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Small-Molecule Inhibition of Human Immunodeficiency Virus Type 1 Infection by Virus Capsid Destabilization▽

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Human immunodeficiency virus type 1 (HIV-1) infection is dependent on the proper disassembly of the viral capsid, or "uncoating," in target cells. The HIV-1 capsid consists of a conical multimeric complex of the viral capsid protein (CA) arranged in a hexagonal lattice. Mutations in CA that destabilize the viral capsid result in impaired infection owing to defects in reverse transcription in target cells. We describe here the mechanism of action of a small molecule HIV-1 inhibitor, PF-3450074 (PF74), which targets CA. PF74 acts at an early stage of HIV-1 infection and inhibits reverse transcription in target cells. We show that PF74 binds specifically to HIV-1 particles, and substitutions in CA that confer resistance to the compound prevent binding. A single point mutation in CA that stabilizes the HIV-1 core also conferred strong resistance to the virus without inhibiting compound binding. Treatment of HIV-1 particles or purified cores with PF74 destabilized the viral capsid *in vitro*. Furthermore, the compound induced the rapid dissolution of the HIV-1 capsid in target cells. PF74 antiviral activity was promoted by binding of the host protein cyclophilin A to the HIV-1 capsid, and PF74 and cyclosporine exhibited mutual antagonism. Our data suggest that PF74 triggers premature HIV-1 uncoating in target cells, thereby mimicking the activity of the retrovirus restriction factor TRIM5α. This study highlights uncoating as a step in the HIV-1 life cycle that is susceptible to small molecule intervention.

Human immunodeficiency virus type 1 (HIV-1) infection leads to depletion of T helper cells and immune dysfunction, resulting in immunodeficiency and thus vulnerability to opportunistic infections. Formerly a death sentence, infection by HIV-1 can be managed effectively by treatment with multiple drugs targeting the viral enzymes, reverse transcriptase and protease. Such combination therapy results in a dramatic reduction in the level of circulating virus and minimizes the emergence of drug-resistant viral mutants. However, even the most effective current therapy fails to eliminate the virus, and incomplete patient adherence to therapy promotes the emergence of drug-resistant mutants. These issues have promoted the development of antiviral compounds targeting novel steps in infection, including fusion and integration. Historically, the virus-specific enzymes have been preferred targets for therapy due to the facility of biochemical assays for screening. Nonetheless, any viral function that is essential for replication may be considered as a potential target for antiviral drug development.

Retroviruses such as HIV-1 contain an internal viral RNA-protein complex surrounded by a protein shell, termed the capsid, which consists of a polymer of the viral protein CA arranged in a hexameric lattice (12). These components collectively comprise the viral core. Upon virus fusion with a susceptible host cell, the core is released into the target cell. Although the ensuing stages are poorly understood at a molecular level, the capsid is thought to undergo a controlled disassembly reaction in order for reverse transcription of the viral genome to occur (10). This process, termed uncoating, appears to be perturbed by restrictive host factors that target CA, including simian TRIM5α proteins, and by mutations in CA that alter the structure and/or stability of the capsid (9, 10, 23, 30, 33). Premature uncoating results in attenuated reverse transcription in target cells (21, 27). Another host protein, cyclophilin A (CypA), interacts with HIV-1 CA in target cells and facilitates infection by an unknown mechanism (19, 31). CypA also potentiates restriction of HIV-1 by simian TRIM5α proteins, which target the viral capsid upon penetration into target cells and lead to impaired reverse transcription. Although the molecular mechanism of TRIM5α-dependent restriction has not been fully defined, one attractive model involves acceleration of virus uncoating in target cells (26, 27).

Here we describe the mechanism of action of a newly identified HIV-1 inhibitor that targets the CA protein. We show that the compound destabilizes the viral capsid. The study identifies a novel antiviral mechanism, highlights CA as a viable target for antiviral therapy, and introduces a novel pharmacologic probe for analyzing the early postentry events in HIV-1 infection.

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MATERIALS AND METHODS

Viruses and cell lines. The full-length HIV-1 molecular clone R9 (11), and its corresponding CA mutant derivatives (10), were used in these experiments. The 5Mut derivative contains five substitutions in the CA coding sequence, resulting in the following codon changes: Q67H, K70R, H87P, T107N, and L111I. For experiments involving SIV, a full-length molecular clone of SIVmac239 containing a frameshift in the Env coding sequence was used, and particles were pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) by cotransfec-

tion of 293T cells with the proviral plasmid and pHCMV-G (37). LacZ-transducing MLV reporter particles were produced by transfection of 293T cells with the expression vector pCIG3B (4), expressing B-tropic MLV Gag-Pol, together with pCL-lacZ (20) and pHCMV-G. To generate stable CypA-depleted cells, TZM-bl reporter cells (34) were transduced with pGIPZ-based lentiviral vectors encoding short hairpin RNAs (shRNAs) specific for human TRIM5α and CypA. Stable transduced cell populations were selected with puromycin and subsequently used in assays of HIV-1 infection. Functional depletion of CypA was confirmed by titration of the cyclosporine (Cs)-dependent HIV-1 CA mutant A92E, which is not attenuated in CypA-depleted cells (22).

PCR quantification of reverse transcription. HeLa-P4 cells were inoculated with DNase I-treated stocks of Env-defective HIV-1 (R9.Env−) pseudotyped with VSV-G in the presence or absence of PF74 (10 μM) or Efavirenz (5 nM). After 8 h of culture, the cells were harvested and DNA isolated with a DNeasy Blood & Tissue kit (Qiagen). HIV-1 DNA in the samples was quantified by real-time PCR using primers specific for products formed after the second strand transfer—5′-AGCAGCTGCTTTTTGCCTGTACT-3′ and 5′-CCTGC GTCGAGAGATCTCCTCTGG-3′—with SYBR green detection in a Stratagene MX3000p instrument.

Antiviral assays. Stock solutions of PF74 were prepared at 10 mM in dimethyl sulfoxide (DMSO). For antiviral assays up to a 10 μM final concentration, the compound was diluted to 1 mM in DMSO and added directly to prediluted viruses to the desired final concentrations. Infection assays were performed in HeLa-P4 (HeLa-CD4/LTR-lacZ) (5) or TZM-bl (34) reporter cells. Infections were quantified following visualization of infected cells by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining, as previously reported (36). Control assays with DMSO demonstrated no effect of the solvent at the final concentrations present in the PF74-containing assays.

Assays of HIV-1 capsid stability. HIV-1 cores were purified as previously described (15). In experiments involving treatment with PF74, concentrated virus suspensions were incubated with or without the compound at the final concentrations for 1 h at room temperature and subsequently cooled to 4°C prior to the ultracentrifugation through a layer containing 1% Triton X-100. Fractions were harvested from the top of the gradient, and the CA protein in each fraction was quantified by p24 enzyme-linked immunosorbent assay (ELISA). The recovery of core-associated CA protein was determined as a percentage of the total CA protein in each gradient. Uncoating of purified cores was assayed as previously described (1).

Assay of HIV-1 uncoating in target cells. The effect of PF74 on HIV-1 capsid stability in target cells was analyzed by density gradient separation of cell lysates, as previously described (27). Briefly, cultures of 5×10^6 CrFK cells in 100-mm dishes were inoculated with 10 ml of VSV-G-pseudotyped wild-type or 5Mut HIV-1 particles (corresponding to about 5×10^6 cpm of reverse transcriptase activity). The virus was preincubated with PF74 (10 μM) or carrier DMSO (0.1%) for 30 min at room temperature and then added to cultures (prewashed with phosphate-buffered saline [PBS]) for 30 min at 4°C, after which the cultures were incubated at 37°C for 4 h. Cultures were treated with Pronase E (Sigma) in PBS to detach cells from the dish. After lysis with hypotonic buffer, the cell lysate was loaded onto a 10-ml 30 to 50% sucrose gradient with a 1-ml 50% sucrose cushion at the bottom. Tubes were centrifuged in a Beckman SW32.1 Ti rotor at 32,000 rpm for 16 h at 4°C. After centrifugation, the pellet was dissolved in 0.5% Triton X-100 in PBS. Both pellet and cell lysate were analyzed for CA content by p24 ELISA. The percentage of pelleted CA was calculated as the total amount of CA in the pellet divided by the total quantity of CA in the supernatant and the pellet.

RESULTS

A series of novel HIV-1 inhibitors was identified in a high-throughput antiviral assay encompassing the full HIV-1 replication cycle (3). PF-3450074 (henceforth referred to as PF74) (Fig. 1A) and a cluster of structurally related compounds inhibited HIV-1 replication at submicromolar concentrations by targeting a postentry stage prior to reverse transcription. These compounds exhibited no inhibitory activity toward HIV-1 protease or reverse transcriptase activity in biochemical assays. Furthermore, HIV-1 mutants resistant to reverse transcriptase inhibitors remained sensitive to PF74. PF74 inhibited a wide variety of HIV-1 isolates with similar potency. Selection for drug resistance in culture identified a series of five substitutions in CA that collectively conferred strong resistance upon introduction into an infectious molecular clone (3a). Thus, PF74 inhibits HIV-1 infection at an early step in the virus life cycle, apparently by targeting the viral capsid.

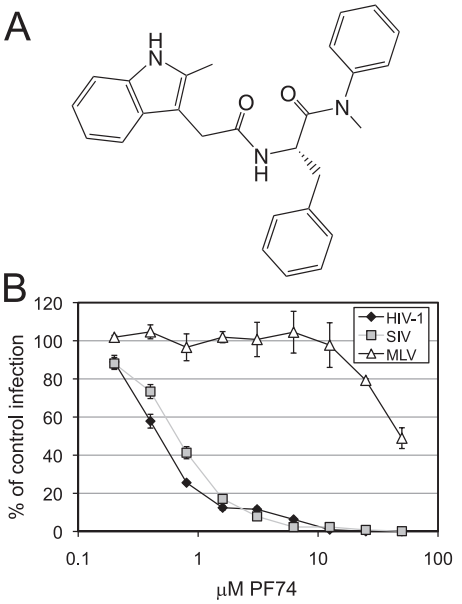

FIG. 1. PF74 selectively inhibits infection by HIV-1. (A) Chemical structure of PF74. (B) Selectivity of PF74 antiviral activity. VSV-G-pseudotyped HIV-1, SIV, and lacZ-transducing MLV vector particles were inoculated on HeLa-P4 indicator cells in the presence of the indicated concentrations of PF74. In the case of HIV-1 and SIV, expression of viral Tat protein in the target cells transactivates an LTR-lacZ reporter, resulting in the expression of β-galactosidase. For the MLV vector particles, transduction of the *lacZ*-containing vector results in expression of β-galactosidase. Two days after inoculation, the cultures were fixed and stained with X-Gal, and blue cells were enumerated by digital image processing. Shown is the percentage of infection at the corresponding inhibitor concentrations, with error bars representing the standard deviations of the means of the triplicate determinations. The results are representative of four independent experiments.

Specificity of retroviral inhibition by PF74. To examine whether inhibition of infection extends to retroviruses besides HIV-1, we assayed infection by HIV-1, simian immunodeficiency virus (SIVmac), and murine leukemia virus (MLV) in the presence of various concentrations of PF74. To eliminate potential differences arising from distinct virus-receptor interactions used by the viruses, each of viruses was pseudotyped by the VSV-G, thus ensuring that all of the viruses use the same endocytic entry pathway and limiting infection to a single cycle. HIV-1 and SIVmac were both markedly inhibited by PF74 concentrations in the low micromolar range (Fig. 1B). In contrast, PF74 inhibited MLV infection by only 30% at a concentration of 6 μM; however, no additional inhibition observed at concentrations as high as 100 μM (Fig. 1B). These results demonstrate the selectivity of PF74 antiviral activity.

PF74 binds specifically to HIV-1 particles. PF74 binds to recombinant CA *in vitro* with micromolar affinity, occupying a binding pocket in the amino-terminal domain of the two-domain protein (3a). To determine whether the compound binds to the assembled CA protein within virions, we incubated con-

centrated HIV-1 particles with ^{3}H-labeled PF74 and subsequently purified the particles by density gradient ultracentrifugation to remove the unbound compound. The gradients were fractionated, and virus particles in each fraction were pelleted. The levels of CA protein and radioactivity in each pellet were then quantified. ^{3}H-PF74 bound to wild-type HIV-1 particles but not to mutant HIV-1 particles containing five amino acid substitutions (Q67H, K70R, H87P, T107N, and L111I) in CA that collectively confer strong resistance to the compound (Fig. 2A and B). These data suggest that the antiviral activity of PF74 depends on its ability to associate with CA in the context of the mature HIV-1 particle.

PF74 inhibits HIV-1 reverse transcription in target cells. To more precisely define the stage in infection targeted by PF74, we analyzed the accumulation of reverse transcribed viral DNA in target cells inoculated with HIV-1 in the presence or absence of PF74. Quantitative PCR analysis of HIV-1 cDNA accumulation revealed that addition of the inhibitor resulted in a marked reduction in late products of reverse transcription (Fig. 2C). These results demonstrate that PF74 inhibits a step in HIV-1 infection prior to completion of reverse transcription.

PF74 destabilizes the HIV-1 capsid. Mutations in CA that either stabilize or destabilize the HIV-1 capsid attenuate infection, indicating that the stability of the capsid must be properly balanced for productive uncoating in target cells (10). Therefore, we reasoned that binding of PF74 to CA within HIV-1 particles could inhibit infection by either stabilizing or destabilizing the viral capsid lattice. To test this, we purified cores from HIV-1 particles after incubation with the compound. In this assay, positive and negative effects on capsid stability are reflected in an increase or decrease, respectively, in the levels of CA protein cosedimenting with the viral cores (10). Analysis of the gradient fractions revealed that ca. 20% of the virion-associated CA protein was recovered in the dense fractions of the gradient from the untreated wild-type virions (Fig. 3A). In contrast, treatment of the virions with PF74 resulted in a marked decrease in the recovery of core-associated CA protein in a dose-dependent manner (Fig. 3A and B). The decreased recovery of CA in the cores from the drug-treated wild-type HIV-1 particles indicates that the compound destabilizes the viral capsid. As a control, we performed the analysis of the PF74-resistant HIV-1 mutant. The drug-resistant particles exhibited only a 10% decrease in CA recovery upon treatment with the compound (Fig. 3B). Thus, the destabilization of the virus capsid by PF74 was specific for wild-type HIV-1.

We next tested the effects of PF74 on the uncoating of purified HIV-1 cores *in vitro*. Native viral cores were isolated by centrifugation of a suspension of concentrated virions through a detergent layer into a linear sucrose density gradient at 4°C, and the cores were recovered from the gradient fractions. Samples of purified cores were subsequently incubated at 37°C in the presence or absence of PF74, followed by separation of core-associated and soluble CA protein by ultracentrifugation. PF74 potently stimulated the uncoating of wild-type HIV-1 cores *in vitro*, with an ~100% increase observed at 0.25 μM (Fig. 3C). This difference was statistically significant ($P = 0.027$). In contrast, no significant effect of PF74 was observed on the extent of uncoating of cores purified from the PF74-resistant 5Mut virus. However, much higher concentrations of the compound (10 to 20 μM) were capable of stimulating the uncoating of the mutant cores (data not shown).

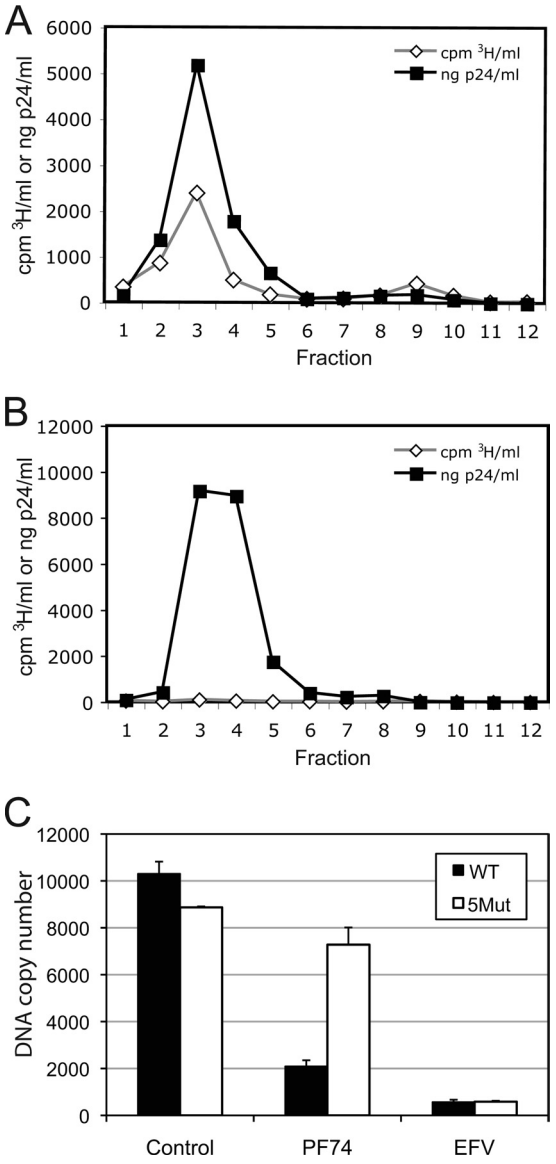

FIG. 2. PF74 binds HIV-1 particles specifically and inhibits an early postentry stage of infection. Concentrated wild-type (A) or PF74-resistant mutant (B) HIV-1 particles were incubated with ^{3}H-PF74 for 3 h at room temperature and then subjected to equilibrium sucrose gradient ultracentrifugation. The gradients were fractionated, and the particles in each fraction were pelleted by ultracentrifugation. The radioactivity and CA protein in the pellets were quantified by scintillation counting and antigen-capture ELISA, respectively. (C) PF74 treatment inhibits HIV-1 reverse transcription in target cells. Cells were inoculated with HIV-1 particles in the presence of PF74 (10 μM), and DNA was harvested and assayed by qPCR for late reverse transcription products. EFV: cultures containing HIV-1 with the RT inhibitor Efavirenz (EFV) to serve as a control for contaminating plasmid DNA. Shown are the mean values of triplicate determinations, with error bars depicting one standard deviation. The results in this figure are representative of at least three independent experiments.

We also tested the effects of PF74 on the stability of the incoming HIV-1 core in target cells. To do this, we used an approach previously developed for the analysis of retrovirus

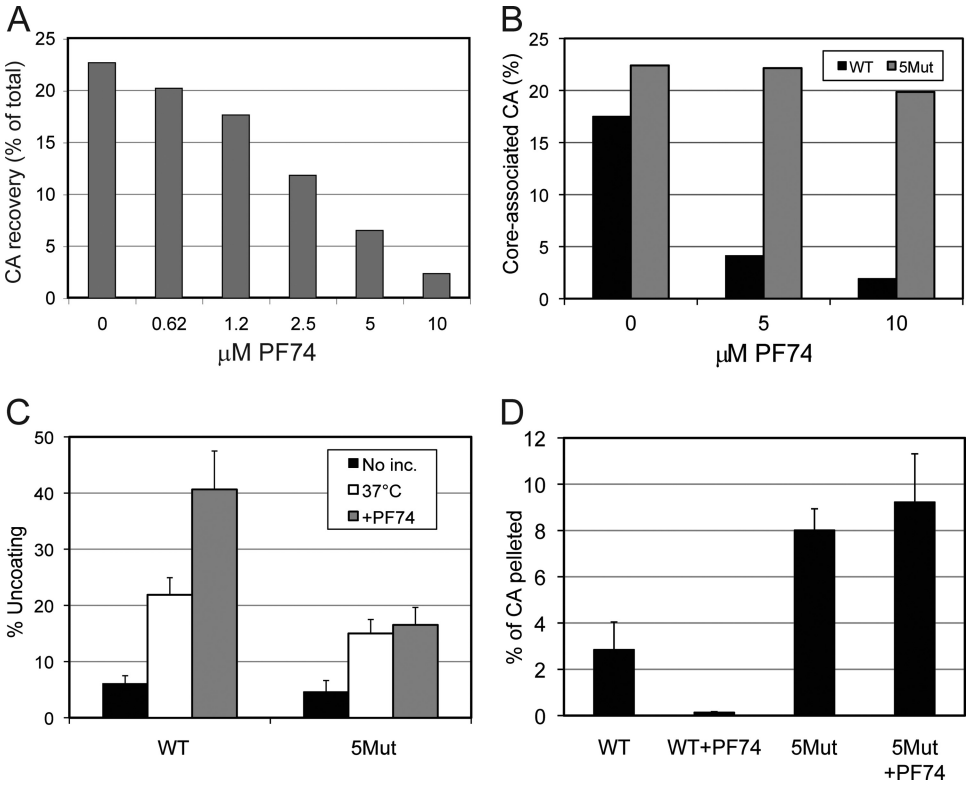

FIG. 3. PF74 destabilizes the HIV-1 capsid. (A) Treatment of HIV-1 with PF74 reduces the level of CA recovered upon purification of HIV-1 cores. Concentrated HIV-1 particles were incubated with or without PF74 then subjected to ultracentrifugation through a layer containing Triton X-100. Fractions were collected from the gradient and analyzed for CA concentrations by ELISA. Shown is the quantity of CA in the fractions of each gradient corresponding to the density of viral cores, expressed as a percentage of the total CA protein in the gradient. (B) Analysis of the effects of 5 and 10 μM PF74 on CA recovery from wild-type and resistant (5Mut) HIV-1 particles. (C) Effects of PF74 on uncoating of HIV-1 cores. Purified wild-type and 5Mut cores were incubated at 37°C in the presence or absence of PF74 (0.25 μM). Wild-type cores were incubated for 30 min, and the 5Mut cores were incubated at 70 min due to their slower uncoating kinetics. After incubation, the extent of uncoating was determined by quantifying the percentage of CA released into soluble form. Shown are the mean values of triplicate parallel determinations from a single experiment. (D) Effect of PF74 on pelletable CA following virus entry into cells. Cultures of CrFK cells were inoculated with VSV-G-pseudotyped HIV-1 in the presence or absence of PF74 (10 μM). Four hours after inoculation, the cells were harvested and lysed, the lysates subjected to ultracentrifugation, and CA in the supernatants and pellets was quantified by ELISA. Shown are the mean values of triplicate parallel determinations, with error bars depicting the standard deviations. All experiments in this figure were performed at least three times, with similar outcomes.

restriction by TRIM5α (27). Cultures of the highly permissive cell line, CrFK, were inoculated with VSV-G-pseudotyped HIV-1 in the presence or absence of PF74 (10 μM). Four hours later, the cells were harvested with Pronase to remove surface-bound particles and subjected to hypotonic lysis and density gradient fractionation by ultracentrifugation. Analysis of the levels of CA in the pellet and supernatant revealed that PF74 markedly reduced the level of pelletable CA in the lysates of cells inoculated with the wild-type virus (Fig. 3D). In contrast, no significant effect was observed on the PF74-resistant 5Mut virus, which exhibited an overall greater level of pelletable CA protein. Collectively, the results in Fig. 3 demonstrate that PF74 induces the premature dissolution of the HIV-1 capsid *in vitro* and in target cells.

HIV-1 sensitivity to PF74 is modulated by mutations altering the intrinsic stability of the viral capsid. In a previous study, mutations in CA that alter the intrinsic stability of the viral capsid were associated with impaired HIV-1 infectivity (10, 36). To test whether PF74 antiviral activity is modulated by changes in intrinsic capsid stability, we assayed PF74 inhibition of a panel of HIV-1 CA mutants exhibiting alterations in capsid stability (10). Although impaired for infection relative to wild-type HIV-1, the mutant viruses retain a sufficient level of infectivity to quantify inhibition by PF74. The hyperstable capsid mutant E45A exhibited reduced sensitivity to inhibition by PF74 relative to wild-type HIV-1 (Fig. 4A). In contrast, the P38A and Q219A mutants, which contain unstable capsids, were somewhat more sensitive to inhibition by PF74 than the wild type, exhibiting a greater reduction of infection at both low and high inhibitor concentrations. Like the E45A mutant, the Q63A/Q67A mutant was less sensitive to PF74 than wild-type HIV-1. This mutant was previously shown to exhibit unstable cores *in vitro* (10); however, unlike the other unstable mutants, Q63A/Q67A particles are competent for reverse transcription in target cells and are impaired at nuclear import (8). Furthermore, Q63A/Q67A preintegration complexes recovered from cells retained elevated levels of CA protein, indicating that this mutant undergoes incomplete or delayed uncoating in target cells (8). To determine whether altered binding of the compound could explain the changes in drug sensitivity, we assayed the binding of the drug to a subset of these mutants. We observed differences in the extent of com-

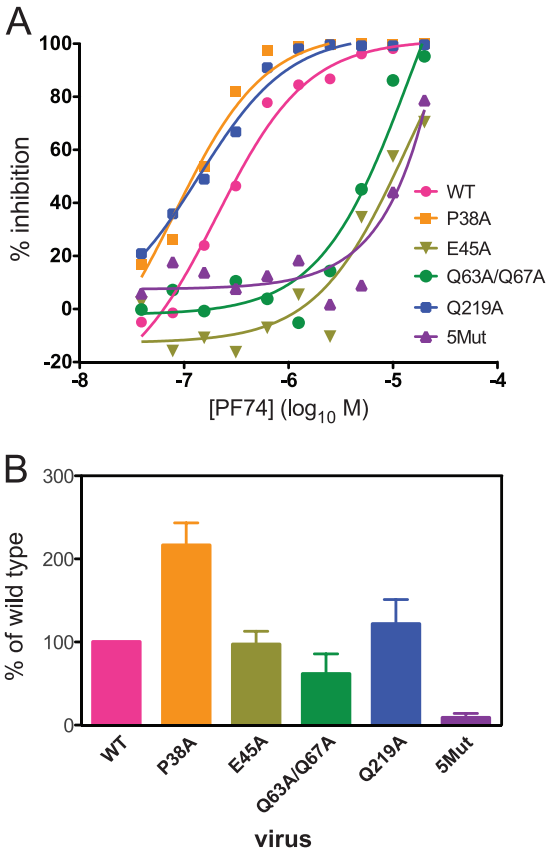

FIG. 4. Mutations that alter HIV-1 core stability modulate HIV-1 sensitivity to PF74. (A) Wild-type and mutant HIV-1 viruses were titrated on HeLa-P4 cells in the presence of various concentrations of PF74. For each virus, infection at each concentration of PF74 was calculated as a percentage of the corresponding untreated virus infection. The experiment was performed more than three times, with similar results. (B) Binding of ^{3}H-PF74 to the indicated HIV-1 mutant particles was tested. Samples of concentrated virus particles were incubated with the radiolabeled compound, pelleted, resuspended in PBS, and then repelleted through a 20% sucrose cushion. The pelleted particles were dissolved in sodium dodecyl sulfate-containing sample buffer, and the levels of ^{3}H and CA protein were quantified by liquid scintillation counting and ELISA, respectively. The ratios of the radioactive signals to the CA protein were calculated and expressed as a percentage of the wild-type value. Shown are the mean values obtained from averaging the data from at least six independent experiments, with error bars representing one standard deviation.

pound binding to some of the mutants (Fig. 4B). Specifically, the P38A mutant bound approximately twice as much of the PF74 as wild-type HIV-1 particles, potentially contributing to the increased sensitivity of this mutant to the inhibitor. The inhibitor bound to E45A and Q219A mutant particles to an extent comparable to that of the wild type. In contrast, a small but significant decrease ($P = 0.013$) in binding to Q63A/Q67A particles was observed relative to the wild type, potentially contributing to the reduced sensitivity of this mutant. The observation that E45A and Q219A mutant particles bind PF74 as efficiently as wild-type HIV-1 indicates that the altered sensitivity of these viruses to the inhibitor is likely owing to their changes in intrinsic capsid stability. The E45A mutant containing hyperstable cores was resistant to the compound, while the Q219A mutant with unstable cores was hypersensitive to the inhibitor.

PF74 antiviral activity is promoted by cyclophilin A binding to the viral capsid. The cytoplasmic host protein CypA binds specifically to HIV-1 CA and promotes infection by acting at an early postentry step. Although the precise mechanism by which CypA acts is unknown, one model involves binding to the incoming viral core and protection from an intrinsic antiviral restriction (reviewed in references 19 and 31). To determine whether CypA-CA interactions are involved in the antiviral mechanism of PF74, we tested the effects of Cs on PF74 antiviral efficacy. Cs acts by binding to CypA and inhibiting its association with CA. The MacSynergy II three-dimensional (3D) model of Prichard and Shipman produces predictions of synergy or antagonism by comparing the data generated against a prediction for additivity, which is revealed as a flat surface in a 3D plot. Synergistic interactions cause a positive volume of synergy and antagonistic interactions produce a negative volume of antagonism. Volumes greater than 25 μM^2 are considered to indicate moderate synergy or antagonism and volumes > 100 μM^2 indicate strong synergy. Highly synergistic combinations such as when nucleoside and non-nucleoside reverse transcriptase inhibitors are combined produce synergy volumes of 80 to 200 μM^2, whereas highly antagonistic combinations such as d4T and ribavirin are reported to have negative volumes of 300 to 600 μM^2 (6, 7, 16). The interaction between PF74 and Cs produced a clear negative volume (-129 μM^2), creating a bowl shape covering the majority of combinations tested (Fig. 5A and B). These data indicate strong antagonism between the antiviral activity of PF74 and Cs and suggest that the antiviral activity of PF74 is enhanced by CypA.

To confirm that the antagonistic effect of Cs on PF74 antiviral activity is due to Cs inhibition of CypA binding to CA, we tested the antiviral efficacy of PF74 in target cells depleted for CypA expression by RNA interference. Inhibition by PF74 in shRNA-transduced CypA-depleted cells was less effective than in the corresponding control cells transduced with the empty lentiviral vector (Fig. 5C). Finally, HIV-1 CA mutants (G89V and P90A) with reduced affinity for CypA exhibited reduced sensitivity to PF74 (Fig. 5D). We conclude that binding of CypA to CA in target cells promotes inhibition of infection by PF74.

DISCUSSION

In this study, we analyzed the mechanism of action of a novel small molecule inhibitor of HIV-1 infection. PF74 inhibits HIV-1 infection by targeting an early postentry step in infection, leading to attenuated reverse transcription. The compound targets the viral capsid, as recently demonstrated by identification of resistance mutations in CA (3a), and by our demonstration that PF74 binds specifically to wild-type HIV-1 but not to the inhibitor-resistant mutant. Our data indicate that PF74 inhibits HIV-1 infection by triggering premature uncoating in target cells. Treatment of HIV-1 particles with PF74 resulted in a decrease in the recovery of CA upon purification of viral cores, and mutations conferring resistance to the compound reduced this effect. PF74 also potently stimulated the uncoating of purified HIV-1 cores *in vitro* and induced CA solubilization in target cells. Furthermore, sensitiv-

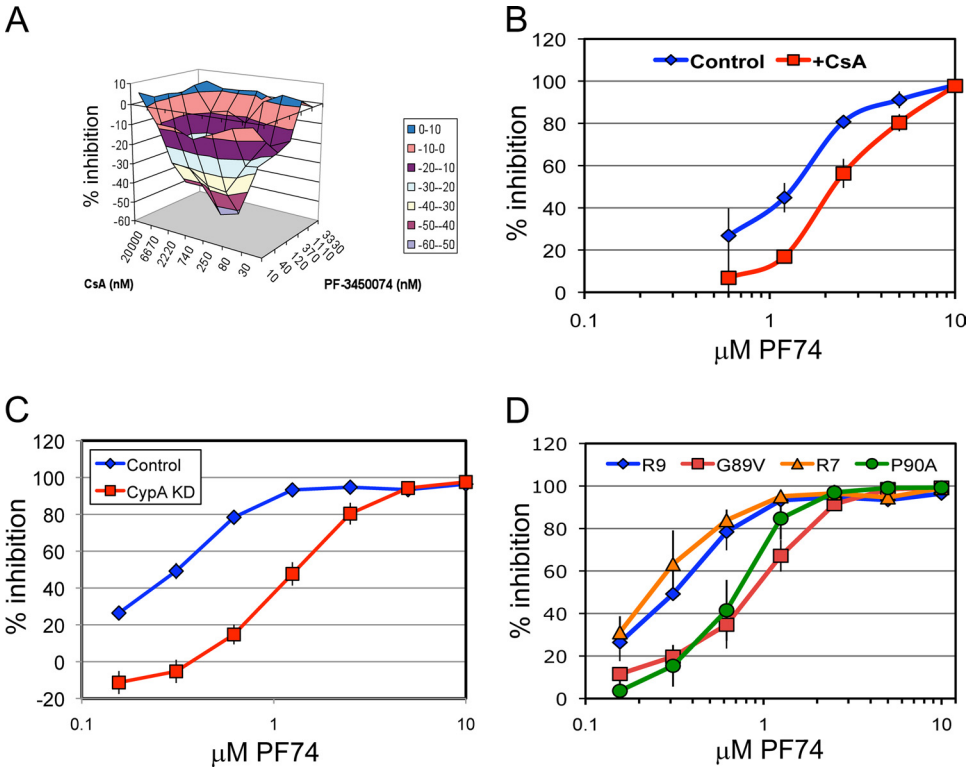

FIG. 5. Dependence of PF74 antiviral activity on CypA-CA interactions. (A) Macsynergy plot from analysis of Cs and PF74. The bowl-shaped surface indicates antagonism between the two inhibitors. This experiment was performed twice, with each assay performed in quadruplicate. The two experiments yielded similar outcomes. (B to D) Antiviral activity of PF74 was determined against HIV-1 in the presence or absence of 5 μM Cs (B), in CypA-depleted cells (C), and against the G89V and P90A CA mutants impaired for CypA binding (D). In panel D, R9 represents the wild-type control for G89V, whereas R7 is the control for the P90A mutant. Shown are data from representative experiments; each of the experiments in panels B to D was performed a minimum of thrice. The error bars shown in panels B to D represent the standard deviations of triplicate determinations.

ity to PF74 was modulated by mutations that alter the intrinsic stability of the viral capsid. Specifically, a mutation (Q219A) that reduced capsid stability conferred increased sensitivity to the inhibitor, whereas a mutation (E45A) that stabilizes the core reduced drug efficacy. Neither mutation detectably altered the ability of HIV-1 particles to associate with PF74, suggesting that the resistance of these viruses to the PF74 is independent of effects on compound binding. Intriguingly, infection by the mutant Q63A/Q67A was also markedly resistant to PF74. This mutant, while containing intrinsically unstable capsids, was found to undergo efficient reverse transcription in target cells. Preintegration complexes isolated from cells inoculated with Q63A/Q67A particles exhibited elevated levels of CA relative to wild type, indicative of delayed uncoating in target cells. The resistance of this mutant may therefore also result from an inefficient uncoating in the cell. Recent studies have also demonstrated that the E45A and Q63A/Q67A mutant viruses are impaired for infection of mitotically arrested cells (35) and are less dependent on the nuclear transporter TNPO3 (17), pointing to a link between HIV-1 uncoating and nuclear entry. While further studies are necessary to determine whether impaired HIV-1 uncoating is sufficient to confer resistance to PF74, the inverse correlation between capsid stability and PF74 sensitivity, coupled with the decreased level of CA associated with cores purified from PF74-treated virions, further support a link between destabilization of the viral capsid by PF74 and its antiviral activity. The effect of PF74 on HIV-1 infection is similar to that observed with HIV-1 mutants containing unstable capsids, the majority of which exhibit inefficient reverse transcription in target cells.

Two other inhibitors targeting the HIV-1 capsid protein have been previously described. The small molecule CAP-1 inhibits HIV-1 capsid assembly *in vitro* and reduces the infectivity of HIV-1 particles when present at high concentrations during particle formation (14, 29). CAP-1 does not appear to target uncoating but acts at a late stage of the virus replication cycle. CAI, a dodecapeptide identified as a CA ligand by phage display (25), inhibits the assembly of mature and immature HIV-1 particles *in vitro*. The peptide was recently rendered cell-permeable by chemical stapling, and the modified peptide inhibited HIV-1 at both early and late stages of replication (38). The cell-permeable peptide inhibited HIV-1 particle production and inhibited infection at micromolar concentrations. None of these previous studies reported the identification of HIV-1 resistance mutations, which is critical for unambiguous identification of the drug target.

The dependence of PF74 antiviral potency on CypA binding to CA is noteworthy and has implications for the mechanism of CypA action in HIV-1 infection. Currently, the mechanism by which CypA facilitates HIV-1 infection of human cells is un-

known, although recent findings have suggested that CypA binding to CA may shield the core from a postentry restriction in human cells (32), or modulates uncoating (18). The putative restriction is not mediated by TRIM5α, since depletion of this protein from human cells did not affect the requirement for CypA-CA association in HIV-1 replication (13, 24). Remarkably, restriction by the rhesus macaque TRIM5α protein is potentiated by host CypA (2, 13, 28), suggesting that the CypA-CA renders the capsid more vulnerable to the inhibitory effects of simian TRIM5α. Our finding that CypA also promotes HIV-1 inhibition by PF74 suggests that CypA binding may prime the viral core for uncoating in target cells, thus rendering the virus more susceptible to the destabilizing effects of PF74 as well as restrictive TRIM5α proteins. Furthermore, the dependence of both PF74 antiviral activity and TRIM5α restriction on CypA-CA interactions suggests a mechanistic similarity between these two HIV-1 inhibitors.

Our study revealed two mechanisms for generating HIV-1 resistance to PF74: (i) loss of binding of the compound and (ii) stabilization of the viral capsid. The resistant mutant virus encoding 5 amino acid changes in CA was selected by continuous culture in the presence of the drug. This mutant was markedly reduced in its ability to bind to PF74 (Fig. 2). In contrast, the core-stabilizing mutants E45A and Q63A/Q67A retained the ability to bind the drug and yet were highly resistant in single-cycle infection assays, suggesting that resistance can also be engendered via capsid stabilization without a loss of drug binding (Fig. 3). These mutants were previously shown to be impaired for replication in culture and exhibit poor infectivity in single-cycle assays (10). Thus, resistance to PF74 inhibition by capsid stabilization may initially be accompanied by a strong replicative fitness cost, which could be compensated for by additional mutations. Clinical resistance to the class of inhibitors may therefore be more likely to result from mutations that prevent binding of the inhibitor. Further analysis of the resistance profile to PF74 and related compounds will likely yield additional insights into the relationships between drug binding, capsid destabilization, and antiviral activity.

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