HIV-1 Resistance to the Capsid-Targeting Inhibitor PF74 Results in Altered Dependence on Host Factors Required for Virus Nuclear Entry

Jing Zhou, Amanda J. Price, Upul D. Halambage, Leo C. James, Christopher Aiken

Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; MRC Laboratory of Molecular Biology, Cambridge Biomedical Campus, Cambridge, United Kingdom

ABSTRACT

During HIV-1 infection of cells, the viral capsid plays critical roles in reverse transcription and nuclear entry of the virus. The capsid-targeting small molecule PF74 inhibits HIV-1 at early stages of infection. HIV-1 resistance to PF74 is complex, requiring multiple amino acid substitutions in the viral CA protein. Here we report the identification and analysis of a novel PF74-resistant mutant encoding amino acid changes in both domains of CA, three of which are near the pocket where PF74 binds. Interestingly, the mutant virus retained partial PF74 binding, and its replication was stimulated by the compound. The mutant capsid structure was not significantly perturbed by binding of PF74; rather, the mutations inhibited capsid interactions with CPSF6 and Nup153 and altered HIV-1 dependence on these host factors and on TNPO3. Moreover, the replication of the mutant virus was markedly impaired in activated primary CD4+ T cells and macrophages. Our results suggest that HIV-1 escapes a capsid-targeting small molecule inhibitor by altering the virus’s dependence on host factors normally required for entry into the nucleus. They further imply that clinical resistance to inhibitors targeting the PF74 binding pocket is likely to be strongly limited by functional constraints on HIV-1 evolution.

IMPORTANCE

The HIV-1 capsid plays critical roles in early steps of infection and is an attractive target for therapy. Here we show that selection for resistance to a capsid-targeting small molecule inhibitor can result in viral dependence on the compound. The mutant virus was debilitated in primary T cells and macrophages—cellular targets of infection in vivo. The mutations also altered the virus’s dependence on cellular factors that are normally required for HIV-1 entry into the nucleus. This work provides new information regarding mechanisms of HIV-1 resistance that should be useful in efforts to develop clinically useful drugs targeting the HIV-1 capsid.
additional effects of the substitutions may promote resistance (34). Moreover, resistance can also be conferred by substitutions in the NTD that do not eliminate binding of the compound, such as E45A and Q63A/Q67A (32). These substitutions reduce HIV-1 infectivity and alter the dependence of HIV-1 on host factors important for virus nuclear entry, including TNPO3, Nup153, and RanBP2 (13). These data suggest that resistance to PF74 is complex and may depend on the interaction of the viral capsid with host factors. Indeed, binding of the host protein cyclophilin A to the incoming viral capsid contributes to the antiviral activity of PF74 (32). In the present study, we identified a novel PF74-resistant HIV-1 mutant, the replication of which is stimulated by the compound. We showed that the mutant virus is less dependent on expression of host factors required for nuclear entry of wild-type (WT) HIV-1.

MATERIALS AND METHODS

Viruses and cells. Viruses were produced from the R9 molecular clone and mutant derivatives (35). Virus stocks were produced by transfection of cloned HIV-1 proviral DNA by a calcium phosphate method. The viruses were assayed for p24 concentrations and/or reverse transcriptase (RT) activity prior to being frozen in aliquots at −80°C. CA mutants were generated by PCR segment overlap extension of the BssHII-ApaI fragment in R9. Mutant clones were confirmed by sequencing of the PCR-amplified regions. Compounds (BI-2 and PF74) were synthesized by the Chemical Synthesis Core, Vanderbilt Institute for Chemical Biology, Vanderbilt University. [3H]-PF74 was produced by tritium exchange reaction with iodo-PF74, as described previously (34). CEM T cells and primary T cell cultures were cultivated in RPMI 1640 medium containing 10% fetal bovine serum, penicillin, and streptomycin. Primary T cells and monocytes were purified from normal human blood obtained from healthy donors. All healthy blood donors provided written informed consent under a protocol approved by the Vanderbilt University Institutional Review Board. Mononuclear cells were first purified over Ficoll-Paque Plus (GE Healthcare), after which T cells were purified with a CD4+ isolation kit (Invitrogen) and activated with Dynabeads human T-activator CD3/Dextram (Life Technologies). T cells were cultured in medium containing 30 U/ml recombinant interleukin-2 (obtained from the NIH AIDS Research and Reference Reagent Program) for 6 days prior to HIV-1 infection. Monocytes were purified by negative selection and differentiated into macrophages in medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF), as previously described (34). Cells were cultured in 96-well plates and inoculated with HIV-1. On subsequent days, half of the culture supernatants were collected for virus quantification, and the cultures were replenished with an equal volume of fresh medium. Single-cycle infectivity assays to measure HIV-1 infectivity and antiviral inhibition were performed by luciferase assay in TZM-bl reporter cells, as previously described (34).

Selection for PF74-resistant HIV-1. Cultures of CEM cells (40,000 cells) were inoculated with wild-type HIV-1. One day later, the culture medium was replaced with fresh medium containing 0.31 μM PF74. Cultures were inspected regularly for signs of virus replication, and virus production was monitored periodically by p24 enzyme-linked immunosorbent assay (ELISA). When substantial HIV-1 growth was observed, the culture supernatant was transferred to uninfected cells, and the PF74 concentration doubled. This process was repeated until virus replication was detected in the presence of 5 μM PF74, after which the virus RNA was isolated and converted to cDNA, and the CA region was amplified by PCR with primers spanning the BssHII and Apal sites in R9. The product was sequenced and transferred into R9 to produce 4Mut.

PF74 binding assays and CA hexamer structure determination. Virus binding assays were performed with [3H]-PF74 and concentrated HIV-1 particles as previously described (32, 34). CA hexamers were generated from recombinant mutant CA proteins expressed in Escherichia coli, as described previously (34, 36). The affinities of PF74 for purified CA hexamers were determined by equilibrium dialysis and isothermal titration calorimetry (ITC), as reported previously (30, 34). The affinity of Nup153 and CPSF6 for CA hexamers was determined by ITC (30, 37). 4Mut CA hexamers were crystallized in the presence of PF74, and the structures were determined as reported previously (30).

Depletion of cellular Nup153, RanBP2, and TNPO3 by RNA interference. Lentiviral short hairpin RNA (shRNA) clones were purchased from Sigma (Mission shRNA) and purified over CsCl gradients. Lentiviral particles were produced by cotransfection of 293T cells with the lentiviral clone, the Gag-Pol expression construct pSAX2 (38), and pHCMV-G encoding vesicular stomatitis virus glycoprotein G (VSV-G) (39). Cells were transduced with the lentivirus vectors, selected in puromycin, and challenged with HIV-1 as previously described (40). Depletion of the relevant host proteins was confirmed by quantitative analysis of total cellular RNA by quantitative RT-PCR.

ITC. ITC experiments were conducted as previously described (30). Proteins were dialyzed against ITC buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl), and in each case, the hexamer was used in the syringe and binding partner (peptide, protein, or drug) in the cell. The typical concentrations used were 700 to 1,000 μM hexamer titrated against 70 to 100 μM peptide (CPSF6 and Nup153) or protein (CypA) and 200 μM hexamer titrated against 30 μM PF74. ITC experiments were conducted on a BioCal ITC-200 calorimeter, and data were analyzed using Origin data analysis software (MicroCal).

Crystallization, data collection, structure determination, and refinement. Crystals of 4Mut hexamer grew at 17°C in sitting drops and were prepared by mixing protein solution (0.6 mM 4Mut hexamer in 50 mM Tris [pH 8.0]) with reservoir solution (0.1 M NaCl, 12% [wt/vol] polyethylene glycol [PEG] 4000, 0.1 M Tris [pH 8.5], 4% [vol/vol] formamide) in a 1:1 mix. Crystals of 4Mut-hexamer–PF74 grew at 17°C in sitting drops and were prepared by coconcentrating the protein with a 2-fold molar excess of PF74 to a final concentration of 0.6 mM hexamer (in 50 mM Tris [pH 8.0], 100 mM NaCl) before mixing with reservoir solution (0.2 M potassium thiocyanate, 8% [wt/vol] PEG 20,000, 8% [vol/vol] PEG 550 monomethyl ether [MME], 0.1 M Tris [pH 8.5], 3% 1,4-dioxane) in a 1:1 mix. Crystals were cryoprotected with 20% methylpentanediol, before being flash frozen in liquid nitrogen. Data were collected in house on a Pilatus detector. Crystal data collection and refinement statistics are provided in Table 1. The data sets were processed using the CCP4 program suite (41). Data were indexed and scaled in MOSFLM and SCALA, respectively. The structure was determined by molecular replacement in Phaser using HIV-1 CA hexamer pdb 3H47 as a model. Structural figures were prepared using PyMOL (MacPyMOL Molecular Graphics System, 2009, DeLano Scientific LLC).

Protein structure accession numbers. Protein structures have been deposited in the Protein Data Bank under PDB ID codes 4XRO (4Mut Hex) and 4XRQ (4Mut Hex:PF74).

RESULTS
To identify novel HIV-1 mutations conferring resistance to PF74, we performed a long-term serial passage experiment in CEM cells, a human T cell line that is permissive to HIV-1 replication. To select for resistance, we progressively increased the concentration of PF74 at each passage, maintaining each culture until HIV-1 particles accumulated to an appreciable extent in the culture, as detected by p24 ELISA (Fig. 1A). After 226 days, a virus was recovered, the region of the genome encoding CA was amplified by RT-PCR, and its sequence was determined. The virus contained four substitutions in CA, encoding Ser41 to Ala (S41A), Gln67 to His (Q67H), Val165 to Ile (V165I), and Leu172 to Ile (V172I) (Fig. 1A). Sequence analysis of viruses recovered at earlier points in the selection process demonstrated that the mutations accumulated in the order S41A, V165I/L172I, and Q67H. Transfer of the...
ment of the gag gene containing the four substitutions into a wild-type HIV-1 molecular clone yielded a viral clone that we named “4Mut” to distinguish it from the previously reported PF74-resistant virus containing 5 changes in CA (5Mut) (29). 4Mut and 5Mut viruses are genetically distinct, as they contain only the Q67H substitution in common, and only 4Mut contains substitutions in the carboxy-terminal domain of CA. Single-cycle infection assays in the presence of a range of inhibitor concentrations revealed that infection by the 4Mut virus was not inhibited in the presence of one, two, and three substitutions and assayed infectivity and inhibition by PF74 (Fig. 3). While none of the double mutants was as resistant to PF74 as 4Mut, several of the triple mutants retained substantial resistance. Specifically, infection by the S41A Q67H V172I (41/67/172) mutant was inhibited by approximately 50% at a PF74 concentration of 10 µM, and the S41A Q67H V165I (41/67/165) mutant virus was inhibited by 50% at 5 µM PF74. In contrast, the S41A V165I V172I (41/165/172) and Q67H V165I V172I (67/165/172) mutants were inhibited by 50% at 2 µM PF74. However, only the 4Mut virus was strongly enhanced by PF74. These results demonstrate that the resistance to PF74 exhibited by 4Mut requires at least three of its amino acid substitutions and that its dependence on the compound requires all four changes.

Table 1. Data collection and refinement statistics for molecular replacement

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a Single crystal was used for each structure. Values for the highest-resolution shell are shown in parentheses.
b RMSD, root mean square deviation.

4Mut, we created a set of mutant viruses containing combinations of one, two, and three substitutions and assayed infectivity and inhibition by PF74 (Fig. 3A). In the absence of inhibitor, the mutant viruses varied between 50 and 120% infectivity relative to the wild type; however, the 4Mut virus was only 20% as infectious (Fig. 3A). While none of the double mutants was as resistant to PF74 as 4Mut, several of the triple mutants retained substantial resistance. Specifically, infection by the S41A Q67H V172I (41/67/172) mutant was inhibited by approximately 50% at a PF74 concentration of 10 µM, and the S41A Q67H V165I (41/67/165) mutant virus was inhibited by 50% at 5 µM PF74. In contrast, the S41A V165I V172I (41/165/172) and Q67H V165I V172I (67/165/172) mutants were inhibited by 50% at 2 µM PF74. However, only the 4Mut virus was strongly enhanced by PF74. These results demonstrate that the resistance to PF74 exhibited by 4Mut requires at least three of its amino acid substitutions and that its dependence on the compound requires all four changes.

**PF74 binds at an interface between subunits in the CA hexamer.** We previously reported that the highly resistant 5Mut virus particles exhibit reduced capacity for binding to PF74 (32). The observed enhancement of 4Mut infection by PF74 suggested that the virus retained the ability to bind the inhibitor. To test this, we incubated concentrated wild-type, 5Mut, and 4Mut particles with 3H-PF74, pelleted the particles through a sucrose cushion to separate them from the unbound compound, and quantified the viral

![Image](http://jvi.asm.org/ on August 30, 2017 by guest)
PF74 stimulates replication of 4Mut HIV-1 in T cells. The wild-type and 4Mut HIV-1 strains were inoculated into cultures of the CEM T cell line, and PF74 was added the following day to the concentrations (micromolar) shown in the legends. Cultures were sampled at the indicated times, and HIV-1 replication was monitored by quantification of reverse transcriptase activity in culture supernatants.

FIG 2 PF74 stimulates replication of 4Mut HIV-1 in T cells. The wild-type and 4Mut HIV-1 strains were inoculated into cultures of the CEM T cell line, and PF74 was added the following day to the concentrations (micromolar) shown in the legends. Cultures were sampled at the indicated times, and HIV-1 replication was monitored by quantification of reverse transcriptase activity in culture supernatants.

PF74 Resistance via Altered Host Factor Dependence

PF74 inhibition of HIV-1 infection is partially dependent on binding of the host protein cyclophilin A (CypA) to the incoming viral capsid in the target cell (32). Ablation of CypA binding at the time of infection, either by addition of cyclosporine (CsA), mutation of the CypA-binding loop in CA, or depletion of CypA from target cells, results in a 4-fold decrease in PF74 antiviral potency (32). To determine whether CypA plays a role in PF74 stimulation of 4Mut infection, we titrated PF74 in HIV-1 infection assays in the presence and absence of CsA. CsA totally ablated the PF74 enhancement of 4Mut infection, yet the virus remained resistant to PF74 (Fig. 5A). Addition of the G89V substitution to 4Mut, which abolishes CypA binding, resulted in a virus that was not enhanced by PF74 and was poorly infectious (data not shown). Collectively, these results indicate that CypA contributes to the observed enhancement of 4Mut infection induced by PF74.

Recently, another HIV-1 inhibitor, BI-2, was shown to bind the same pocket in CA as PF74. BI-2 is less potent an inhibitor than PF74, with a 50% inhibitory concentration (IC50) of approximately 5 μM (28). Unlike PF74, which destabilized HIV-1 cores in vitro, BI-2 stabilizes the viral capsid (28), although a recent report concluded that both compounds promote premature HIV-1 uncoating in target cells (33). BI-2 does not inhibit reverse transcription in target cells, suggesting that the compound selectively inhibits nuclear entry and/or integration. Resistance to BI-2 can be conferred by a single amino acid substitution at Thr107 in the CA binding site. We asked whether BI-2 would stimulate infection by 4Mut. Titration of BI-2 in single-cycle infection assays of 4Mut revealed a dose-dependent enhancement at concentrations up to 20 μM, which was prevented by addition of CsA (Fig. 5B). We conclude that 4Mut is resistant to both PF74 and BI-2 and that both inhibitors can enhance infection of the virus.

PF74 binding does not perturb the structure of the 4Mut hexamer. In previous studies, we and others showed that PF74 binds with higher affinity to a disulfide-stabilized (14C 45C double cysteine mutant) assembled CA hexamer versus dissociated CA subunits, consistent with the functional binding site of the compound being the NTD-CTD intersubunit interface within the assembled capsid (30, 31). Structural analysis of the hexamer-PF74 complex demonstrated that PF74 does not significantly perturb the hexamer structure, despite the observation that PF74 appears to accelerate dissociation of the viral capsid following entry into target cells (32, 33). The 4Mut substitutions Q67H, V165L, and L172I reside at positions near the NTD-CTD interface, suggesting they could alter the structure of the hexamer. Additionally, we speculated that PF74 binding might alter the 4Mut hexamer structure, potentially restoring infectivity of the virus. To test these hypotheses, we determined the structure of the disulfide-stabilized 4Mut CA hexamer with and without bound PF74 by X-ray crystallography. The mutations did not significantly alter the interhexamer interface structure relative to the wild-type CA hexamer (Fig. 6A), suggesting that the reduced infectivity of the 4Mut is not a consequence of altered capsid structure. Additionally, binding of PF74 to 4Mut did not significantly perturb the structure of the hexamer (Fig. 6B), suggesting that the enhancement of infection is not a consequence of compound-induced structural alterations. However, superposition of the structure of a CA-binding peptide de-
depletions on infectivity. As reported in several studies, depletion of Nup153, RanBP2, and TNPO3 rendered cells less permissive to infection by 4Mut and 5Mut, although to a lesser extent than the wild type. Unexpectedly, RanBP2 depletion appeared to reduce permissiveness to 4Mut and 5Mut, although to a lesser extent than the wild type. Unsurprisingly, the inhibitor may act by altering host factor binding. To test this, we assayed infection of Nup153, RanBP2, and TNPO3 rendered cells less permissive to the wild-type virus, while the N74D mutant was less dependent on these proteins (Fig. 7). Nup153 and TNPO3 depletions also reduced infection by 4Mut and 5Mut, although to a lesser extent than the wild type. Unexpectedly, RanBP2 depletion appeared to increase permissiveness to 4Mut, 5Mut, and N74D, although a rather large variation in the extent was observed between individual experiments. We also observed a strong decrease in cell viability in the transient RanBP2 knockdowns, consistent with a recent report (42). Overall, the effects of the host factor depletions on 4Mut and 5Mut viruses resembled those observed for N74D. Collectively, our results indicate that infection by 4Mut and 5Mut viruses exhibits decreased dependence on Nup153, RanBP2, and TNPO3 relative to wild-type HIV-1.

**4Mut amino acid substitutions result in impaired binding of CPSF6 and Nup153 peptides.** Amino acid changes in CA can alter the interaction of the viral capsid with host factors involved in nuclear entry, potentially leading to utilization of alternate pathways of infection. The decreased dependence of 4Mut on TNPO3,
PF74 Resistance via Altered Host Factor Dependence

Nup153, and RanBP2 suggested that the amino acid changes may impair interactions with these host factors. TNPO3 dependence of infection has been linked to an opposing effect of this protein on the host CPSF6 protein, which can restrict HIV-1 infection when present in the cytoplasm (43). To examine the relationship between PF74 resistance and binding of host factors, we tested the affinity of stabilized CA hexamers containing the 4Mut and 5Mut substitutions for CPSF6 and Nup153 peptides and for CypA (Fig. 8). Both hexamers bound CPSF6 peptide with decreased affinity compared to the wild type, in particular 5Mut, which had a >50-fold reduction in binding (2.8 mM versus 50 μM). Decreased affinities were also observed for Nup153 peptide (176 μM and 100 μM for 4Mut and 5Mut, respectively, versus 49 μM for the wild type). In contrast, 4Mut substitutions did not alter the affinity for recombinant CypA, while 5Mut hexamer exhibited a 3-fold decreased affinity (41 μM versus 12 μM for WT and 4Mut). The latter effect is likely due to the H87P substitution in 5Mut CA, which results in the loss of a hydrogen bond between H87 in capsid and N71 in CypA and may alter the conformation of the CypA binding loop, as has been reported for a substitution at the adjacent position 86 (44).

**Replication of 4Mut is impaired in primary T cells and macrophages.** We recently reported that the PF74-resistant 5Mut virus is impaired for replication in human macrophages (34). The altered dependence of 4Mut and 5Mut infection on expression of TNPO3 and RanBP2 prompted us to examine the fitness of 4Mut in primary targets of HIV-1 replication in vivo—i.e., CD4+ T cells and macrophages. Activated primary CD4+ T cells supported robust replication of wild-type X4- and R5-tropic HIV-1 (Fig. 9A). In contrast, and as observed with the CEM T cell line, 4Mut was markedly attenuated, as was 5Mut. Addition of PF74 or BI-2 promoted 4Mut replication in the cells (Fig. 9B). In macrophages, in contrast, replication of the 4Mut virus (rendered CCR5 dependent by replacement of the NL4-3 env by that of HIV-1.Bal) was severely impaired and was not stimulated by PF74 or BI-2 (Fig. 9C). Surprisingly, replication of the N74D mutant, which we had included as a control, was markedly stimulated by PF74 and BI-2 in macrophages at drug concentrations that were only moderately inhibitory toward the wild-type virus. We conclude that the 4Mut virus, while exhibiting features of the N74D mutant in terms of host factor requirement, responds differently to capsid-targeting inhibitors in macrophages.

**DISCUSSION**

Several capsid-targeting HIV-1 inhibitors have been reported to date. Of these, PF74 represents an attractive compound owing to its specificity and mechanism of action, yet its potency is too low for clinical development. Nonetheless, the PF74-binding site in viral capsid is an attractive target for therapeutic development, and studies of PF74 mechanism and resistance are important to further understand the nature of the target.

In the present study, we identified a novel HIV-1 CA mutant that is resistant to PF74 yet only moderately reduced in binding. Remarkably, the infectivity of the mutant was stimulated by both PF74 and BI-2. The virus exhibits approximately 20-fold resistance to the inhibitor, and all four of the mutations were required for this level of resistance. Unlike the previously identified 5Mut
virus, 4Mut encodes two amino acid substitutions in the CA NTD and two in the CTD. These results add further support for the NTD-CTD interface as the functional target of PF74. Only one of the substitutions, Q67H, was observed in the 5Mut virus. Therefore, S41A, V165I, and L172I represent novel mutations contributing to PF74 resistance. By deconstructing the 4Mut substitutions, we showed that combinations of as few as three of the substitutions (S41A, Q67H, and either V165I or L172I) confer strong resistance to PF74. We conclude that a minimum of three mutations are required for functional PF74 resistance, consistent with our recent analysis of the 5Mut virus (34).

Initial studies had reported that PF74 inhibits HIV-1 reverse transcription at high concentrations, likely owing to its capsid-destabilizing activity. More recently, we and others reported that at moderate concentrations, PF74 antiviral action was correlated with reduction in accumulation of 2-long terminal repeat (2-LTR) circles (30, 45). Unfortunately, our efforts to determine whether PF74 stimulates reverse transcription or nuclear entry of 4Mut failed to demonstrate a significant enhancement effect, likely owing to insufficiency of quantitative PCR in quantifying small differences in DNA copy number. Nonetheless, by analogy to inhibition of the wild-type virus, we suspect that the compound likely stimulates 4Mut infection by acting at nuclear entry and/or integration.

Resistance of 4Mut to PF74 could result from reduced binding of the inhibitor to the 4Mut capsid. However, the affinity of PF74 for recombinant CA hexamers was only moderately reduced (by ~4-fold) by the 4Mut substitutions. This slight reduction in affinity seems unlikely to account for drug resistance given that 4Mut is resistant to PF74 at doses up to 5 µM, a concentration that is 10 times the $K_d$. In contrast, the 4Mut substitutions significantly reduced the affinity for cofactor peptides, lowering the affinity to CPSF6 by 16-fold. We suggest that the resistance of 4Mut is due to its independence from these cofactors: while PF74 binds, competition with host factors is inconsequential.

Infection by HIV-1 is dependent on the cellular factors Nup153, RanBP2, and TNPO3, which promote HIV-1 nuclear entry and/or integration (19, 46). HIV-1 dependence on TNPO3 and Nup153 is altered by substitutions in the PF74 binding site (13, 40, 47), suggesting that the compound competes with host factor binding. Studies in our laboratory and others also suggest that HIV-1 sensitivity to PF74 is dependent on the ability of the virus to engage these host proteins. Depletion of TNPO3 and Nup153 reduces cellular permissiveness to HIV-1 infection, and the residual infectivity is less sensitive to inhibition by PF74 (40, 47). Moreover, the E45A substitution in CA reduces HIV-1 infectivity and dependence on both TNPO3 and Nup153 (13). Infection by E45A is resistant to PF74, yet, the mutant virus retains full ability to bind to the inhibitor (32). Addition of the second-site suppressor R132T to E45A partially rescues infectivity (48) and TNPO3 dependence (40) as well as sensitivity to inhibition of PF74 (48). Like E45A, the Q63A Q67A mutant also exhibits resistance to PF74 and altered host factor dependence. Thus, HIV-1 inhibition by PF74 appears related to the pattern of host factor

![FIG 6](image_url)

**FIG 6** The 4Mut substitutions have no significant effect on the structure of the PF74 binding pocket as revealed by X-ray crystallography. (A) Superposition of wild-type (pink) with 4Mut (gray) hexamer structures determined in the absence of PF74. (B) Superposition of 4Mut ± PF74 (apo hexamer in gray, complex in yellow, and PF74 in orange). (C) Superposition of the wild-type–CPSF6 complex (teal and green) with the 4Mut apo hexamer (gray). The substitution of His for Gln67 removes a hydrogen bond with the peptide.

![FIG 7](image_url)

**FIG 7** Infection by PF74-resistant viruses exhibits reduced dependence on the host factors Nup153, RanBP2, and TNPO3. Infection was assayed in cells depleted of the host factors by RNA interference using lentiviral delivery of shRNAs. Shown are the mean values from four independent experiments, with error bars representing 1 standard deviation.
FIG 8 4Mut and 5Mut substitutions reduce the affinity of CA hexamers for CPSF6 and Nup153 peptides. Interactions between PF74, CPSF6 peptide, Nup153 peptide, and CypA protein and the corresponding CA hexamers were determined by isothermal titration calorimetry at 20°C. The measured affinity is shown for each panel.
FIG 9 Replication of PF74-resistant HIV-1 is impaired in activated primary human T cells and monocyte-derived macrophages. Shown are the mean values from duplicate parallel growth curves, which exhibited close agreement. (A) Replication of X4-tropic (top row) and R5-tropic (bottom row) viruses in activated CD4+ T cells from three different donors cultured in the absence of PF74. (B) Replication of X4-tropic (top row) and R5-tropic (bottom row) 4Mut viruses in the presence of no drug (circles), 20 μM BI-2 (triangles), and 2 μM PF74 (squares). (C) Replication of R5-tropic CA mutants in monocyte-derived macrophages purified from three donors in the absence and presence of 1.25 μM PF74 and 2.5 μM BI-2.
utilization. The phenotype of PF74-resistant viruses 4Mut and 5Mut further supports a connection between host factor utilization and viral sensitivity to PF74. Both of these viruses exhibited decreased dependence on TNPO3, Nup153, and Nup358. With respect to 5Mut, we previously showed that PF74 sensitivity was correlated with binding of the compound, yet this correlation was not strict (34). In the present study, we observed that, like the E45A mutant, 4Mut is resistant to PF74, despite retaining significant capacity to bind the inhibitor. We conclude that resistance to PF74 does not require complete loss of binding and likely results from altered HIV-1 dependence on host factors.

We also observed that the 4Mut capsid substitutions resulted in impaired HIV-1 replication in activated primary T cells and macrophages. PF74 and BI-2 partially rescued 4Mut replication in T cells but not macrophages. We recently reported that the PF74-resistant 5Mut virus is also impaired for replication in macrophages (34). Thus, HIV-1 acquisition of PF74 resistance appears to result in loss of macrophage tropism. Macrophages are terminally differentiated nondividing cells and may exhibit more stringent host factor requirements for HIV-1 infection (49) or more stringent uncoating requirements. Efficient replication in macrophages may also require cloaking of the viral DNA from DNA sensors, such as cGAS, which appears to limit innate cellular responses to HIV-1 (22). It will be interesting to test the responses of macrophages and dendritic cells to a variety of HIV-1 CA mutants, including PF74-resistant viruses.

HIV-1 drug resistance is frequently the result of reduced compound binding to its viral target, although other mechanisms contribute (reviewed in references 50 and 51). We have found that in the presence of PF74, HIV-1 evolves to also become less dependent upon host proteins that bind the viral capsid. While a reduction in compound binding was observed for both 4Mut and 5Mut viruses, our previous demonstration that the PF74-resistant E45A mutant retains full binding of PF74 suggests that altered host factor utilization can be sufficient for resistance (32). Nonetheless, the impaired ability of 4Mut and 5Mut to replicate in primary T cells and macrophages suggests that host factor utilization is restricted in vivo, consistent with a study of immune escape CA variants (21). To date, no escape viruses or mutants that prevent PF74 binding without an associated fitness cost in primary cells have been reported. Based on these observations and the high conservation of the PF74 binding site among primary HIV-1 isolates (52), we predict that HIV-1 will have more difficulty escaping capsid-targeting antivirals in vivo than might be at first assumed from the emergence of PF74-resistant mutants in vitro.

The presence of the S41A mutation in 4Mut is intriguing. S41A does not overlap the PF74 binding site. However, this change has previously been shown to compensate for a loss of viral fitness resulting from immune escape in vivo, and it reverts a cyclosporine-dependent replication phenotype exhibited by some CA mutants (53). Moreover, S41A renders HIV-1 resistant to a CPSF6-dependent restriction exhibited by such mutants (21). Thus, this substitution appears to subtly alter the interaction of HIV-1 with host factors that may be restrictive in some viral contexts.

We observed that cyclosporine inhibited the enhancement of 4Mut infectivity by PF74, suggesting that infectivity enhancement is dependent on binding of cyclophilin A to the viral capsid. However, cyclosporine did not enhance 4Mut infectivity in the absence of PF74, indicating that the virus’s limited infectivity does not result from cyclophilin A-dependent restriction. Cyclophilin A binding to the viral capsid potentiates the ability of PF74 to inhibit infection by wild-type HIV-1, yet the mechanism by which this occurs is not yet clear. Cyclophilin A has previously been shown to influence the dependence of HIV-1 on host factors that the virus exploits to enter the nucleus (19). We suggest that interaction of CypA with the viral capsid restricts the nuclear entry pathway to a specific route that cannot be efficiently utilized by 4Mut and that PF74, by reducing the binding of CPSF6 and Nup153, releases the virus to utilize an alternative nuclear entry pathway. CypA has also been reported to stabilize the viral capsid (40), which could also directly regulate nuclear entry by controlling the uncoating process.

The PF74 enhancement phenotype is reminiscent of the previously described WIN compound-resistant picornavirus mutants that acquired dependence on the inhibitor (54, 55) and on the dependence of specific HIV-1 CA mutants on cyclosporine (56, 57). In the former case, drug dependence was attributed to altered capsid stability conferred by the mutants. We have previously shown that PF74 can destabilize the viral capsid (32). Preliminary studies in our laboratory suggest that the 4Mut particles contain hyperstable capsids; thus, the observed enhancement of infection may result from the ability of the compound to reverse capsid hyperstability, thus facilitating proper uncoating in target cells and, consequently, the dependence on host factors. Our results reported herein further underscore the complex interplay between host factor engagement, capsid stability, and inhibition of infection by capsid-targeting small molecules.

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