**Toward Structurally Novel and Metabolically Stable HIV-1 Capsid-Targeting Small Molecules**

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**Abstract:** HIV-1 capsid protein (CA) plays an important role in many steps of viral replication and represents an appealing antiviral target. Several CA-targeting small molecules of various chemotypes have been studied, but the peptidomimetic PF74 has drawn particular interest due to its potent antiviral activity, well-characterized binding mode, and unique mechanism of action. Importantly, PF74 competes against important host factors for binding, conferring highly desirable antiviral phenotypes. However, further development of PF74 is hindered by its prohibitively poor metabolic stability, which necessitates the search for structurally novel and metabolically stable chemotypes. We have conducted a pharmacophore-based shape similarity search for compounds mimicking PF74. We report herein the analog synthesis and structure-activity relationship (SAR) of two hits from the search, and a third hit designed via molecular hybridization. All analogs were characterized for their effect on CA hexamer stability, antiviral activity, and cytotoxicity. These assays identified three active compounds that moderately stabilize CA hexamer and inhibit HIV-1. The most potent analog (10) inhibited HIV-1 comparably to PF74 but demonstrated drastically improved metabolic stability in liver microsomes (31 min vs. 0.7 min t 1/2). Collectively, the current studies identified a structurally novel and metabolically stable PF74-like chemotype for targeting HIV-1 CA.

**Keywords:** HIV-1; capsid-targeting antivirals; PF74; metabolic stability

1. Introduction

Human immunodeficiency virus 1 (HIV-1) encodes a Gag polyprotein which contains multiple protein domains for viral assembly and release: matrix (p17 MA), capsid (p24 CA), nucleocapsid (p7 NC), p6 and spacer peptides Sp1 and Sp2 [1]. Gag polyproteins assemble to form the immature viral capsid core, which upon protease cleavage rearranges into a fullerene-shaped mature capsid core [2] comprising approximately 250 CA hexamers and exactly 12 asymmetrically-distributed CA pentamers [3]. CA plays essential roles in viral assembly and multiple events during viral replication [4,5]. CA-CA interactions drive the assembly and disassembly of viral capsid, and capsid core stability is important for reverse transcription, nuclear entry, and cloaking of the viral DNA product from host nucleic acid sensing.
mechanisms in the cytosol [6]. CA interacts with many cellular factors [7,8], including TRIM5α [9,10], cleavage and polyadenylation specific factor 6 (CPSF6) [11,12] nucleoporins 153 [13–15] and 358 [16–18] (NUP153, NUP358), MxB [19,20], and Cyclophilin A (CypA) [21–23]. These interactions enable early viral replication steps, including uncoating, cytoplasmic trafficking, reverse transcription, nuclear transport, site of integration, and the evasion of innate immunity [24]. Hence, CA-targeting small molecules could confer both early and late stage antiviral phenotypes by perturbing the stability of viral capsid core and interfering with important CA-host interactions.

The CA structure [25–27] is mostly helical (Figure 1A): it comprises 7 helices at the N-terminal domain (CA_NT_D) and 4 helices at the C-terminal domain (CA_CTD). Interactions between one CA_NT_D and the CA_NT_D and CA_CTD of an adjacent monomer form the molecular basis of stable hexameric lattice and virus-host interactions. Previous structural studies with CA-bound compounds have revealed a small molecule binding sites [28], such as the particularly interesting PF74 binding pocket. This pocket is formed primarily by H3 and H4 of the CA_NT_D (cyan), and the H8 and H9 of the CA_CTD of an adjacent monomer (green) (Figure 1A). In addition to accommodating the binding [25,29,30] of PF74 and BI-2, a smaller CA-targeting molecule (Figure 1B), the same pocket is also used by important cellular factors, including Nup153, a nucleoporin important for nuclear transport of viral preintegration complexes (PICs); and CPSF6 which transports HIV-1 PICs to transcriptionally active chromatin [31]. PF74 is a well-characterized [32] peptidomimetic built around a phenylalanine core, and capped with an aniline moiety at the carboxylate end and an indole-3-acetic acid at the amino end (Figure 1B). All three components, the core, the aniline moiety (right) and the indole acid moiety (left), provide key molecular interactions for the binding of PF74 [25]. Consistent with the binding site and mode, PF74 displayed a concentration-dependent bimodal mechanism [32,33] of action: at lower concentrations it competes against host factors including CPSF6 and NUP153 to affect nuclear entry; and at higher concentrations it blocks uncoating and reverse transcription, presumably by altering inter-hexamer interactions [25].

Despite the attractive antiviral profile and the unique bimodal mechanism of action, PF74 is not a viable drug candidate due primarily to its prohibitively poor metabolic stability [34]. In human liver microsomes (HLMs), the half-life (t_1/2) of PF74 was less than 1 min [35,36]. This major deficiency strongly necessitates targeted efforts to search for structurally novel and metabolically stable small molecules capable of binding to the PF74 binding pocket. Along this line, the Zhan and Liu group recently reported PF74-like compounds where an easier and synthetically more accessible 1,2,3-triazole ring was substituted for the indole moiety [35,36]. However, such compounds were considerably (>10-fold) less potent than PF74 in antiviral assays and were essentially as unstable in HLMs [35,36]. Our current work features a pharmacophore-based shape similarity search approach based on PF74 (Figure 2A). Molecular similarity [37,38] is an important concept in medicinal chemistry and drug discovery. Hit generation using similarity search is based on the premise that similar chemical structure confers similar biological activity. In our search, we extracted the 3D conformation of PF74 from reported co-crystal structure (PDB: 4XFZ [25]) and defined 7 pharmacophore points (Figure 2A) according to its mode of binding, including two H-bond donors (D1 and D2), two H-bond acceptors (A1 and A2), as well as three hydrophobic moieties (H1, H2 and H3). This 3D pharmacophore was then used to screen against a subset of the ZINC database [39] (100K compounds) using the program PHASE [40,41]. Hit ranking was based on the number of pharmacophore points satisfied and the lead like properties as predicted by the Lipinski’s rule of 5 [42]. From this similarity search we selected two hits (2 and 22) for analog synthesis and SAR (Figure 2B). Molecular hybridization [43] between hit 2 and PF74 also generated hit 11, which was also subjected to analog synthesis (Figure 2B). We report herein the analog synthesis and SAR on all three hits. In the end, our best compound inhibited HIV-1 with an EC_50 of 1.6 µM, and more important exhibited a half-life (t_1/2) 44-fold longer than PF74.
From this similarity search we selected two hits (pharmacophore points satisfied and the lead like properties as predicted by the Lipinski’s rule of 5). This 3D pharmacophore was then used to screen against a subset of the ZINC database (39) (D1 and D2), two H-bond acceptors (A1 and A2), as well as three hydrophobic moieties (H1, H2 and H3). This resulted in a hit list of 100K compounds using the program PHASE (40,41). Hit ranking was based on the number of similar chemical structures. In our search, we extracted the 3D conformation of H3. 

Considerably (> 10-fold) less potent than PF74, triazole ring was substituted for the indole moiety (35,36). However, such compounds were not viable drug candidates due primarily to their poor metabolic stability (34). In human liver microsomes (HLMs), the half-life (t1/2) of PF74 (pointed to with an arrow) was less than 1 min (35,36). This major deficiency strongly necessitates targeted efforts to search for structurally novel and metabolically stable small molecules capable of binding to the CA dimer (Protein Data Bank code: 4XFZ [25]). The pocket formed around H3 and H4 of the CA dimer (cyan), and H8 and H9 of the adjacent CA (green) accommodates the binding of multiple ligands, including host factors Nup153 and CPSF6, and small molecules PF74 and BI-2. Shown in the pocket is PF74 (pointed to with an arrow); (B) chemical structures of PF74 and BI-2.

The general synthetic strategy for major analogs (Schemes 1 and 2) and the synthesis for others is outlined in supporting information (Schemes S1 and S2). Detailed synthetic procedures and compound characterization data are included in Supplemental Materials.

Figure 1. Structure of HIV-1 capsid protein (CA) and a key CA ligand binding pocket. (A) The structure of CA dimer (Protein Data Bank code: 4XFZ [25]). The pocket formed around H3 and H4 of the CA (cyan), and H8 and H9 of the adjacent CA (green) accommodates the binding of multiple ligands, including host factors Nup153 and CPSF6, and small molecules PF74 and BI-2. Shown in the pocket is PF74 (pointed to with an arrow); (B) chemical structures of PF74 and BI-2.

Figure 2. Generation of novel compounds targeting the PF74 binding pocket. (A) Initial hits generated via a pharmacophore and shape similarity search; (B) SAR of two selected hits (2 and 22), as well as a new hit (11) from the hybridization between 2 and PF74. Compounds 3–10 are variants of 2, with modifications in the circled regions.
2. Materials and Methods

All analogs were synthesized using procedures described in Schemes 1 and 2, fully characterized with $^1$H NMR, $^{13}$C NMR, and HRMS, and displayed a purity of ≥95% as determined by HPLC. Detailed synthetic procedures and compound characterization data are included in Supplemental Materials.

The general synthetic strategy for major analogs (1–39) tested in this work is described here (Schemes 1 and 2) and the synthesis for others is outlined in supporting information (Schemes S1 and S2). Commercially available 1H-benzo[d] [1,3] oxazine-2,4-dione (a) was reacted with glycine methyl ester hydrochloride in the presence of Et$_3$N in DMF to yield intermediate (b). Further treatment of intermediate (b) with ethyl chloroformate in pyridine and then with NaOH in MeOH/H$_2$O afforded the carboxylic acid intermediate (c). A subsequent amide coupling of carboxylic acid intermediate (c) with different L-amino acid methyl ester hydrochlorides in the presence of HATU and DIPEA in DMF furnished acid intermediates (d) after hydrolysis under LiOH in MeOH/H$_2$O. To synthesize varied hit 2 analogs, carboxylic acid intermediates (d) were further coupled with L-proline methyl ester hydrochloride in the presence of PyAOP and DIPEA to produce compounds 2–4, which upon hydrolysis afforded 5–7. Another coupling with various amines delivered analogs 8–10. Likewise, acid intermediates (d) were treated with various anilines using HATU and DIPEA in DMF to produce hit 11 analogs 11–21.

The synthesis of hit 22 analogs 22–39 was achieved with a straightforward approach described in Scheme 2. Hydrazides (e) reacted with ethyl isothiocyanatoacetate in ACN to give thiosemihydrazides (f) that were converted to triazole acid derivatives (g) through in situ cyclization and hydrolysis when treated with aqueous NaOH. Amide coupling of these triazole acid derivatives (g) with various amines in the presence of HATU and DIPEA in DMF resulted in analogs 22–39.
TZM-GFP cells are a modified version of TZM-bl cells and contain an integrated nlsGFP reporter gene under the transcriptional control of the HIV-1 long terminal repeat (LTR) [44,45]. TZM-GFP cells were kindly provided by Dr. Marc Johnson (University of Missouri-Columbia, Columbia, MO) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone). HEK293-FT cells were also cultured in DMEM supplemented with 10% FBS. MT-2 cells were grown in RPMI supplemented with 10% heat-inactivated FBS. All cells were grown at 37 °C and maintained in humidified atmosphere containing 5% CO₂.

2.2. Method Details

2.2.1. Thermal Shift Assays (TSAs) to Screen Compounds for Effect on HIV-1 CA Hexamer Stability

Compounds were screened for their effect on CA hexamer stability using purified covalently-crosslinked hexameric CA₁₁₄C/E₄₅C/W₁₈₄A/M₁₈₅A (CA121). CA121 cloned in a pET11a expression plasmid was provided by Dr. Owen Pornillos (University of Virginia, Charlottesville, VA). Protein was expressed in E. coli BL21(DE3)RIL and purified as reported previously [26]. The TSA was conducted as previously described [46–48] using the PikoReal Real-Time PCR (Thermo Fisher Scientific, Waltham, MA, USA) or the QuantStudio 3 Real-Time PCR (Thermo Fisher Scientific) systems. Each reaction contained 10 μL of 15 μM CA121 (7.5 μM final concentration) in 50 mM sodium phosphate buffer (pH 8.0), 10 μL of 2× Sypro Orange Protein Gel Stain (Life Technologies, Carlsbad, CA, USA) in 50 mM sodium phosphate buffer (pH 8.0) and 0.2 μL of DMSO (control) or compound. Compounds were tested at a final concentration of 20 μM. The plate was heated from 25 to 95 °C with a heating rate of 0.2 °C/10 s. The fluorescence intensity was measured with an Ex range of 475–500 nm and Em range of 520–590 nm. The differences in the melting temperature (ΔTm) of CA hexamer in DMSO (T0) verses in the presence of compound (Tm) were calculated using the following formula: ΔTm = Tm − T0.

2.2.2. Virus Production

The wild-type laboratory HIV-1 strain, HIV-1NL₄-3 [49], was produced using a pNL4-3 vector that was obtained through the NIH AIDS Reagent Program. HIV-1NL₄-3 was generated by transfecting HEK 293FT cells in a T75 flask with 10 μg of the pNL4-3 vector and FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA). Supernatant was harvested 48–72 h post-transfection and transferred to MT2 cells for viral propagation. Virus was harvested when syncytia formation was observed, which took 3–5 days. The viral supernatant was then concentrated using 8% w/v PEG 8000 overnight at 4 °C, followed by centrifugation for 40 min at 3500 rpm. The resulting viral-containing pellet was concentrated 10-fold by resuspension in DMEM without FBS and stored at −80 °C.

2.2.3. Anti-HIV-1 and Cytotoxicity Assays

Anti-HIV-1 activity of PF74 and related analogs was examined in TZM-GFP cells. The potency of HIV-1 inhibition by a compound was based on its inhibitory effect on viral LTR-activated GFP expression compared with that of compound-free (DMSO) controls. Briefly, TZM-GFP cells were plated at density of 1 × 10⁴ cells per well in a 96-well plate. 24 h later, media was replaced with increasing concentrations of compound. 24 h post treatment, cells were exposed to an HIV-1 strain (MOI = 1). After incubation for 48 h, anti-HIV-1 activity was assessed by counting the number of GFP positive
cells on a Cytation™ 5 Imaging Reader (BioTek, Winooski, VT, USA) and 50% effective concentration (EC\textsubscript{50}) values were determined.

Cytotoxicity of each compound was also determined in TZM-GFP cells. Cells were plated at a density of 1 × 10^4 cells per well in a 96-well plate and were continuously exposed to increasing concentrations of a compound for 72 h. The number of viable cells in each well was determined using a Cell Proliferation Kit II (XTT), and 50% cytotoxicity concentration (CC\textsubscript{50}) values were determined. All the cell-based assays were conducted in duplicate of at least two independent experiments and the average values were determined.

For calculation of EC\textsubscript{50} and CC\textsubscript{50} dose response curves, values were plotted in GraphPad Prism 5 and analyzed with the log (inhibitor) vs. normalized response—Variable slope equation. Final values were calculated in each independent assay and the average values were determined. Statistical analysis (calculation of standard deviation) was performed using Microsoft Excel.

2.3. Microsomal Stability Assay

The in vitro microsomal stability assay was conducted in duplicate in mouse and human liver microsomal systems, which were supplemented with nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Briefly, a compound (1 µM final concentration) was pre-incubated, in the absence or presence of 0.5 µM Cobicistat (CYP 3A inhibitor, purchased from medchemexpress.com and verified with LCMS), with the reaction mixture containing liver microsomal protein (0.5 mg/mL final concentration) and MgCl\textsubscript{2} (1 mM final concentration) in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C for 15 min. The reaction was initiated by addition of 1 mM NADPH, followed by incubation at 37 °C. A negative control was performed in parallel in the absence of NADPH to measure any chemical instability or non-NADPH dependent enzymatic degradation for each compound. At various time points (0, 5, 15, 30 and 60 min), 1 volume of reaction aliquot was taken and quenched with 3 volumes of acetonitrile containing an appropriate internal standard and 0.1% formic acid. The samples were then vortexed and centrifuged at 15,000 rpm for 5 min at 4 °C. The supernatants were collected and analyzed by LC-MS/MS to determine the in vitro metabolic half-life (t\textsubscript{1/2}).

2.4. Molecular Modeling

Molecular modeling was performed using the Schrödinger small molecule drug discovery suite 2019-1 [50]. The crystal structure of native HIV-1 capsid protein in complex with PF74 [25] was retrieved from the protein data bank (PDB code: 4XFZ [25]). The above structure was analyzed using Maestro [51] (Schrödinger; LLC: New York, NY, USA) and subjected to a docking protocol that involves several steps including preparing protein of interest, grid generation, ligand preparation, and docking. The crystal structure was refined using the protein preparation wizard [52] (Schrödinger; LLC: New York, NY, USA,) wherein missing hydrogen atoms, side chains, and loops were added using Prime and minimized using the OPLS 3e force field [53] to optimize the hydrogen bonding network and converge the heavy atoms to an rmsd of 0.3 Å. The receptor grid generation tool in Maestro (Schrödinger; LLC: New York, NY, USA) was used to define an active site around the native ligand PF74 to cover all the residues within 12 Å. All the compounds were drawn using Maestro and subjected to Lig Prep to generate conformers, possible protonation at pH of 7 ± 2 that serves as an input for docking process. All the dockings were performed using Glide XP [54] (Glide: version 8.2) with the van der Waals radii of nonpolar atoms for each of the ligands were scaled by a factor of 0.8. The solutions were further refined by post docking and minimization under implicit solvent to account for protein flexibility. The residue numbers of HIV-1 capsid protein used in the discussion and the figures were based on the native HIV-1 capsid protein.

3. Results

All analogs synthesized for each hit were assessed first with a biophysical protein stability assay, or thermal shift assay, where the effect of the compound was measured by the change in protein melting point compared to the DMSO control (ΔTm). A positive value in ΔTm indicates a stabilizing
effect on the protein and a negative value indicates a destabilizing effect. Of note, the target CA protein is in a covalently crosslinked hexameric state. Thus, the \( \Delta Tm \) values likely reflect local changes that may affect stabilization and exclude inter-hexamer effects, which are important correlates of overall capsid core stability. To simplify presentation of the data, we refer to the effects of these compounds as stabilization or destabilization of “CA hexamer.” All compounds were then screened at 20 \( \mu \)M against HIV-1 in a cell-based assay to determine antiviral activity. Compounds demonstrating significant inhibition were further tested in a dose-response fashion for antiviral EC\(_{50}\) values. All compounds were tested for cytotoxicity either by screening at 100 or 50 \( \mu \)M, or determination of CC\(_{50}\) values. The benchmark compound PF74 was resynthesized and tested in these assays (\( 1, \Delta Tm = 7.4 \) °C, EC\(_{50} = 0.61 \) \( \mu \)M, CC\(_{50} = 76 \) \( \mu \)M). The best compound (10) was also tested for liver microsomal stability. Molecular modeling was performed for a few selected compounds to help understand the SAR.

3.1. SAR of Hit 2 (\( R^1 \) and \( R^2 \))

Hit 2 showed only a weak CA hexamer stabilizing effect (\( \Delta Tm = 0.5 \) °C) with no significant antiviral activity at 20 \( \mu \)M and no cytotoxicity at 100 \( \mu \)M (Table 1). Removing the isopropyl group at \( R^1 \) (compound 3) resulted in the complete loss of the CA hexamer stabilizing effect, whereas replacing it with an indole-7-methyl (compound 4) did not yield significant differences in all three assays. When the ester was hydrolyzed to acid (compounds 5-7), only the analog with an indole-7-methyl at \( R^1 \) (compound 5) weakly stabilized CA hexamer (\( \Delta Tm = 0.8 \) °C); no such effect was observed when \( R^1 \) was a benzyl methyl thioether (compound 6) or a bulky alkyl (compound 7). Amidation of the acid with an aniline yielded more structurally elaborate analogs 8–10. Of these, the analogs with a benzyl methyl thioether (compound 8) or an indole-7-methyl (compound 9) at \( R^1 \) showed only weak CA hexamer stabilization (\( \Delta Tm = 0.5 \) °C for both), whereas a much stronger CA hexamer stabilizing effect was observed with compound 10 which bears a benzyl moiety at \( R^1 \) (\( \Delta Tm = 2.5 \) °C). More importantly, compound 10 inhibited HIV-1 with a potency (EC\(_{50} = 1.6 \) \( \mu \)M) only 2.5-fold less than PF74 (EC\(_{50} = 0.61 \) \( \mu \)M). In addition, compound 10 (CC\(_{50} > 100 \) \( \mu \)M) was less cytotoxic than PF74 (CC\(_{50} = 76 \) \( \mu \)M).

Table 1. Anti-HIV-1 activity, cytotoxicity, and CA hexamer stability profiles of 2–10 (\( R^1, R^2 \)).

| Compd | \( R^1 \) | \( R^2 \) | EC\(_{50} \) (\( \mu \)M) \(^{[a]}\) | CC\(_{50} \) (\( \mu \)M) \(^{[b]}\) | TSA \( \Delta Tm \) (°C) \(^{[c]}\) |
|-------|---------|---------|-----------------|-----------------|-----------------|
| 1 (PF74) | – | – | 0.61 ± 0.2 | 76 ± 9 | 7.4 |
| 2 | – | | >20 | >100 | 0.5 |
| 3 | H | | >20 | >100 | 0 |
| 4 | | | >20 | <100 | 0.5 |
| 5 | | | >20 | >100 | 0.8 |
| 6 | | | >20 | >100 | 0 |
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These observations strongly indicate that 1) pyridine at R5 destabilizes the CA hexamer whereas a methyl group at the para position of the phenyl ring at R7 reinforces the destabilizing effect (31 vs. 22) but does not impact the stabilizing effect was not impacted by functional group substitution at either R5 or R7. Finally, N-methyl methyl thioether (compound 3 did not show activity in either assay. Overall, SARs around R1 and R3 within this series strongly indicate that both the benzyl group at R1 and the methyl group at R3 are important for potency.

Table 1. Anti-HIV-1 activity, cytotoxicity, and CA hexamer stability profiles of 11–21 (R1, R3, and R4).

| Compd | R1 | R3 | R4 | EC50 (μM) [a] | CC50 (μM) [b] | TSA ΔTm (°C) [c] |
|-------|----|----|----|-------------|-------------|-----------------|
| 1 (PF74) | – | – | – | 0.61 ± 0.2 | 76 ± 9 | 7.4 |
| 11 | Me | H | | 6.9 ± 0.8 | >100 | 2.7 |
| 12 | H | H | | >20 | <100 | 0 |
| 13 | Me | F | | 8.0 ± 1.3 | >100 | 2.4 |
| 14 | Me | H | | >20 | >100 | 0 |
| 15 | H | H | | >20 | >100 | 0.7 |
| 16 | Me | Me | | >20 | >100 | 0 |
| 17 | H | H | | >20 | >100 | 0 |
| 18 | Me | H | | >20 | >100 | 0.5 |

[a] Concentration of compound inhibiting HIV-1 replication by 50%, expressed as the mean ± standard deviation from at least two independent experiments. [b] Concentration of compound causing 50% cell death, expressed as the mean ± standard deviation from at least two independent experiments. [c] TSA: thermal shift assay. ΔTm: change of CA crosslinked hexamer melting point in the presence of compound minus DMSO control.

3.2. SAR of Hit 11 (R1, R3, and R4)

Hit 11 was designed from hit 2 and PF74 via molecular hybridization. As a result, the skeleton of 11 is very similar to that of PF74, except that the indole moiety was replaced with a bioisosteric quinazoline-2, 4-dione moiety. A general SAR observation was that when R1 was a bulky alkyl group, compounds (14–21) did not show significant antiviral activity at 20 μM or cytotoxicity at 100 μM (Table 2), though most analogs weakly stabilized CA hexamer (ΔTm = 0.5–0.7 °C). However, when R1 was a benzyl group to mimic PF74, the activity profiles changed substantially (analogs 11–13) and demonstrated a strong dependence on R3. When R3 was a methyl group, analog 11 (ΔTm = 2.7 °C, EC50 = 6.9 μM) and analog 13 (ΔTm = 2.4 °C, EC50 = 8.0 μM) both moderately stabilized CA hexamer and inhibited HIV-1, without observed cytotoxicity (CC50 >100 μM). By contrast, when R3 was a hydrogen, the resulting analog (12) did not show activity in either assay. Overall, SARs around R1 and R3 within this series strongly indicate that both the benzyl group at R1 and the methyl group at R3 are important for potency.
Table 2. Cont.

| Compd | R₁ | R₃ | R₄ | EC₅₀ (μM) | CC₅₀ (μM) | TSA ΔTm (°C) |
|-------|----|----|----|-----------|-----------|--------------|
| 19    |    | H  | H  | >20       | >100      | 0.6          |
| 20    |    | Me | Cl | >20       | >100      | 0.5          |
| 21    |    | H  | H  | >20       | >100      | 0.6          |

* Concentration of compound inhibiting HIV-1 replication by 50%, expressed as the mean ± standard deviation from at least two independent experiments. b Concentration of compound causing 50% cell death, expressed as the mean ± standard deviation from at least two independent experiments. c TSA: thermal shift assay. ΔTm: change of CA crosslinked hexamer melting point in the presence of compound minus DMSO control.

3.3. SAR of Hit 22 (R⁵, R⁶, and R⁷)

Compound 22 is structurally quite different from PF74 and the other two hits (2 and 11), though it satisfied multiple pharmacophore points (Figure 2A), and hence, could be similar to PF74 in 3D shape. In addition, the 1,2,4-trazole is a well-known bioisostere [55] of carboxamide, which renders 22 functionally similar to PF74. The SARs of this series were centered around the two aromatic rings (R⁵ and R⁷). The most prominent observation was that analogs within this series did not significantly inhibit HIV-1 at 20 μM, and did not show cytotoxicity at 100 μM (Table 3). However, with the exception of 23, 32, and 33, all analogs demonstrated an impact on CA hexamer stability, which essentially validates our hit generation approach as the shape similarity search is target-based. Very interestingly, a weak CA hexamer destabilizing effect, rather than stabilizing effect, was observed with hit 22 (ΔTm = −0.5 °C). A similar destabilizing effect was also observed with the only other analog featuring a pyridine ring at R⁵ (compound 31), though the effect was significantly more prominent (ΔTm = −1.2 °C). All other analogs feature either a phenyl or a biphenyl ring at R⁵, and in contrast, produced CA hexamer stabilizing effect (ΔTm = 0.5–0.9 °C). This weak stabilizing effect was not impacted by functional group substitution at either R⁵ or R⁷. Finally, N-methyl substitution of analog 23 (ΔTm = 0 °C) yielded a compound 39 stabilizing CA hexamer (ΔTm = 1.2 °C). These observations strongly indicate that (1) pyridine at R⁵ destabilizes the CA hexamer whereas a phenyl or biphenyl moiety at R⁵ stabilizes the CA hexamer; (2) a methyl group at the para position of the phenyl ring at R⁷ reinforces the destabilizing effect (31 vs. 22) but does not impact the stabilizing effect (28 vs. 24, 30 vs. 36); and (3) a methyl group at R⁶ confers a CA hexamer stabilizing effect (39 vs. 23).

Table 3. Anti-HIV-1 activity, cytotoxicity, and CA hexamer stability profiles of 22–39 (R⁵, R⁶, and R⁷).

| Compd | R⁵ | R⁶ | R⁷ | EC₅₀ (μM) | CC₅₀ (μM) | TSA ΔTm (°C) |
|-------|----|----|----|-----------|-----------|--------------|
| 22    |    | H  | H  | >20       | >100      | −0.5         |
| 23    |    | H  | H  | >20       | >100      | 0            |
| 24    |    | H  | H  | >20       | >100      | 0.7          |
| 25    |    | H  | H  | >20       | >100      | 0.6          |
Metabolic stability is a major absorption, distribution, metabolism and excretion (ADME) property.

### 3.4. Metabolic Stability

To characterize the drug-like property of 10, our most potent antiviral compound from the current work, we conducted metabolic stability assays in both HLMs and mouse liver microsomes (MLMs). Metabolic stability is a major absorption, distribution, metabolism and excretion (ADME) property that profoundly impacts drug bioavailability [56]. Peptidomimetics are particularly susceptible to phase I metabolism, presumably because they are good substrates [57] for liver metabolizing enzyme subfamily cytochrome P450 3A (CYP3A), which is responsible for the metabolism of at least 50% of all current drugs [58]. It is known that PF74 is a severely flawed antiviral lead due to its prohibitively low metabolic stability [34]. This was confirmed in our metabolic stability assays, where the half-life (t1/2) of PF74 is less than 1 min in both HLMs and MLMs (Table 4). By contrast, our compound 10 was decisively more stable, particularly in HLMs where its half-life (t1/2 = 31 min) was 44-fold longer than

| Compd | R3 | R6 | R7 | EC50 (μM)[a] | CC50 (μM)[b] | TSA ΔTm (°C)[c] |
|-------|----|----|----|--------------|--------------|----------------|
| 26    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.7            |
| 27    | ![CN](image) | H  | ![CN](image) | >20          | ~100         | 0.7            |
| 28    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.7            |
| 29    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.7            |
| 30    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.8            |
| 31    | ![CN](image) | H  | ![CN](image) | >20          | >100         | -1.2           |
| 32    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0              |
| 33    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0              |
| 34    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.6            |
| 35    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.5            |
| 36    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.8            |
| 37    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 1.0            |
| 38    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.9            |
| 39    | ![CN](image) | Me | ![CN](image) | >20          | >50          | 1.2            |

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[a] Concentration of compound inhibiting HIV-1 replication by 50%, expressed as the mean ± standard deviation from at least two independent experiments.
[b] Concentration of compound causing 50% cell death, expressed as the mean ± standard deviation from at least two independent experiments.
[c] TSA: thermal shift assay. ΔTm: change of CA crosslinked hexamer melting point in the presence of compound minus DMSO control.

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### Table 3. Cont.

| Compd | R3   | R6 | R7 | EC50 (μM) [a] | CC50 (μM) [b] | TSA ΔTm (°C) [c] |
|-------|------|----|----|--------------|--------------|----------------|
| 36    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.8            |
| 37    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 1.0            |
| 38    | ![CN](image) | H  | ![CN](image) | >20          | >100         | 0.9            |
| 39    | ![CN](image) | Me | ![CN](image) | >20          | >50          | 1.2            |
that of PF74 ($t_{1/2} = 0.7$ min). When tested in combination with a CYP3A inhibitor Cobicistat (Cobi) [59], 10 still exhibited significantly longer half-life than that of PF74. A $t_{1/2} >30$ min in HLM generally indicates good in vivo metabolic stability and oral bioavailability. Collectively, these observations support our compound 10 as a viable antiviral hit, and corroborate the hypothesis that the poor metabolic stability of PF74 is due to CYP3A-mediated phase I metabolism.

### Table 4. Phase I metabolic stability in liver microsomes $t_{1/2}$ (min).

| Compound | HLM $^a$ | HLM $^a$ (+Cobi $^c$) | MLM $^b$ | MLM $^b$ (+Cobi $^c$) |
|----------|----------|----------------------|----------|----------------------|
| PF74     | 0.7      | 91                   | 0.6      | 34                   |
| 10       | 31       | >120                 | 2.9      | 85                   |
| Verapamil| 15       | –                    | 4.2      | –                    |

$^a$ HLM: human liver microsome; $^b$ MLM: mouse liver microsome; $^c$ Microsomal stability measured in the presence of CYP3A inhibitor Cobi.

### 3.5. Molecular Modeling

To understand some of the aforementioned SAR, we performed molecular modeling with selected compounds based on the co-crystal structure of native HIV-1 capsid protein bound to PF74 (PDB code: 4XFZ [25]). Compound 11 (Figure 3A) bearing a quinazoline-2,4(1H,3H)-dione core in place of the indole ring of PF74 interacted with the same key residues in the CA hexamer as PF74. Observed key interactions included (i) hydrogen-bonding between N57 and the NH and carbonyl groups of the phenylalanine fragment on compound 11, between K70 and the carbonyl next to the phenylalanine fragment of 11, and between Q63 and the free NH of the quinazoline-2,4(1H,3H)-dione core of 11; (ii) cation-π interactions between protonated K70 and quinazoline-2,4(1H,3H)-dione aromatic ring. However, despite sharing the same binding site and similar key interactions, compound 11 was significantly less potent than PF74. In the meantime, a complete loss in potency was observed for leucine derived analog 14, which could be attributed to its shifting away from the binding site (circled), possibly in a binding mode shown in Figure 3B, and hence the loss of key interactions. This observation signified the importance of the phenylalanine core of these compounds for potency. In particular, the benzyl group of the phenylalanine core could play an important role in keeping a molecule inside the PF74-bound cavity through hydrophobic interactions [25] with surrounding residues L56, M66, and L69, as evident from the observed binding mode for compound 11 (Figure 3A). This was consistent with the significant increase in the potency of compound 10 as well, which bears a benzyl group and an additional L-proline core. Like the PF74 backbone, the benzyl group of the phenylalanine fragment forces compound 10 to nestle in the cavity in such a way that the aniline ring of the L-proline core is extended to the adjacent CA$_{NTD}$ domain, resulting in an additional hydrogen-bonding between the carbonyl of the L-proline and NH$_2$ of N53, along with the typical H-bonding with N57, Q63, and K70 (Figure 3C). Additionally, pyrrolidine core of L-proline is oriented in such a way that it has maximum interaction with A105, T107, and Y130. Expectedly, introduction of an L-cysteine derived core having an elongated backbone in place of phenylalanine in compound 8 forces the entire molecule out of the cavity, diminishing it potency (Figure 3D). Compound 8 was found to be closer to the adjacent CA$_{CTD}$ and CA$_{NTD}$ domains, interacting with P34, R173, Q179, and K182 through H-bonding.
Figure 3. Docking poses of key ligands based on native HIV-1 capsid protein bound to PF74 (PDB code: 4XFZ [25]). (A) Predicted binding mode of 11. Glide score (kcal/mol): −6.3. (B) Predicted binding mode of 14 away from the preferred binding site (circled). Glide score (kcal/mol): −4.2. (C) Predicted binding and fitting of L-proline analog 10 within the preferred PF74 binding cavity. Glide score (kcal/mol): −6.3. (D) Predicted binding and fitting of L-cysteine derivative analog 8 away from the preferred PF74 binding site. Glide score (kcal/mol): −5.2. Ligands numbers 8, 10, 11, and 14 are in pink. In Figure A and D, H-bond and cation-π interactions are depicted as black dotted lines and double headed arrow, respectively. In (A) and (B), the capsid protein chain is colored cyan and key residues around binding site are colored olive. In (C) and (D), CANTD around helices H3 and H4 in chain A are colored cyan, and CACTD around helices H8 and H9 in the adjacent chain B are colored green. The nitrogen, oxygen, and sulfur atoms are colored blue, red, and yellow, respectively.

4. Discussion

Despite the approval of many HIV-1 antiviral regimens [60], a curative therapy remains elusive and HIV-1 continues to pose a global healthcare challenge. There is a need to develop new classes of HIV-1 drugs with distinct mechanisms of action to manage HIV-1 strains resistant to current drugs. The multifunctional HIV-1 CA represents an attractive target for novel antiviral discovery. PF74 is a CA-targeting small molecule which binds to a unique pocket between a viral CANTD and the adjacent CACTD, and competes against a few host factors important for viral replication. The antiviral profile of PF74 and its mode of CA binding are well characterized. However, PF74 is not a viable antiviral lead as it suffers from extremely low metabolic stability. Aiming to identify mechanistically similar yet structurally distinct small molecules with improved metabolic stability, we performed a pharmacophore-based shape similarity search based on PF74. Subsequently, we conducted analog synthesis and SAR for two hits (2 and 22) generated from the shape similarity search, as well as a third hit (11) designed via molecular hybridization. Overall, most of analogs exhibited weak yet discernible...
impacts on the stability of CA hexamer, which largely validates our hit generation approach. Three of the analogs (10, 11, and 13) showed moderate CA hexamer stabilizing effects and significant anti-HIV-1 activities. Particularly, compound 10 inhibited HIV-1 with an EC\textsubscript{50} of 1.6 µM, which is only 2.5-fold less potent than PF74. More importantly, compound 10 demonstrated drastically improved metabolic stability over PF74 in HLMs (1/2 = 31 min for 10 vs. 0.7 min for PF74). Molecular modeling indicates that our compound 10 binds comfortably in the PF74 binding pocket. Collectively, our data support 10 as a potent and metabolically stable HIV-1 CA-targeting antiviral lead.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/4/452/s1, Scheme S1: Synthesis of intermediates b–d, and compounds 2–21, Scheme S2: Synthesis of intermediates f–g and compounds 22–39.

Author Contributions: S.G.S. and Z.W. conceptualized the research. S.K.V.V., R.L.S., and L.W. designed, synthesized and characterized all compounds. J.K. performed the shape similarity search. R.L.S. conducted molecular docking. J.X. performed the metabolic stability assays. M.C.C., K.A.K., H.D., H.Z., and P.R.T. performed T.S.A., antiviral and cytotoxicity assays. R.L.S. and Z.W. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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