New Small-Molecule Inhibitor Class Targeting Human Immunodeficiency Virus Type 1 Virion Maturation

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A new small-molecule inhibitor class that targets virion maturation was identified from a human immunodeficiency virus type 1 (HIV-1) antiviral screen. PF-46396, a representative molecule, exhibits antiviral activity against HIV-1 laboratory strains and clinical isolates in T-cell lines and peripheral blood mononuclear cells (PBMCs). PF-46396 specifically inhibits the processing of capsid (CA)/spacer peptide 1 (SP1) (p25), resulting in the accumulation of CA/SP1 (p25) precursor proteins and blocked maturation of the viral core particle. Viral variants resistant to PF-46396 contain a single amino acid substitution in HIV-1 CA sequences (CAI201V), distal to the CA/SP1 cleavage site in the primary structure, which we demonstrate is sufficient to confer significant resistance to PF-46396 and 3-O-(3',3'-dimethylsucinyl) betulinic acid (DSB), a previously described maturation inhibitor. Conversely, a single amino substitution in SP1 (SP1A1V), which was previously associated with DSB in vitro resistance, was sufficient to confer resistance to DSB and PF-46396. Further, the CAI201V substitution restored CA/SP1 processing in HIV-1-infected cells treated with PF-46396 or DSB. Our results demonstrate that PF-46396 acts through a mechanism that is similar to DSB to inhibit the maturation of HIV-1 virions. To our knowledge, PF-46396 represents the first small-molecule HIV-1 maturation inhibitor that is distinct in chemical class from betulinic acid-derived maturation inhibitors (e.g., DSB), demonstrating that molecules of diverse chemical classes can inhibit this mechanism.

Despite the wide-ranging success of highly active retroviral therapies against human immunodeficiency virus type 1 (HIV-1), there still remain several powerful drivers to discover and develop new classes of HIV inhibitors. First and foremost is the continued acquisition of HIV-1 resistance to currently administered antiretroviral drugs. The discovery of compounds that inhibit the replication of HIV-1 via new mechanisms offers the best hope of generating drugs that are active against all HIV-1 variants in the clinic. In principle, viral mutations conferring resistance to any existing drug classes would not confer cross-resistance to drugs targeting a new mechanism. Also, as the current standard of care requires lifelong therapy for HIV-infected individuals, it is imperative that highly active retroviral therapy-related adverse effects and toxicity are minimized with new drugs.

Recently, two new classes of antiretroviral medications have been approved for use in patients for whom previous HIV treatment regimens have failed; the CCR5 [chemokine (C-C motif) receptor 5] inhibitor maraviroc (9, 11) and the HIV-1 integrase (IN) inhibitor raltegravir (22). Additional novel mechanism drug classes will add similar value to the treatment of HIV-1 by further expanding the possibilities for combination regimens and providing a wider range of options for treatment of experienced patients failing first- and second-line therapies.

Virion maturation represents one new antiviral mechanism that is currently being evaluated in the clinic (20, 21). During maturation of the HIV-1 viral particle, the Gag polyprotein undergoes several cleavage events mediated by the viral protease that yield the individual structural proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and the p6 protein. In addition, there are spacer peptides that must be excised from between CA and NC (spacer peptide 1 [SP1]) and from between NC and p6 (SP2). Although the functions of the spacer peptides have not been clearly delineated, recent evidence suggests that SP1 stabilizes CA hexamers (25). Gag cleavage events occur in a sequential fashion as dictated by the relative kinetics of proteolysis at each site. The last of these proteolytic events cleaves at the CA/SP1 junction to convert p25 Gag to the mature p24 CA protein. This is a critical step in the replication cycle of HIV-1, as only in its mature form is CA capable of forming the higher-order complexes that comprise the mature viral core and in the absence of this maturation, viral particles remain noninfectious. Currently, two HIV-1 inhibitors [3-O-(3',3'-dimethylsucinyl) betulinic acid (DSB/PA-457/bevirimat) and MPC-9055] targeting HIV-1 virion maturation are being evaluated in clinical trials (3, 4, 14, 16). The hallmark of this class of inhibitors is the accumulation of the CA/SP1 (p25) precursor in cells and HIV-1 viral particles in the presence of the compounds. However, to date, all of the
Gag maturation inhibitors reported belong to the same general chemical class of betulinic acid derivatives.

In this report, we describe a specific small-molecule inhibitor of HIV-1 Gag maturation, PF-46396 {1-[2-(4-tert-butylphenyl)-2-(2,3-dihydro-1H-inden-2-ylamino)ethyl]-3-(trifluoromethyl)pyridin-2(1H)-one}. This pyridone-based compound was first discovered as a hit from our HIV-1 high-throughput full-replication screen that incorporates all of the HIV-1 targets required for viral replication in cell culture (8). This approach allowed us to screen for multiple targets in the context of a full replication cycle and to identify compounds directed against new HIV-1 mechanisms. We demonstrate that PF-46396 is a late stage (postintegration) HIV-1 inhibitor by showing that the compound inhibits the production of infectious viral particles by HIV-1 producer cells and that the compound does not inhibit early events in HIV-1 replication. We present mechanism-of-action studies demonstrating that PF-46396 does not inhibit HIV protease but interferes specifically with the cleavage of the CA/SP1 (p25) Gag precursor to the mature CA (p24) protein and show a correlation between this mechanism and the antiretroviral activity of the compound. Consistent with these mechanistic data, we show that selection of HIV-1 isolates that are resistant to PF-46396 yields a mutation in the C-terminal region of HIV-1 CA. This single mutation is sufficient to cause significant loss of susceptibility to the compound and displays cross-resistance to the previously reported HIV-1 Gag maturation inhibitor, DSB. Conversely, we demonstrate that a mutation selected for resistance to DSB is cross-resistant to PF-46396.

Our data show that PF-46396 is a specific Gag maturation inhibitor that is clearly differentiated from previously reported compounds that share this mechanism of action. The structure and, in particular, the low molecular weight of PF-46396 show that this mechanism of action can be prospected through a more diverse set of chemical structures than previously represented in the literature.

**Materials and Methods**

**Cells and virus.** HeLa CD4 LTR/beta-Gal, MT-2, PM1, CEM-SS, and HEK 293 cells were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD. The HeLa CD4 LTR/beta-Gal cells and HEK 293 cells were propagated in Dulbecco’s modified Eagle medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT). MT-2, CEM-SS, and PM1 cells were propagated in RPMI 1640 medium (RPMI) (Invitrogen Life Technologies) containing 10% FBS (HyClone). The pNL4-3 HIV-1 infectious molecular clone and HIV-1 IIIB, HIV-1 RF, HIV-1 92HT594, HIV-1 92HT594 BA-L, HIV-1 92BR017, HIV-1 93TH074, HIV-1 92BR004, HIV-1 92HT596, HIV-1 92KW009, HIV-1 98IN012, HIV-1 CMU06, HIV-1 ROJO, HIV-1 WEJO, HIV-1 91US005, HIV-1 91US056, HIV-1 92US072, HIV-1 92US076, HIV-1 92US660, HIV-1 96USHIPS7, HIV-1 92BR014, HIV-1 92BR002, HIV-1 92BR030, HIV-1 93BR023, HIV-1 92HT014, HIV-1 92HT026, and HIV-1 93BR029 viruses were also obtained through the National Institutes of Health AIDS Research and Reference Research Program.

**Compounds.** Efavirenz (EFV) was kindly provided by DuPont Merck Pharmaceutical Company (Wilmington, DE). Nelfinavir (NFV), DSB, and PF-46396 of Health AIDS Research and Reference Reagent Program. Nelfinavir (NFV), DSB, and PF-46396 were synthesized by Pfizer Inc. (San Diego, CA).

**CPE assays.** In cytopathic effect (CPE) assays, host cells were infected with HIV-1 NL-3 or HIV-1 IIIB at a multiplicity of infection (MOI) of 0.08 or with HIV-1 RF at an MOI of 0.25 to 0.819 or mock infected with medium only and added at 2 × 10⁴ cells per well into 96-well plates containing half-log dilutions of test compounds. Six days later, 50 μl of 2.3% (2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) (1 mg/ml XTT tetrazolium, 0.02 mM phenazine methosulphate) was added to the wells, and the plate was reincubated for 4 hours. Viability, as determined by the amount of XTT formazan produced, was quantitated spectrophotometrically by absorbance at 450 nm (24). Data from CPE assays were expressed as percentages of formazan produced in compound-treated cells compared to formazan produced in wells of uninfected, compound-free cells. The 50% effective concentration (EC½) was calculated as the concentration of compound that affected a decrease in the percentage of formazan production in infected, compound-treated cells to 50% of that produced by uninfected, compound-free cells. The 50% cytotoxicity concentration (CC½) was calculated as the concentration of compound that decreased the percentage of formazan produced in uninfected, compound-treated cells to 50% of that produced in uninfected, compound-free cells. The therapeutic index (TI) was calculated by dividing the CC½ value by the EC½.

**HIV-1 p24 assays.** PM1 cells were infected with HIV-1 NL-3 or HIV-1 BA-L virus or MT-2 cells were infected with HIV-1 92HT594 or HIV-1 92HT594 using an MOI of 0.16 for 2 h. Infected cultures were then washed with RPMI and resuspended in 5 ml of RPMI at a final cell density of 2 × 10⁵ cells/ml and added at 1 × 10⁶ cells per well into 96-well plates containing half-log dilutions of test compounds. Five days after infection, virus replication was measured by quantifying HIV-1 p24 antigen present in the supernatants of infected cell cultures using the Coulter HIV-1 p24 antigen assay kit (Beckman Coulter, Miami, FL) according to the manufacturer’s protocol. The EC½ was calculated as the concentration of compound that affected a decrease in p24 production in the supernatants of infected, compound-treated cells to 50% of that produced in the supernatants of infected, compound-free cells. The CC½ value was measured in PM1 cells using the XTT dye reduction method described above.

**Clinical isolate antiviral assays (Southern Research Institute).** HIV-1 clinical isolates 92TH593, 92TH594, WEJO, 91US006, 91US076, 92US072, 92US056, 92US060, 96USHIPS7, 92BR014, 92BR002, 92BR030, 92BR021, 92BR023, 92HT014, and 92HT026 were obtained from the NIH AIDS Research and Reference Research Program. Fresh human peripheral blood mononuclear cells (PBMCs), seronegative for HIV and hepatitis B virus, were isolated from blood samples from screened donors (Biological Specialty Corporation; Colmar, PA) using lymphocyte separation medium (Cellogro [Mediathec, Inc.]; density, 1.078 ± 0.002 g/ml) following the manufacturer’s instructions. Cells were stimulated by incubation in 4 ml of 25 mM lipopolysaccharide (PHA; Sigma) for 48 to 72 h. Mitogenic stimulation was maintained by the addition of 20 U/ml recombinant human interleukin 2 (R&D Systems, Inc.) to the culture medium. PHA-stimulated PBMCs from at least two donors were pooled, diluted in fresh medium, and added to 96-well plates at 5 × 10⁴ cells/well. Cells were infected (final MOI of 0.1) in the presence of nine different concentrations of test compounds (triplicate wells/ concentration) and incubated for 7 days. To determine the level of virus inhibition, cell-free supernatant samples were collected and analyzed for level of reverse transcriptase activity as previously described (7). Following removal of supernatant samples, compound cytotoxicity was measured by the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (CellTiter 96 reagent; Promega) following the manufacturer’s instructions.

**Clinical isolate antiviral assays (Pfizer).** HIV-1 strains NL4-3, IIIB, 92HT599, 92BR017, 93TH074, 92BR004, 92HT596, 92KW009, 98IN012, CMU06, 93BR025, and 93BR029 were obtained from the NIH AIDS Research and Reference Research Program. Frozen stocks of PBMCs were used and prepared as described below. PHA-stimulated PBMCs were rapidly thawed and resuspended in 50 ml of warm cell culture medium. For antiviral assays, a predetermined input of virus stock per 1.0 × 10⁶ cells was used to infect PBMCs depending on the strain of virus used and based on their respective 50% tissue culture infective dose. Infection was performed by incubating the cells with virus at 37°C in a humidified 5% (vol/vol) CO₂ incubator for 1 h. The cells were then pelleted by centrifugation at 225 × g for 5 min and resuspended in medium containing 10 mg/ml interleukin 2 (R&D Systems). The cells were then plated out, to give a final cell density of 4 × 10⁵ cells/ml, into preprepared 96-well plates containing test compounds. Assay plates were incubated for 7 days at 37°C. Quantitation of virus was carried out by measuring viral reverse transcriptase activity in 20 μl of supernatant transferred from the PBMC assay plates using Quan-T-RT assay kits according to the manufacturer’s protocol (Amersham) and read on a Trilux scintillation counter.

**Isolation and preparation of frozen PBMCs.** PBMCs were prepared from buffy coats (National Blood Service, Tooting, London, United Kingdom) pooled from three or four HIV-1 and hepatitis B virus-seronegative donors. Separation of PBMCs was achieved by centrifugation of blood diluted 1:1 in phosphate-buffered saline (PBS) through a Ficoll-Paque gradient for 30 min at 1,000 × g. The PBMCs were harvested at the Ficoll-plasma interface and washed twice in cold PBS by centrifugation. Contaminating erythrocytes were lysed by the addition of 9 ml sterile water to the PBMC pellet followed immediately by 1 ml of 10×...
PF-46396 were selected as described previously (5). Briefly, MT-2 cells (10^6)
in the presence of compound relative to that observed for the no-compound control.
Viral variants resistant to PF-46396 were selected as described previously (5). Briefly, MT-2 cells (10^6) were infected with HIV-1 NL4-3 single-cycle infectious cDNA (pNL4-3deltalEnv) and an NL4-3 envelope expression vector using Lipofectamine Plus according to the manufacturer’s protocol (Invitrogen Life Technologies). Half-log dilutions of test compounds were added to HeLa CD4 LTR/beta-Gal target cells, seeded in 96-well plates at a cell density of 1 x 10^5 cells per well in Dulbecco’s modified Eagle medium containing 10% FBS. Compound-treated or compound-free target cells were then infected with the HIV-1 single-cycle infectious virus at an MOI of 0.03. Seventy-two hours after infection of the HeLa CD4 LTR/beta-Gal target cells, viral infection was monitored by measuring the induction of the beta-galactosidase reporter gene present in the HeLa CD4 LTR/beta-Gal target cells using the Dual-Light System according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Data from the reporter gene measurements were expressed as a percentage of reporter gene activity in infected compound-treated cells relative to that of infected, compound-free cells. Each EC50 value was calculated as the concentration of compound that affected a decrease in the percentage of the virally encoded reporter gene activity in infected, compound-treated cells to 50% of that produced in infected, compound-free cells.

**Virus production assays.** Envelope-deleted NL4-3 cDNA (pNL4-3deltalEnv) was cotransfected into HEK 293 cells with an HIV envelope expression vector using Lipofectamine Plus according to the manufacturer’s protocol (Invitrogen Life Technologies) as previously described (5). The compounds (PF-46396, NFV, or EFV) were then added to transfected cell cultures at 2 x 10^6 effective concentrations (Kd EC50), which corresponded to concentrations of 10 μM, 0.14 μM, and 0.01 μM for PF-46396, NFV, and EFV, respectively, 3 h after transfection. The supernatants of transfected cells were then harvested 72 h after transfection. Infectious virus production was subsequently quantified by measuring the induction of the beta-galactosidase reporter gene after various dilutions of the supernatants of transfected cells were incubated in the presence of HeLa CD4 LTR/beta-Gal indicator cells for 72 h. Results are presented as percentages of inhibition of reporter signal in infected HeLa CD4 LTR/beta-Gal cells in the presence of compound relative to that observed for the no-compound control.

**Selection and characterization of resistant virus.** Viral variants resistant to PF-46396 were selected as described previously (5). Briefly, MT-2 cells (10^6) were infected with HIV-1 NL4-3 at an MOI of 0.01 and then cultured in RPMI containing 10% FBS and PF-46396 at an initial concentration of 3 μM. The cultures were monitored daily by microscopic observation for viral replication, and infectivity titers were then calculated as the concentration of the cells in the culture displaying CPE. Supernatants from the infected cultures were removed and transferred to fresh MT-2 cell cultures containing PF-46396 to produce a tenfold-higher concentration than the previous culture. This process was repeated (10 serial passages) until viral replication was observed in MT-2 cell cultures containing PF-46396 concentrations of 10 μM (>5-fold higher than the mean EC50 determined in CPE assays using HIV-1 NL4-3 and MT-2 cells). Virus cDNAs were amplified from infected cells by PCR, and individual clones were subjected to sequence analysis. Compound-selected mutations were identified by comparing the sequences of the viral cDNA clones derived from compound-treated cells to that of virus propagated in parallel in the absence of compound.

To construct NL4-3 recombinant virus containing the CAI201V amino acid substitution identified in the serial passage studies, a 503-bp restriction fragment (SpeI-ApaI) of Gag was subcloned from the amplified cDNA of a resistant viral isolate into wild-type pNL4-3 using standard molecular biology techniques. Sequencing confirmed that the compound exhibited bona fide antiviral activity.

**RESULTS**

**In vitro antiviral activity of PF-46396 in T-cell lines.** PF-46396 was identified in a high-throughput antiviral screen by Cao et al. (8). The structure of PF-46396 is shown in Fig. 1A along with the structure of DSB (Fig. 1B) for comparison. To confirm that the compound exhibited bona fide antiviral activity, PF-46396 was evaluated in antiviral assays using different HIV-1 laboratory strains or clinical isolates and T-cell lines (Table 1). PF-46396 exhibited antiviral activity against the HIV-1 laboratory strains HIV-1 NL4-3 and HIV-1 IIIB in MT-2 cells with EC50s of 0.36 μM (TI of 53) and 0.017 μM (TI of 1,118), respectively. In PM1 cells, PF-46396 inhibited the replication of HIV-NL4-3 and HIV-1 Ba-L with EC50s of 0.030 μM.
CC50 value determined for each cell line in each experiment. ND, not determined.

**TABLE 1. In vitro antiviral activity of PF-46396 against different HIV-1 laboratory strains**

<table>
<thead>
<tr>
<th>HIV-1 strain</th>
<th>Cell line</th>
<th>Assay</th>
<th>EC50 (μM)</th>
<th>CC50 (μM)</th>
<th>TI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL4-3</td>
<td>MT-2</td>
<td>CPE</td>
<td>0.36 ± 0.20</td>
<td>19 ± 3.0</td>
<td>53</td>
</tr>
<tr>
<td>HIV-1</td>
<td>PM1</td>
<td>p24</td>
<td>0.030</td>
<td>19</td>
<td>633</td>
</tr>
<tr>
<td>HIV-1</td>
<td>PM1</td>
<td>p24</td>
<td>0.017 (0.017)</td>
<td>19 ± 3.0</td>
<td>1,118</td>
</tr>
<tr>
<td>Ba-L</td>
<td>PM1</td>
<td>CPE</td>
<td>5.3</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>RF</td>
<td>CEM-SS</td>
<td>CPE</td>
<td>&gt;20</td>
<td>20 ± 1.9</td>
<td>ND</td>
</tr>
<tr>
<td>MT-2</td>
<td>CPE</td>
<td></td>
<td>&gt;16, &gt;15</td>
<td>16, 16</td>
<td>ND</td>
</tr>
<tr>
<td>92HT594</td>
<td>MT-2</td>
<td>p24</td>
<td>0.24</td>
<td>19 ± 3.0</td>
<td>79</td>
</tr>
<tr>
<td>92HT599</td>
<td>MT-2</td>
<td>p24</td>
<td>0.21</td>
<td>19 ± 3.0</td>
<td>90</td>
</tr>
</tbody>
</table>

a Antiviral activity was determined in CPE assays after infection of MT-2 or CEM-SS cells with HIV-1 NL4-3, HIV-1 RF, or HIV-1 IIIB or by measuring p24 production 5 days after infection of PM1 or MT-2 cells with HIV-1 NL4-3, HIV-1 Ba-L, HIV-1 92HT594, or HIV-1 92HT599 as described in Materials and Methods. The EC50 and CC50 values represent the means ± standard deviations from three to five experiments, means with individual values from two experiments (for HIV-1 IIIB EC50 values), or individual values.

b TI, therapeutic index calculated by dividing the mean EC50 into the mean CC50 value determined for each cell line in each experiment. ND, not determined.

μM (TI of 633) and 5.3 μM (TI of 4), respectively. PF-46396 showed antiviral activity against the HIV-1 clinical isolates 92HT594 and 92HT599 in MT-2 cells with EC50s of 0.24 μM (TI of 79) and 0.21 μM (TI of 90), respectively. In contrast, PF-46396 did not exhibit measurable antiviral activity against HIV-1 RF in CEM-SS cells or in MT-2 cells up to concentrations that were cytotoxic (Table 1).

**In vitro antiviral activity of PF-46396 against HIV-1 clinical isolates in PBMCs.** To confirm that PF-46396 inhibited HIV-1 replication in primary cells, PF-46396 was evaluated in vitro antiviral assays using HIV-1 clinical isolates and PBMCs. PF-46396 exhibited antiviral activity against 17/27 HIV-1 clinical isolates tested with EC50s ranging from 0.005 to 7 μM and TI values ranging from 2 to 3,400 (Table 2). PF-46396 did not exhibit measurable antiviral activity against 10 isolates up to concentrations that were cytotoxic (CC50 measured in PBMCs, 17 μM). The antiviral activity of PF-46396 did not appear to be dependent on HIV-1 clade or tropism (CCR5 versus CXCR4 [chemokine (C-X-C motif) receptor 4]). These data show that PF-46396 exhibits activity against several HIV-1 primary isolates in PBMCs and suggest that the compound does not target the CCR5 or CXCR4 receptor.

**HIV-1 single-cycle infection and virus production assays.** In an initial effort to determine the mechanism of action for PF-46396, the compound was evaluated in HIV-1 single-cycle infection assays along with two control compounds (NFV and EFV). In such assays, HeLa CD4 LTR/beta-Gal cells are infected with single-cycle infectious HIV-1 virions. Reporter gene expression from infected HeLa CD4 LTR/beta-Gal cells is dependent on viral entry, reverse transcription, integration of viral cDNA, and HIV-1 Tat-mediated gene expression. Consistent with the expected results, EFV, a nonnucleoside reverse transcriptase inhibitor, exhibited potent activity in the assay (EC50, 0.001 μM) (Table 3). Alternatively, NFV, an HIV-1 protease inhibitor that acts late in infection, was not active in the assay (EC50 of >1 μM) (Table 3). Similar to the results observed for NFV, PF-46396 did not exhibit activity in the single-cycle infection assay (EC50 of >10 μM) (Table 3), suggesting that the compound targets a postintegration step in the HIV-1 replication cycle.

To confirm that PF-46396 targets a postintegration step in the HIV-1 replication cycle, the compound was tested in virus production assays. In such assays, pNL4-3 infectious cDNAs are transfected into HEK 293 cells in the presence or absence of single-cycle infection (EC50 of >10 μM) (Table 3), suggesting that the compound targets a postintegration step in the HIV-1 replication cycle.

**TABLE 2. In vitro antiviral activity of PF-46396 against different HIV-1 clinical isolates or laboratory strains in PBMCs**

<table>
<thead>
<tr>
<th>HIV-1 isolate</th>
<th>Clade</th>
<th>Receptor</th>
<th>EC50 (μM)</th>
<th>TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIB</td>
<td>X4</td>
<td></td>
<td>0.32</td>
<td>53</td>
</tr>
<tr>
<td>NL4-3</td>
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<tr>
<td>ROJO</td>
<td>X4</td>
<td></td>
<td>&gt;17</td>
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<tr>
<td>WEOJO</td>
<td>X4</td>
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<tr>
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<td>R5</td>
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<td>&gt;17</td>
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</table>

a Antiviral activity was determined by measuring reverse transcriptase activity 7 days after infection of PBMCs with HIV-1 clinical isolates as described in Materials and Methods.

b Clade and receptor assignments based on designations from the NIH AIDS Research and Reference Reagent Program or our collaborator for the study (Southern Research Institute, Fredrick, MD). Receptor abbreviations: X4, CCR5; R5, CXCR4.

c TI, therapeutic index calculated by dividing the mean EC50 into the mean CC50 value determined in PBMCs (17 μM).

**TABLE 3. In vitro antiviral activity of PF-46396 in HIV-1 single-cycle infection and virus production assays**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (μM) in single-cycle infection</th>
<th>Virus production (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-46396</td>
<td>&gt;10</td>
<td>92 (91, 93)</td>
</tr>
<tr>
<td>EFV</td>
<td>0.001 ± 0.0001</td>
<td>24 (18, 30)</td>
</tr>
<tr>
<td>NFV</td>
<td>&gt;1</td>
<td>76 (74, 78)</td>
</tr>
</tbody>
</table>

a Antiviral activity was determined after infection of HeLa CD4 LTR/beta-Gal cells with HIV-1 single-cycle infectious viruses as described in Materials and Methods. Values represent the mean EC50 ± standard deviation or representative EC50 values from three experiments.

b The production of HIV-1 infectious virions was measured as described in Materials and Methods. Results are presented as percent inhibition of infectious virus production in the presence of 2X EC50 of the compound relative to the no-compound control and represent the mean of two independent experiments (individual values shown in parentheses). PF-46396, EFV, and NFV were present at concentrations of 10 μM, 0.01 μM, and 0.14 μM, respectively.
of 2× EC₉₀ concentrations of compound. Infectious virus production is then quantified in the supernatants of transfected cells as described in Materials and Methods. As shown in Table 3, PF-46396 inhibited virus production 92% compared to that observed in the absence of compound. NFV inhibited infectious virus production by 76%, while EFV showed only marginal inhibition (24%) in the assay. It is likely that the marginal inhibition observed for EFV resulted from residual compound present in the transfection supernatant carried over into the infectivity assay. None of the compounds had a significant effect on the level of p24 (data not shown), indicating that release of Gag particles was not affected. These data show that similar to an HIV-1 protease inhibitor (NFV), PF-46396 acts late in the HIV-1 replication cycle to inhibit the production of infectious virions.

**PF-46396 inhibits CA/SP1 processing.** Small-molecule inhibitors that inhibit virion maturation, a hallmark of which is the inhibition of CA/SP1 processing, have been reported (3, 14, 27). To determine whether PF-46396 interfered with HIV-1 Gag proteolytic processing, Gag protein products derived from virions produced in the presence of PF-46396 were examined using Western blot analysis and compared to that observed for virions produced in the presence of DSB (an inhibitor of virion maturation), NFV, or EFV. Figure 2A indicates the temporal nature of Gag processing and the major products expected of each proteolytic step (17). As shown in Fig. 2B, Gag products derived from virions produced in the presence of 2× EC₉₀ concentrations of PF-46396 or DSB displayed a specific accumulation of p25, which corresponds to the CA/SP1 precursor protein, compared to untreated virions. In contrast, multiple uncleaved Gag precursor proteins were detected in virions produced in the presence of NFV, while Gag processing was not affected in virions produced in the presence of EFV. While processing was clearly altered, the overall yield of Gag protein, and thus viral particles, by the producer cells did not appear to be significantly affected by PF-46396. These data demonstrate that PF-46396 inhibits CA/SP1 processing and suggest that the compound acts via a mechanism similar to that of the virion maturation inhibitor, DSB. Consistent with these observations, PF-46396 was not active in an in vitro HIV-1 protease assay (data not shown).

**A single amino acid substitution in HIV-1 CA sequences confers resistance to PF-46396.** To identify the target of PF-46396, viral variants resistant to PF-46396 were selected in in vitro serial passage experiments as described in Materials and Methods. Sequence analysis of the Gag coding region revealed a single amino acid substitution, I201V, in CA. Recombinant NL4-3 virus was then generated encoding the I201V mutation in CA (CAI201V) or the A1V substitution in SP1 (SP1A1V), which was previously described to confer resistance to DSB (14), and evaluated in reporter gene-based susceptibility assays with PF-46396, DSB, NFV, and EFV as described in Materials and Methods. Similar reporter signals were measured for wild-type NL4-3 and the CAI201V mutant virus in the reporter gene-based infection assays (in the absence of compound), suggesting that the I201V substitution does not significantly impair viral replication (data not shown). However, more detailed studies are required to accurately determine whether the I201V substitution affects replication fitness. The results of the susceptibility assays showed that PF-46396 exhibited >49-

![Fig. 2. PF-46396 specifically inhibits the processing of CA/SP1 Gag precursor proteins. (A) Schematic diagram of HIV Gag proteolytic processing. With the parent Gag protein, p55, at the top, the downward arrows indicate the overall order of proteolytic cleavage of HIV-1 Gag by the viral protease, as dictated by the kinetics of cleavage at each site. The numbers in parentheses indicate the molecular weights of various cleavage products, and the box indicates the specific event that is inhibited by PF-46396 and DSB. (B) Western blot analysis of Gag protein processing. Virions produced after transfection of HEK 293 cells with HIV-1 NL4-3 infectious cDNAs in the presence or absence (No Comp) of 2× EC₉₀ concentrations of compound (PF-46396, 10 μM; NFV, 0.14 μM; EFV, 0.01 μM; DSB, 0.32 μM) were isolated, and virion-associated Gag proteins were analyzed by Western blot analysis. Supernatants from mock-transfected cells (cell control) were also treated under the same protocol to demonstrate that detected protein products were virion specific. The positions of CA/SP1 (p25) precursor and mature CA (p24) proteins are indicated by arrows to the right of the blot. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the blot.
fold and >16-fold reduction in antiviral activity against the CAI201V and SP1A1V mutant viruses, respectively, compared to the wild-type virus (Table 4). Similarly, DSB showed 32-fold and >111-fold reduction in antiviral activity against the CAI201V and SP1A1V mutant viruses, respectively (Table 4). The control compounds EFV and NFV exhibited comparable antiviral activity against the wild-type virus and the CAI201V and SP1A1V mutant viruses. These data demonstrate that single amino acid substitutions in CA (I201V) and SP1 (A1V) confer resistance to PF-46396 and DSB. Furthermore, these data strongly suggest that PF-46396 and DSB act via similar mechanisms to inhibit HIV-1 replication.

The CAI201V mutation restores CA/SP1 cleavage in the presence of PF-46396. Gag cleavage products derived from the wild-type (Fig. 3A) or CAI201V mutant (Fig. 3B) viruses produced in the presence of different concentrations of PF-46396 or DSB or in the absence of compound were analyzed by Western blot analysis as described in Materials and Methods. As shown in Fig. 3A, the accumulation of the p25 (CA/SP1) precursor in wild-type virions increases with increasing concentrations of either PF-46396 or DSB and appears to correlate with inhibition of infectious virus production. Alternatively, significant p25 accumulation was not observed in virions containing the CAI201V substitution in the presence of PF-46396 or DSB (Fig. 3B). This correlated with the reduction in antiviral activity exhibited by the compounds against the CAI201V mutant virus (Fig. 3B and Table 4). These data demonstrate that the I201V substitution in CA restores CA/SP1 processing in the presence of PF-46396 or DSB and suggest that this forms the basis for the observed resistance to the antiviral activity of the compounds.

DISCUSSION

PF-46396 represents a new class of small-molecule HIV-1 virion maturation inhibitors. We demonstrate that PF-46396 specifically blocks an event late in the HIV replication cycle to inhibit infectious virus production. Treatment of HIV-1-infected cells with PF-46396 results in the accumulation of a CA/SP1 precursor protein (p25), demonstrating that the compound directly or indirectly inhibits Gag processing. Viral variants selected in vitro that are resistant to PF-46396 contain a single amino acid substitution (I201V) in CA sequences. In susceptibility assays using recombinant viruses, the I201V amino acid substitution in HIV-1 CA was sufficient to confer resistance to PF-46396 and DSB, a previously described HIV-1 maturation inhibitor. In addition, a single amino acid substitution in SP1 (A1V), which was originally described as a substitution conferring resistance to DSB (14), is sufficient to confer resistance to PF-46396 and DSB. These data provide strong genetic evidence that PF-46396 and DSB act via the same mechanism to inhibit HIV-1 replication. Furthermore, we show that the I201V substitution in CA restores CA/SP1 (p25) processing in the presence of PF-46396 or DSB, confirming that inhibition of Gag maturation represents the antiviral mechanism of action of PF-46396.

Although it is clear that PF-46396 and previously reported

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**TABLE 4.** Single amino acid substitutions in HIV-1 capsid and SP1 confer resistance to PF-46396 and DSB.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ (µM) [fold change]$^a$</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>PF-46396</td>
<td>0.206 ± 0.15</td>
</tr>
<tr>
<td>DSB</td>
<td>0.009 ± 0.004</td>
</tr>
<tr>
<td>EFV</td>
<td>0.0006 ± 0.0004</td>
</tr>
<tr>
<td>NFV</td>
<td>0.016 ± 0.006</td>
</tr>
</tbody>
</table>

$^a$ EC$_{50}$ values (reported in micromolar) were determined for PF-46396, DSB, EFV, and NFV in susceptibility assays with recombinant HIV-1 encoding the CAI201V or SP1A1V single amino acid substitutions or wild-type (WT) virus in MT-2 cells cocultured with HeLa CD4 LTR/beta-Gal indicator cells as described in Materials and Methods. Results represent mean EC$_{50} ±$ standard deviations from three to six experiments. The fold change values, which are shown in brackets in the table, were calculated by dividing the mutant EC$_{50}$ by the wild-type EC$_{50}$.
maturation inhibitors specifically block CA/SP1 processing, the
precise mechanisms of action of these compounds are not known.
Current models suggest that maturation inhibitors inter-
act directly with Gag in the context of higher-order Gag
trimers to inhibit CA/SP1 processing (18). Consistent with
this model, determinants of DSB sensitivity, including resis-
tance mutations, map proximal to the CA and SP1 junction (1,
14, 15, 26, 27). In this study, we identified a single amino acid
substitution in CA (I201V) that confers resistance to PF-46396
and DSB and is distal to the CA-SP1 cleavage site in the
primary structure (30 residues upstream). This is the first
demonstration that a residue distal to the CA-SP1 cleavage site
affect sensitivity to maturation inhibitors of different chemical
classes. In available crystal structures of HIV-1 CA, the last
several residues of the C-terminal domain are too disordered
to be resolved, and there is no existing data on the precleaved
tertiary structure of CA/SP1; thus, we cannot rule out the
possibility that this residue is proximal to the CA-SP1 junction.
While I201V may directly interfere with compound binding, it
is possible that this substitution perturbs the higher-order
structure required for PF-46396 or DSB binding or that it
compensates similarly for allosteric rearrangements that are
induced by either PF-46396 or DSB binding. Alternatively, it is
possible that the I201V substitution affects the kinetics of Gag
processing, thus conferring resistance to maturation inhibitors
indirectly. Further studies will be required to distinguish be-
tween these possible explanations.

We observed a significant variation in PF-46396 antiviral
activity, particularly when the compound was evaluated against
HIV-1 clinical isolates (>3,400-fold range in EC50 measured).
It is possible that this large range of antiviral activity results
from amino acid variation in the target of PF-46396. Recent
studies reported that polymorphisms in HIV-1 Gag sequences,
particularly at residues 6 to 8 in SP1 (i.e., the “QVT motif”),
are associated with significant variations (up to 150-fold) in
DSB antiviral activity (19, 23). However, the variation in PF-
46396 antiviral activity reported in this study appears greater
than that reported for DSB (13, 14, 19). It is possible that
PF-46396 is more sensitive to polymorphisms in Gag, resulting
in greater variation in antiviral activity. We noted that the
published sequence of HIV-1 RF, which showed a significant
reduction in susceptibility to PF-46396, contained the canoni-
cal “QVT” amino acid residues at SP1 positions 6 to 8, while
the published sequence of HIV-1 91US005 (another virus
showing reduced PF-46396 susceptibility) contained polymor-
phisms in this region. The sequences for many of the viruses
used in this study were not publicly available; therefore, it was
not practical or within the scope of this study to complete a full
analysis of the relationship of Gag sequence polymorphisms
and PF-46396 susceptibility. Further studies directly compar-
ing the antiviral activities of DSB and PF-46396 and how such
activities relate to Gag sequences are required to determine
more precisely how the antiviral activity of PF-46396 compares
to DSB across different HIV-1 strains and the determinants for
PF-46396 susceptibility.

To our knowledge, all previously described virion matura-
tion inhibitors have been derivatives of betulinic acid (2, 10).
The identification of PF-46396 demonstrates that diverse
chemical matter can inhibit this target and opens up the pos-
sibility that additional inhibitor classes might be identified. The
synthetic tractability of PF-46396 should enable efforts to gen-
erate diverse analogs and identify molecules with optimal po-
tency and physiochemical properties. Our preliminary efforts
to generate and characterize analogs of PF-46396 have shown
that the series exhibits a definable structure-activity relation-
ship; however, it was observed that compounds with increased
potency were above the acceptable range of lipophilicity (15a)
(LogP, >5; data not shown). This correlation of potency and
lipophilicity, in addition to limited spectrum across clinical
isolates, must be resolved in order for this series to yield a
clinical candidate for HIV therapy. The identification of small-
molecule inhibitors of virion maturation with improved physio-
chemical properties (e.g., reduced lipophilicity and improved
solubility) might better enable mechanism-of-action studies
and provide scaffolds that could be developed into HIV drugs
targeting this mechanism with improved bioavailability and
pharmaceutical properties. A deeper knowledge of this mech-
anism of action and the determinants of drug susceptibility
might also enable a more informed approach to improving the
spectrum of Gag maturation inhibitors.

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