses were produced by dual transfection of two plasmids (3 μg of viral backbone plasmid and 4 μg of viral envelope plasmid) in 6-well plated 293T cells (0.8x10⁵ cells/well) [13]. Viral backbone plasmid is an envelope-deficient HIV-1 pNL4-3-LucR+E- plasmid that carries the luciferase-reporter gene [17]. Viral envelope plasmid is a plasmid expressing either the HIV-1 JR-FL envelope [18,19] or the heterologous envelope from amphotropic murine leukemia virus (AMLV) or the vesicular stomatitis virus glycoprotein (VSV-G). Transfections of these plasmids was carried out via calcium phosphate transfection (Profection Mammalian Transfection System, Promega, Madison, WI) or using the JetPrime transfection reagent (Polypus #712-60) for 5-6 hours. Following the incubation, transfection reagent and the DNA-containing medium was removed, cells were washed with DMEM and replenished with fresh culture media. Supernatants containing produced pseudovirus were collected 48 hours post-transfection, clarified, filtered, aliquoted and stored at -80°C. Mutations in the HIV-1 pNL4-3-LucR+E- plasmid were performed by Genscript (Piscataway, NJ).

Single-round infection assay

The details of the single-round HIV-1 infection assay for detecting viral infectivity have been published previously [17,20,21]. Briefly, U87.CD4.CCR5 (1.2 x 10⁵ cells/well) target cells were seeded in 96-well luminometer-compatible tissue culture plates (Greiner bio-one). After 24 hours incubation at 37°C, a compound or DMSO (vehicle control for the compound, Sigma) were mixed with pseudotyped viruses (normalized to p24 content) and the mixture was added to the target cells and incubated for 48 hours at 37°C. Subsequently, the media was removed from each well, and the cells were lysed by the addition of 50 μl of luciferase lysis buffer (Promega) and one freeze-thaw cycle. A GloMax 96 microplate luminometer (Promega) was used to measure the luciferase activity of each well after the addition of 50 μl of luciferase assay substrate (Promega). Compound-induced effects are manifested as a decrease in infectivity in the target cells (measured as luciferase activity), normalized against the infectivity of virus produced from DMSO (vehicle control) treated cells. The effects of a compound on viral late stage were also ascertained by effect on viral production as monitored by the p24 assay as described above.

Competition ELISA assay

A 96-well ELISA plate was coated with 200 ng of neuraminidase per well for overnight at 4°C. The next day the plate was blocked with 3% BSA for overnight at 4°C. The plates were washed five times with PBST buffer (0.1% Tween in PBS). Biotinylated CPSF6 peptide (final concentration 0.1 μg/ml) in blocking buffer (0.5% BSA in PBS) was added in and incubated for 4 hours. The plate was then washed five times with PBST buffer. Capsid hexamer (final concentration 0.4 μg/ml) with or without a compound were mixed and added to the plate in blocking buffer (0.05% BSA in PBS). The mixture was incubated for overnight at 4°C. Following the overnight incubation, the plate was washed with PBST buffer and 1:5000 dilution of rabbit anti-p24 (Abcam, ab63913) was added for 2 hours at room temperature. After washing the unbound rabbit anti-p24 off the plate with PBST buffer, goat anti-rabbit-HRP at a 1:5000 dilution was added and incubated in the dark for 30 minutes. Optical densities were then obtained at 450 nm in a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific). The binding of capsid hexamer to the CPSF6 peptide in the presence of a compound was normalized to the binding without a compound (only in the presence of DMSO, as vehicle control for all compounds). A non-biotinylated CPSF6 peptide and compound PF-74 were used as positive binding controls for this assay.

Surface plasmon resonance assay

All binding assays were performed on a ProteOn XPR36 SPR Protein Interaction Array System (Bio-Rad Laboratories, Hercules, CA). The instrument temperature was set at 25°C for all kinetic analyses. ProteOn GLH sensor chips were preconditioned with two short pulses each (10 seconds) of 50 mM NaOH, 100 mM HCl, and 0.5% sodium dodecyl sulphate. Then the system was equilibrated with PBS-T buffer (20 mM sodium phosphate, 150 mM NaCl, and 0.005% polysorbate 20, pH 7.4). The surface of a GLH sensor chip was activated with a 1:100 dilution of a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.2 M) and sulfo-N-hydroxysuccinimide (0.05 M). Immediately after chip activation, the HIV-1 disulfide stabilized NL4-3 capsid protein, purified as outlined in Pornillos et al. [22], was prepared at a concentration of 100 μg/ml in 10 mM sodium acetate, pH 5.0 and injected across ligand flow channels for 5 min at a flow rate of 30 μl/min. Then, after unreacted protein had been washed out, excess active ester groups on the sensor surface were capped by a 5 minutes injection of 1 M ethanolamine HCl (pH
8.0) at a flow rate of 5 μl/min. A reference surface was similarly created by immobilizing a non-specific protein (IgG b12 anti HIV-1 gp120; obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 gp120 Monoclonal (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas) and was used as a background to correct non-specific binding.

To prepare a compound for direct binding analysis, compound stock solutions, along with 100% DMSO, and totaling 30 μl was made to a final volume of 1 ml by addition of sample preparation buffer (PBS, pH 7.4). Preparation of analyte in this manner ensured that the concentration of DMSO was matched with that of running buffer with 3% DMSO. Serial dilutions were then prepared in the running buffer (PBS, 3% DMSO, 0.005% polysorbate 20, pH 7.4) and injected at a flow rate of 100 μl/min, for a 1 minute association phase, followed by up to a 5 minutes dissociation phase using the “one shot kinetics” capability of the Proteon instrument [23]. Data were analyzed using the ProteOn Manager Software version 3.0 (Bio-Rad). The responses of a buffer injection and responses from the reference flow cell were subtracted to account for the nonspecific binding and injection artifacts. The equilibrium dissociation constant (K<sub>d</sub>) for the interactions, and derived from a minimum of four experiments, were calculated in ProteOn Manager Version 3.1.0.6 (Bio-Rad, Hercules, CA), using the equilibrium analysis function.

For the mutant analysis, responses at a single concentration of compound, either PF-74 or CX06, were expressed as a percentage of R<sub>max</sub> to take into account differences in molecular weights between the compound and differences in immobilization densities.

In vitro capsid assembly assay

The effect of compounds on the assembly of HIV-1 CA was measured by monitoring turbidity at 350 nm using a modification of the method of our lab and others [15,24,25]. Briefly, 1.0 μl of concentrated compound (5 mM) in 100% DMSO was added to a 74-μl aqueous solution (solution was made by mixing 2 ml of 5 M NaCl with 1 ml of 200 mM NaH2PO4, pH 8.0). To initiate the assembly reaction, 25 μl of purified capsid protein (120 μM) was added. An identical reaction mixture was prepared, omitting the compound (i.e., just DMSO as a vehicle control). Samples were allowed to equilibrate for 2 minutes before reading. Readings were taken at 350 nm every one minute for 39 minutes. Capsid was used at a final concentration of 30 μM.

Nuclear import assay

Fluorescently labeled HIV-1 particles were produced in 293T/17 cells (from ATCC, Manassas, VA) by co-transfecting plasmids expressing the viral genome (pR9ΔEnv), VSV-G glycoprotein (pMD2.G, Add gene, Cat#1259) and the Vpr-NSfGFP plasmid (Francis et al., 2014). Fluorescent viruses were collected at 36 hours post-transfection, filtered through a 0.45 µm filter and quantified for RT activity. TZM-bl cells were infected at MOI of 10 and 36 hours post-infection, filtered through a 0.45 µm filter, and cell-free supernatant was collected and preserved for measurement of green fluorescent protein (GFP) fluorescence in a background to correct non-specific binding.

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and integration inhibition, cells were treated with TMC278 (also referred to as Rilpivirine, obtained via AIDS Research and Reference Reagent Program [29]).

Computational docking analyses

Docking of CX06 into the capsid dimer: Docking calculations were carried out on a CA dimer extracted from PDB 4XPF. The CA protein was prepared by the Protein Preparation Wizard implemented with Maestro (Schrödinger Maestro Version 11.5.011, MM share Version 4.1.011, Release 2018-1, Platform Darwin-x86_64). The Grid box was centered on the co-crystalized PF-74. For validating the glide docking protocol, the original ligand PF-74 was built using the LigPrep tool (Schrödinger Maestro Version 11.5.011) and docked using glide-XP mode. The predicted docked pose matched the original co-ordinates of the co-crystalized PF-74 with an RMS value of 0.3. CX06 was docked using the same glide-XP mode and the top ranked pose was selected as a representation of the predicted binding mode.

Docking of PF-74 and CX06 into the capsid dimer Q63A mutant: Residues Gln63, in both protomers, were mutated to Ala in silico (Schrödinger Maestro Version 11.5.011). The mutated protein was then energy minimized using MacroModel (water as a solvent and OPLS3 force field). The same grid preparation steps and docking protocol were used to dock PF-74 and CX06 into the Q63A CA. The top ranked pose for each was selected as a representation of the predicted binding mode.

RESULTS AND DISCUSSION

Computational design of head group variants of PF-74

To explore modifications of PF-74, as a lead into our overarching goal, we chose to start fairly small and choose a limited number of compounds to design and focused upon the changes to the methylindole head group of PF-74. We first imported the structure of PF-74 bound to the native HIV-1 capsid hexamer structure (PDB ID 4XPF) [2] into Spark Version 10.4 (Cresset®, Litlington, Cambridgeshire, UK; http://www.cresset-group.com/spark/) to derive a field-based high-content pharmacoophere [30,31]. Two protomers, extracted from the native hexameric HIV-1 capsid protein structure, were added as excluded volume to discourage the selection of bioisosteric fragments that would clash with the protein within the interprotomer pocket during the search (Figure 1). Spark searches a database of inducing fragments derived from multiple databases to find non-classical bioisosteres that exhibit similar shape and electronic properties as the region of interest when placed in the context of the final molecule [11]. The results of this search were analyzed and structures that displayed low 2D similarity, while retaining a sufficiently high BIF% value (a factor that indicates how good the replacement is in the context of the conformation of the entire molecule) were favored. Finally, the suggested molecules were analyzed for their predicted ADME properties (absorption, distribution, metabolism, and excretion) and compared to PF-74. The in silico prediction of drug-like metrics was achieved using StarDrop 6.4 (Optibrium, Ltd., Cambridge, UK) [32], implementing the oral non–central nervous system (CNS) drug profile [33], supplemented with an additional parameter for logD. Details for the specific models are provided in the StarDrop Reference Guide from Optibrium and are online at the StarDrop FAQs (http://www.optibrium.com/community/faq/adme-qsar-models). A probabilistic scoring algorithm [34] is then used to combine the model predictions in the oral non-CNS drug profile into an overall score. For reference, scores range from 0 to 1, with 0 suggesting extremely non–drug-like, and 1 suggesting the perfect drug. Following these analyses, four compounds containing 2-(propan-2-yl)-4,5,6,7-tetrahydro-1H-1,3-benzodiazole, 1-(difluoromethyl)-1H-pyrazole, 1-methyl-5-(trifluoromethyl)-1H-pyrazole, and 5-(difluoromethyl)-1-ethyl-1H-pyrazole head groups were chosen for synthesis and evaluation. All of these compounds had improved predicted ADME properties over that of PF-74 (Figure 2). The syntheses of these compounds, termed CX03, CX04, CX05, and CX06, are outlined in supplemental data. PF-74 MilliporeSigma (Burlington, MA) served as a control compound for these studies. All CX compounds were synthesized as racemic mixtures. The metabolic stability of these new compounds were tested and compared to PF-74. Although they did not display a huge increase in stability, all of the CX compounds did have greater half-lives as compared to PF-74, with the greatest increase being 4-fold for CX04 (Supplemental Data, Table 2).

The CX compounds inhibit HIV-1 from distinct genetic subtypes replicating in human primary peripheral blood mononuclear cells

Following successful syntheses, we next sought to quantify the potency of CX compounds against the fully infectious virus. Moreover, this assay was performed using healthy, primary cells to gain further insight into the potential toxicities of the compound towards natural target cells. Additionally, as a key issue in the development of novel HIV drugs is their ability to inhibit the replication of genetically diverse isolates (especially isolates from the most globally prevalent subtypes A, B, and C), we chose to assess the potency of the CX compounds to inhibit the replication of the primary isolates (i.e., not previously passaged in cell lines prior to cloning) from subtypes A (HIV-1_189E802), B (HIV-1_18905), and C (HIV-1_189090), in primary human peripheral blood mononuclear cells (PBMCs) [11,13,15,35]. The toxicity of the CX compounds to the PBMCs using an MTS assay was evaluated in parallel [33]. PF-74 was tested at the same time for comparison, and as to our knowledge, it hasn’t been tested in this particular format, against these particular isolates. The results of these analyses are summarized in Table 1. As can be observed from the IC50 values, the CX compounds displayed antiviral activity against all of the isolates tested, despite their varying degrees of 2D structure dissimilarity (based on Tanimoto similarity scores based on Merck Atom Pairs) [36]. Moreover, the CX compounds demonstrated no toxicity over the concentration range under investigation, in contrast to PF-74 (Supplemental data, Figure 5). Compounds CX03 and CX06 displayed the best potency, with a median IC50 value over the three isolates tested of 5.9 µM, as compared to the median IC50 of PF-74 of 0.9 µM (Table 1). CX03 demonstrates a Spark-derived shape score of 0.948, which is indicative of it being 3-dimensionally the closest to PF-74. Interestingly, CX06 displays the lowest 3D similarity to PF-74, despite retaining almost identical potency as the most similar compound, CX03.

The CX compounds retain target specificity of the parental molecule, PF-74

Having demonstrated antiviral activity of the CX compounds, we next sought to verify that the compounds still bind to HIV-1 CA. We, therefore, overproduced and purified the HIV-1 NL4-3 disulfide stabilized CA hexamer, as outlined previously [22], for use in surface plasmon resonance (SPR) interaction analysis using the ProteOn™ XPR36 Protein Interaction Array System (Bio-Rad, Hercules, CA). Detailed methods are provided in the material and methods section. Briefly, the pure CA hexamer was immobilized to the surface of a GLH to a density of 16,048 RU using standard amine coupling procedures. A reference surface was similarly created using IgG b12 [37] and was matched with the experimental CA hexamer surface density to within 3%. First, we tested the activity of the CA hexamer surface by performing binding analyses using a peptide derived from nucleoporin 153 (NUP153) [38]. The NUP153_{1407-1423} peptide [3] was synthesized by Genscript (Piscataway, NJ) and dissolved in pure DMSO to a concentration of 50 mM. This stock was then used to make a concentration series, diluted to match that of the running buffer for the experiment (PBS, pH 7.4, 0.005% polysorbate 20, 3% DMSO). Figure 1, Panel A shows representative sensorgrams from this analysis. The equilibrium dissociation constant (K_D) for this interaction, and derived from a minimum of four experiments, was calculated in ProteOn Manager Version 3.1.0.6 (Bio-Rad, Hercules, CA), using the equilibrium analysis function and was found to be 202 ± 22 µM. This is in excellent agreement with the

Figure 1 PF-74 bound in the inter-protomer pocket. Positive field surfaces of the compounds are depicted in red; negative field surfaces of the compounds are depicted in blue, and hydrophobic field surfaces of the compounds are depicted in gold. Visualized in Flare Version 2.0 (Cresset®, Litlington, Cambridgeshire, UK; http://www.cresset-group.com/flare/) [30]. The green cross represents a chloride ion present in the structure.

Figure 2 (A) The individual models that comprise the modified oral non-CNS (central nervous system) drug profile and their respective importance to the profile. HIA = human intestinal absorption; hERG (human ether-a-go-go-related gene); IC50 = half-maximal inhibitory concentration; BBB = blood–brain barrier. (B) Plot showing the StarDrop (Optibrium, Ltd., Cambridge, UK)-derived logS versus the score from a multimetric, modified oral non-CNS profile for PF-74 and the CX compounds.

Figure 3 Sensorgrams depicting the interaction of (A) NUP1531407-1423 peptide, (B) PF-74, (C) CX03, (D) CX04, (E) CX05, and (F) CX06, with immobilized HIV-1NL4-3 CA hexamer. Concentration shown: NUP1531407-1423 peptide (450, 225, 113, 56.3, 28.1 and 0µM); PF-74 (20, 5, 1.25, 0.313, 0.0.78 and 0µM); CX03 (225, 56.3, 14.1, 3.52, 1.2 and 0µM); CX04 and CX05 (300, 150, 75, 37.5, 18.8 and 0µM); CX06 (100, 33.3, 11.1, 3.7, 1.2 and 0µM).
Figure 4 CX06 functions in the late and early stages of HIV-1 replication. (A) To assess the effect of CX06 on late-stage events, recombinant virus was produced from 293T cells either in the absence or presence of CX06, and the culture supernatants containing pseudotype stocks were diluted tenfold and then used to infect target cells in the absence of test compound. Compound-induced effects are manifested as a decrease in infectivity in the target cells (measured as luciferase activity), normalized against the infectivity of virus from untreated cells. (B) Effects of CX06 on early-stage events were determined by producing virus via transfection of 293T cells (as described in the materials and methods) for infection of U87.CD4. CCR5 target cells in the absence or presence of various concentrations of CX06. The antiviral effects of CX06 were determined to be due to a specific effect on the virus as the compound was not cytotoxic to 293T or U87.CD4.CCR5 cells over the concentration range tested.

Figure 5 The effect of compound CX06 on viral production. The effects of compound CX06 (20 µM) on the production of HIV-1JR-FL Envelop pseudotyped viruses relative to vehicle DMSO control as shown by p24 quantitation. Data shown are mean values of three individual experiments done in triplicate, with error bars depicting the standard error of the mean (SEM). Statistical significance was assessed by student t-test (p<0.005).

Effects on assembly were identified by incubating the viral producer cells in the absence or presence of various concentrations of CX06. Supernatants containing HIV-1JR-FL envelope pseudotyped virus (which encodes for firefly luciferase as a reporter gene) were then diluted tenfold and used to infect U87-CD4-CXCR4 target cells. Compound-induced effects are manifested as a decrease in infectivity in the target cells, compared with those infected with the virus from untreated cells. Effects on early-stage events were determined by using virus produced in the absence of compound for infection of target cells in the absence or presence of various concentrations of CX06. As can be seen in Figure 4, CX06 displays effects in both the late, assembly

Figure 6 (A) Effect of CX06 on AMLV Envelop pseudotyped HIV-1 infection. (B) Time of addition study. TZM-bl cells were infected with VSV-pseudotyped HIV-1 virus. Drugs Nevirapine (10 µM), Raltegravir (10 µM), PF-74 (2 µM) and CX06 (20 µM) were added at indicated time points. Infectivity was measured by the luciferase assay (Promega) at 48 hpi and normalized to DMSO control. Data shown are mean values of three individual experiments done in triplicate. Error bars = STD. dev.
and early, infective stages of the HIV-1 replication cycle. This dual-stage inhibition profile is shared with the parental molecule PF-74 (Supplemental Data, Figure 7).

**Elucidating the late stage mechanism**

Having demonstrated that CX06 exerts an antiviral effect upon HIV-1 replication at the late stage, we next sought to determine the nature of this block. PF-74 has been demonstrated to also have an antiviral effect in the late, assembly stages of HIV-1 replication, producing misformed virions, rather than reducing the amount of virus produced [6]. Therefore, we chose to first look at the amount of virus produced in the presence of CX06 before looking more closely. Quantification of the virus produced in the presence of 20 µM of the compound indicates that it functions by simply decreasing the amount of virus released from the cells, as judged by p24 content (Figure 5).

**Elucidating the early stage mechanism**

The results shown in Figure 4B, demonstrate that CX06 also exerts an antiviral effect at the early stage of replication. In the assay used, this early stage encompasses every process from the entry of the virus, to the final integration of the proviral DNA into the host genome. Therefore, we next sought to hone in on the exact process or processes that are disrupted in the phase by CX06. To rule out an effect on the entry process as a point of intervention for this compound. To do this, we performed the single round infection assay, but pseudotyped the HIV-1 virions with the amphotropic murine leukemia virus (AMLV) Env. As can be seen in Figure 6A, CX06 inhibited this AMLV Envpseudotyped HIV-1 with almost identical potency as when the HIV-1 Env was used (Figure 4), suggesting that CX06 targets a post-entry process or processes. This finding is corroborated by a time-of-addition study, in which CX06 is only found to be effective within the first 6 hours post-infection (Figure 6B).

The CA-mediated process of uncoating is an essential but poorly understood process but is tightly associated with the process of reverse transcription [39]. As such, a hallmark of the disruption of uncoating in HIV-1 replication is the inability of the virus to initiate and or complete reverse transcription. Unintegrated viral DNA synthesized during HIV-1 infection includes linear and circular forms, and each of these distinct viral cDNAs can be used as a surrogate marker for events surrounding the completion of reverse transcription and for nuclear import of viral DNA during replication [15]. We, therefore, used polymerase chain reaction (PCR)-based detection and quantitation of HIV-1 early, late and 2-long-terminal repeat (LTR) circle reverse transcriptase products, 2-LTR circles, and integrated provirus. Total DNA at 24 h (2-LTR) or 48 h (RUS, USW, integrated provirus) isolated from HIV-1JR-FL Env infected U87.CD4.CX05 cells. Real-time PCR was performed as described in Materials and Methods. Results were normalized to the endogenous control PBGD and reflect the relative fold change compared to DMSO, TMC278, PF-74, and CX06 treatment. Data shown are mean values of three individual experiments done in triplicate, with error bars depicting the standard error of the mean (SEM). Statistical significance was assessed by student t-test (p<0.005). TMC278 was used at a concentration of 1.5 nM, PF-74 was used at 1.5 µM, and CX06 was used at 15 µM. Nuclear import of HIV-1 INsfGFP complexes was visualized in TZM-bl cells at 4 hpi. Infections (MOI2) were treated with vehicle control (DMSO), RT inhibitor Nevirapine (Nevi 10µM) or CA binding drugs PF-74 (5µM) and CX06 (20µM). (E) Representative images, scale bar = 5 µm. (F) Quantification of nuclear INsfGFP complexes. Error bars represent SEM from 4 fields of view.

**Figure 7** CX06 treatment drastically reduces late reverse transcriptase products, 2-LTR circles, and integrated provirus. Total DNA at 24 h (2-LTR) or 48 h (RUS, USW, integrated provirus) isolated from HIV-1JR-FL Env infected U87.CD4.CX05 cells. Real-time PCR was performed as described in Materials and Methods. Results were normalized to the endogenous control PBGD and reflect the relative fold change compared to DMSO, TMC278, PF-74, and CX06 treatment. Data shown are mean values of three individual experiments done in triplicate, with error bars depicting the standard error of the mean (SEM). Statistical significance was assessed by student t-test (p<0.005). TMC278 was used at a concentration of 1.5 nM, PF-74 was used at 1.5 µM, and CX06 was used at 15 µM. Nuclear import of HIV-1 INsfGFP complexes was visualized in TZM-bl cells at 4 hpi. Infections (MOI2) were treated with vehicle control (DMSO), RT inhibitor Nevirapine (Nevi 10µM) or CA binding drugs PF-74 (5µM) and CX06 (20µM). (E) Representative images, scale bar = 5 µm. (F) Quantification of nuclear INsfGFP complexes. Error bars represent SEM from 4 fields of view.
Analysis of HIV-1 nuclear import revealed that PF-74 (2 µM) and CX06 (20 µM) efficiently inhibited the appearance of INsGFP spots in the nucleus, while the RT inhibitor Nevirapine was without effect. This result suggests that both PF-74 and CX06 abrogate CA-dependent steps of virus nuclear import.

Mutation of residues in the inter-protomer pocket reduces the sensitivity of HIV-1 to CX06 by decreasing its interaction with HIV-1 CA

Having demonstrated thus far that CX06 interacts with HIV-1 CA and inhibits HIV-1 replication in a manner consistent with disruption of CA function, we next sought to establish a direct link between its interaction with the CA protein and its antiviral effect. As a first step towards this, we chose to establish whether or not CX06 truly shares the same binding pocket as PF-74. To achieve this, we designed and implemented a competition ELISA using a peptide from CPSF6 [43]. It has been determined structurally and through competitive binding assays, that PF-74 and CPSF6 share a common binding site and that PF-74 interaction inhibits the binding of CPSF6 to CA [2-4]. Therefore, we capitalized on this to determine whether or not CX06 would inhibit CPSF6 interaction with CA (Figure 9). The non-biotinylated CPSF6 peptide CPSF6 molecules used in this assay. As can be seen, CX06 inhibits the binding of CA to CPSF6 to the same degree as PF-74 in this competition ELISA. As such, we can be reasonably confident that CX06 shares the same inter-protomer binding pocket as does CPSF6 and PF-74.

As CX06 and PF-74 share a common binding site, we next sought to establish if they share common interactions with residues within this site and demonstrate that the antiviral effect of CX06 is mediated through its interaction with HIV-1 CA. Therefore, we investigated mutation of residues shown to be contact residues for PF-74, and tested the sensitivity of these mutant viruses to inhibition by CX06. Also, we included two other mutations, P90A and N74D, which have shown to block the interaction of CA with cyclophilin A and to alter nuclear entry pathways, respectively [44,45]. HIV-1 pseudovirus containing CA wild-type (WT), CA N57A, CA Q63A, CA K70A, CA N74D, and CA P90A were produced and equal amounts of p24 were utilized to infect the cell for a single-round infection assay. Figure 10 shows the relative infectivity of these mutants, establishing that they are infectious enough to utilize in this assessment. As shown in Table 2, PF-74 is relatively resistant to these changes, as demonstrated by the minor changes in the IC50 values between wild-type and the mutant viruses. This is consistent with resistant virus selection studies that demonstrated to have a large effect on PF-74 potency, a mutant must have more than a single mutation [6]. CX06 showed a similar tolerance to mutations within the inter-protomer pockets, with the exception of Q63A, whose mutation resulted in an almost 7-fold increase in the IC50 value relative to wild-type. To investigate whether this is simply by reducing the interaction of CX06 with CA, or something more convoluted, we introduced the Q63A mutation into the disulfide stabilized Figure 10

The effect of compound CX06 on RT activity in vitro. Compounds CX06 (15 µM) and PF-74 (1.5 µM) have no effect on RT activity relative to vehicle DMSO control group. TMC278 (1 nM) has significant inhibitory effect on RT activity. Assay was performed using a colorimetric reverse transcriptase assay kit (Roche Diagnostics, IN, USA). Data shown are mean values of three individual experiments done in triplicate, with error bars depicting the standard error of the mean (SEM). Statistical significance was assessed by student t-test (p<0.005).
hexameric CA construct and performed SPR. Figure 11 shows that the mechanism by which the Q63A mutation desensitizes the virus harboring that mutation to CX06 is by reducing the CA protein’s interaction with the compound. Taken together, the discovery that mutation of CA glutamine 63 to alanine increases the half maximal inhibitory concentration of CX06 relative to wild-type, most likely by reducing interaction with the compound, demonstrates that the antiviral effect of CX06 is mediated through the CA.

**CX06 reduces CA assembly and destabilizes the assembled core**

A previous study by Lemke et al. [46], demonstrated that inhibitors that bind to the same pocket on the HIV-1 capsid can have distinct binding modes and mechanism of action. This, coupled with the difference in mutant sensitivity, prompted us to look at the effects of CX06 compounds on both the in vitro assembly of the HIV-1 CA [15] and the in vitro stability of intact viral cores using a previously published methodology [26].

First, we chose to look at whether or not CX06 accelerated CA assembly in vitro. Using the same turbidity assay utilized by Blair et al. [6], we assessed the effect of CX06 in this assay. Figure 12A shows the results of this assay. In line with its previously shown effect of accelerating assembly of HIV-1 CA, PF-74 (red) increased the rate of assembly of HIV-1 CA under the in vitro conditions of the assay as compared to DMSO alone (black). In stark contrast, CX06 in the same assay reduced the multimerization of HIV-1 CA in the assay compared to both PF-74 and DMSO. This reduction in assembly could easily explain the reduced amount of virions produced in the presence of the CX06.

Next, we looked at the effect of CX06 on the assembled mature HIV-1 capsid. Briefly, HIV-1 pseudoviruses produced in 293T cells were labeled with In5GFP and the CA marker cyclophilin A-DsRed (CypA-DsRed) [Francis et al 2016]. Single viral particles were immobilized onto coverslips and mildly permeabilized by saponin to allow core disassembly (loss of CypA-DsRed/CA) in the presence or absence of indicated compounds. Viruses were continuously imaged to measure the loss of CypA-DsRed/CA in puncta, while the number of In5GFP puncta remained stable over time (Supplemental data, Figure 6). Figure 12B shows that, unlike PF-74, compound CX06 accelerated the loss of CypA-DsRed/CA and thus destabilized the HIV-1 core. Destabilization of the conical capsid shell in the cytoplasm would likely inhibit the completion of reverse transcription and block nuclear import [42].

**Table 2: Potency of PF-74 and CX06 against wild-type and CA mutant viruses.**

<table>
<thead>
<tr>
<th>Virus</th>
<th>PF-74 IC₅₀ (µM)</th>
<th>Fold increase</th>
<th>CX06 IC₅₀ (µM)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.22 ± 0.05</td>
<td>1</td>
<td>3.1 ± 0.5</td>
<td>1</td>
</tr>
<tr>
<td>CA N57A</td>
<td>0.25 ± 0.04</td>
<td>1.14</td>
<td>4.3 ± 0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>CA Q63A</td>
<td>0.40 ± 0.05</td>
<td>1.82</td>
<td>21.4 ± 1.4</td>
<td>6.9</td>
</tr>
<tr>
<td>CA K70A</td>
<td>0.25 ± 0.08</td>
<td>1.14</td>
<td>3.2 ± 0.3</td>
<td>1.03</td>
</tr>
<tr>
<td>CA N74D</td>
<td>0.50 ± 0.10</td>
<td>2.3</td>
<td>2.4 ± 0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>CA P90A</td>
<td>0.21 ± 0.04</td>
<td>0.95</td>
<td>3.1 ± 0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

**Figure 11** Reduced interaction of compound CX06 with a capsid hexamer mutant Q63A. The interaction of compound CX06 (25 µM) and control compound PF-74 (10 µM) with wild-type and mutant Q63A capsid hexamer in SPR. Both responses in wild-type and mutant Q63A were normalized to the theoretical Rmax as percent maximal response. Data shown are mean values of three individual experiments done in triplicate, with error bars depicting the standard error of the mean (SEM) (p<0.005).

**Rotational freedom of the pyrazole ring may explain the different affinity and mechanism of action CX06**

To help to rationalize the observed differences between the affinity and mechanism of action of CX06 as compared to PF-74, we performed molecular docking analyses. Compound CX06 was docked into the inter-protomer pocket using Glide-XP mode and its binding pose compared to that of the co-crystal structure of PF-74 [2]. Figure 13 shows the overlay of the CX06 docked model on the co-crystallized PF-74. The two compounds appear to share a number of the same protein contacts (mainly Asn57 and Lys70) but differ significantly in the orientation of the indole ring of PF-74 and the pyrazole ring of CX06. The side chain carbonyl of Gln63 can accept a hydrogen bond (~1.7 Å) from the PF-74 indole ring, whereas this bond is not possible with the pyrazole ring of CX06. Moreover, the hydrogen bond between Gln63 and the PF-74 indole ring appears to restrict the rotation of the indole ring, therefore stabilizing the compound between the two CA protomers. This stabilizing hydrogen bonding interaction is not maintained in the CX06 docking models, and slightly lower energy poses of CX06 show the possible rotation of the CX06 pyrazole ring by 180 degrees. This rotation of CX06 pyrazole moiety causes steric clashes and may be the reason for the destabilization of the CA hexamer by this compound.
Cocklin et al. (2018)
Email: sc349@drexel.edu

Next, we sought to see whether or not molecular modeling could help us understand why the Q63A mutation causes a 7-fold decrease in antiviral potency, despite CX06 not directly interacting with Gln63. After in silico mutation, protein relaxation, and docking, we found that replacing the Gln63 side chain with the smaller Ala methyl side chain generated a hydrophobic sub-pocket at the main inter-protomer pocket. Not having the Gln63 hydrogen bond with the PF-74 allows the rotation of the indole ring, with the benzo-moiety now facing the new hydrophobic sub-pocket (Figure 14A,B), while other protein contacts are maintained (Asn57 and Lys70). However, in case of CX06, interestingly the new sub-pocket appears to force the compound to flip and adopt a different binding mode altogether in order to fill the pocket with a phenyl group (Figure 14C,D). Contacts with Asn57 and Lys70 are maintained with this pose (Figure 14D), however, in a vastly different way to that of the wild-type interaction. It is therefore conceivable that either this different binding mode, or the possibility of dynamically having two binding modes to the Q63A mutated CA, are the reasons behind the reduced interaction affinity and consequent reduction in antiviral potency.

CONCLUSIONS

In summary, we have designed and tested a number of bioisosteric variants of PF-74 and demonstrated antiviral activity and maintenance of target specificity. This demonstrates that the methylindole region of PF-74 is more tolerant to variation than previously appreciated. Mechanism of action studies with a representative compound, CX06, also demonstrate that the head group region of the compounds is a primary determinant of both affinity and mechanism of action. Taken together, this work further highlights the potential of the inter-protomer pocket in the CA hexameric configuration as an attractive target for the design of further inhibitors. The continued exploration of modification to the PF-74 inhibitor scaffold to increase potency and drug-like metrics is ongoing within our research group.

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