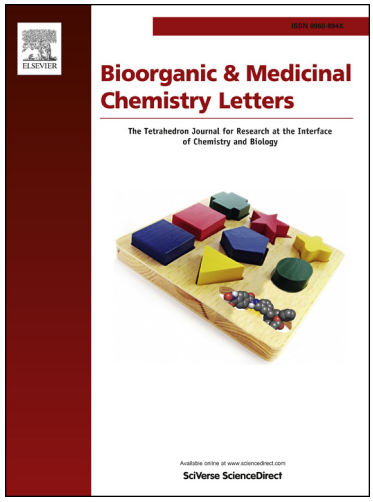

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Identification of a small molecule HIV-1 inhibitor that targets the capsid hexamer

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Abstract

The HIV-1 CA protein is an attractive therapeutic target for the development of new antivirals. An inter-protomer pocket within the hexamer configuration of the CA, which is a binding site for key host dependency factors, is an especially appealing region for small molecule targeting. Using a field-based pharmacophore derived from an inhibitor known to interact with this region, coupled to biochemical and biological assessment, we have identified a new compound that inhibits HIV-1 infection and that targets the assembled CA hexamer.

Keywords: computer-aided drug design; field-based virtual screening; HIV-1 capsid protein; antiviral; surface plasmon resonance

Combination anti-HIV therapy, commonly referred to as highly active antiretroviral therapy (HAART), has led to a dramatic reduction in mortality and morbidity in patients infected with HIV. More than 25 antiretroviral drugs are currently available to treat HIV infection. These therapies are directed against the viral enzymes (reverse transcriptase, viral protease, and integrase). Drugs targeting the process of viral entry into the host cell have also been approved for clinical use. Despite these successes, current therapies for HIV-1 are limited by both the development of multidrug-resistant virus and by significant cumulative drug toxicities. Therefore, the development of new classes of antiretroviral agents with novel modes of action is highly desirable and is a driving force for the pursuit of small-molecule inhibitors of other viral targets. The HIV-1 capsid (CA) protein has recently emerged as an attractive target as it performs essential roles, both regulatory and structural.

The HIV-1 CA performs essential roles both early and late in the life cycle of HIV. The capsid is initially translated as the central region of the Gag polyprotein. As the virus buds, Gag is processed by the viral protease to produce three discrete new proteins—MA, CA, and nucleocapsid (NC)—as well as several smaller spacer peptides. After the capsid has been liberated by proteolytic processing, it rearranges into the conical core structure that surrounds the viral genome at the center of the mature virus.

The HIV-1 capsid shell is composed of about 250 CA hexamers and 12 CA pentamers, comprising about 1500 monomeric CA proteins in all. The multimers interact noncovalently to form the shell's curved surface. CA itself is composed of two domains: the N-terminal domain (CA_{NTD}) and the C-terminal domain (CA_{CTD}). Several structures of the CA have been solved;[1-7] with the most informative being those of disulfide engineered hexameric structure[8, 9] and the very recent native hexameric structure.[10]

To date, the CA protein structure and stability have been demonstrated to be critical for the processes of uncoating, reverse transcription, nuclear entry, selection of the sites of integration, and assembly. Moreover, it is also important for cloaking the DNA product from intracellular immune surveillance. To achieve these functions, HIV-1 CA interacts not only with itself but with host factors including TRIM5α, cleavage and polyadenylation specific factor 6 (CPSF6), nucleoporins 153 and 358 (NUP153, NUP358), MxB, and Cyclophilin A (CypA).

Several chemotypes have been found by several groups, including our own that interact with distinct pockets on the CA protein, each inhibiting by different mechanisms. [11-23] The most widely studied of these compounds, PF-3450074 (abbreviated to PF-74),[24] targets a pocket that is also the binding site for the host cell factors NUP153 and CPSF6.[25] Initial structural studies indicated interactions solely with the monomer[24] but recent investigations have shown that PF-74 makes contacts between two adjacent protomers within the hexamer.[10, 25, 26]

We believe that this inter-protomer pocket, which also serves as an interaction site for host cell factors, is the most attractive site on HIV-1 CA for small molecule targeting. The identification of PF-74, with its submicromolar affinity to this region, supports this assertion. However, PF-74 itself is a poor drug-like chemotype, having a very peptidic nature. Therefore, in this study we sought to identify new compounds that interact in this region, inhibit HIV-1 replication (preferentially at an early post-entry stage) but are less peptide-like and more chemically tractable. Therefore, as a first step towards finding new chemical entities that function as inhibitors of the HIV-1 capsid protein, we employed Field-based 3D similarity searching using Blaze (Cresset, UK) and a high-content pharmacophore[27] based on the crystal structure of PF-74 bound to the CA-NTD (PDB ID 2XDE; **Figure 1**). We have previously used this field-based pharmacophore screening approach successfully to identify new HIV-1 inhibitors.[28]

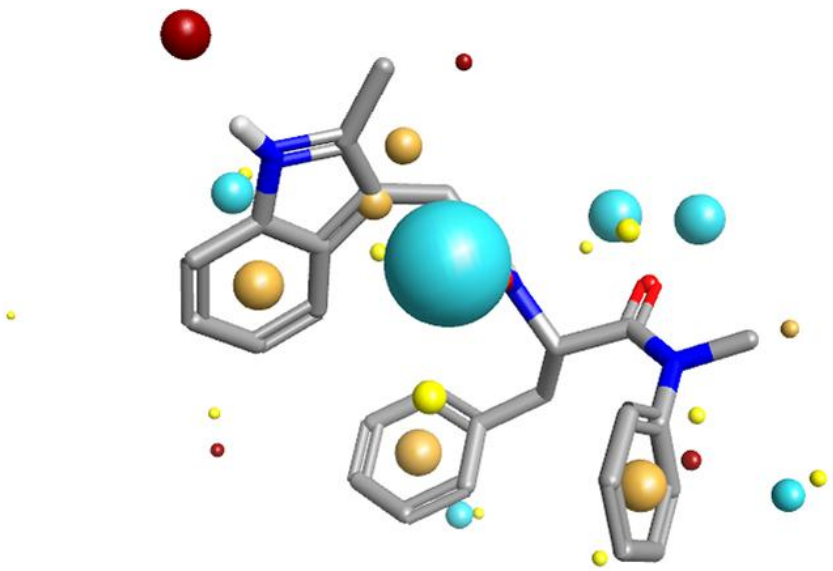


Figure 1: Calculated field point pattern of bioactive conformation of compound PF74 extracted from PDB 2XDE and used for the in silico screen. Blue field points indicate areas where the negative electrostatics are at local maxima; red field points correspond to areas where positive electrostatics are at local maxima. Yellow field points (electrostatically neutral) represent areas where there is high accessible van der Waals surface area; and orange field points represent hydrophobic centroids (areas of high carbon density, centers of rings, near halogens). Oxygen atoms are shown in red, nitrogen in blue. The size of the field points scales with the value of the field at that point in space.

The field point pattern[29] from the conformation of PF-74 in 2XDE was used to query a database of the 100 lowest-energy conformations of approximately 6 million commercially available compounds (600 million comparisons) using Blaze (Cresset, UK).[28, 30] 1000 top scoring (50% field / 50% shape) results from the Blaze field/shape-based virtual screening procedure[28, 30] were compared on score and visually to the field point pattern of PF-74. Fifty-four compounds were chosen from this list and purchased for biological testing using the single-round infection assay.[28] The results of this analysis are shown in **Figure 2**.

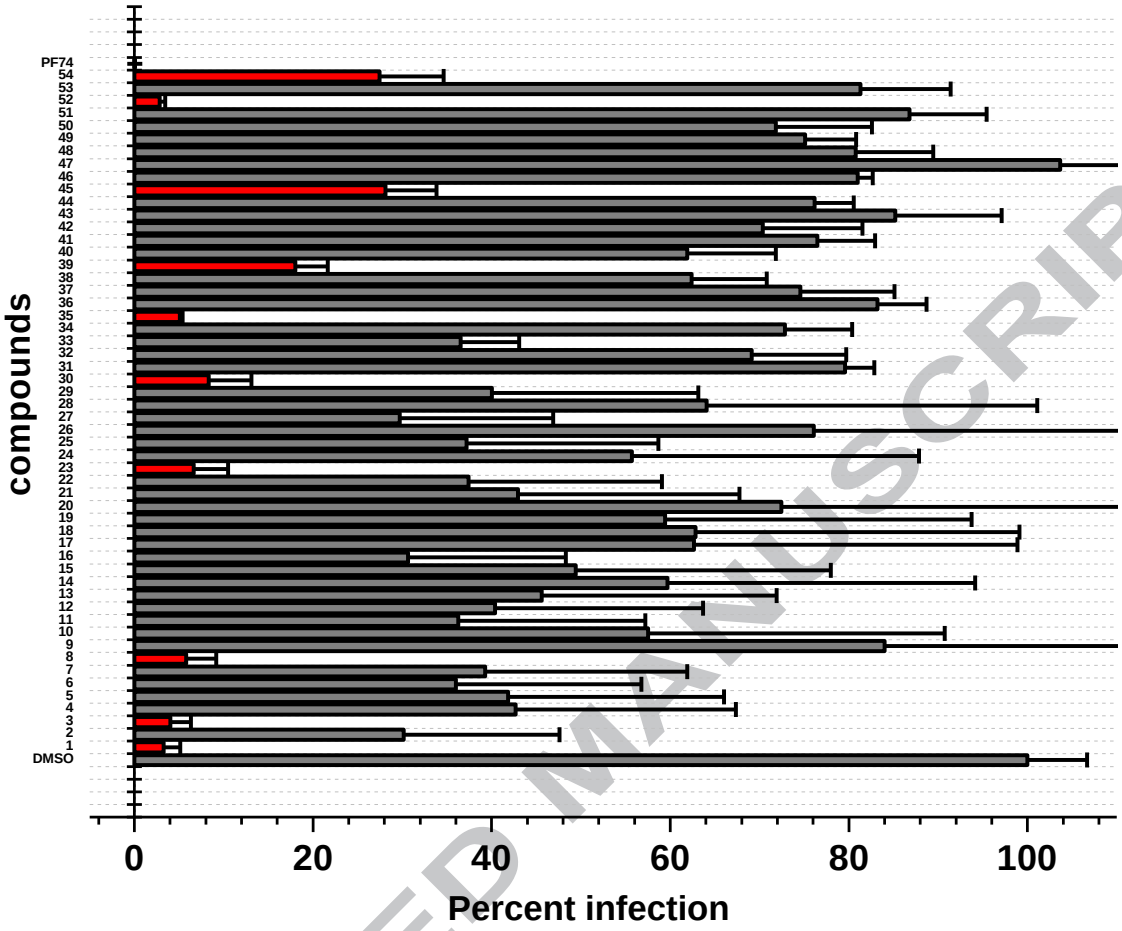


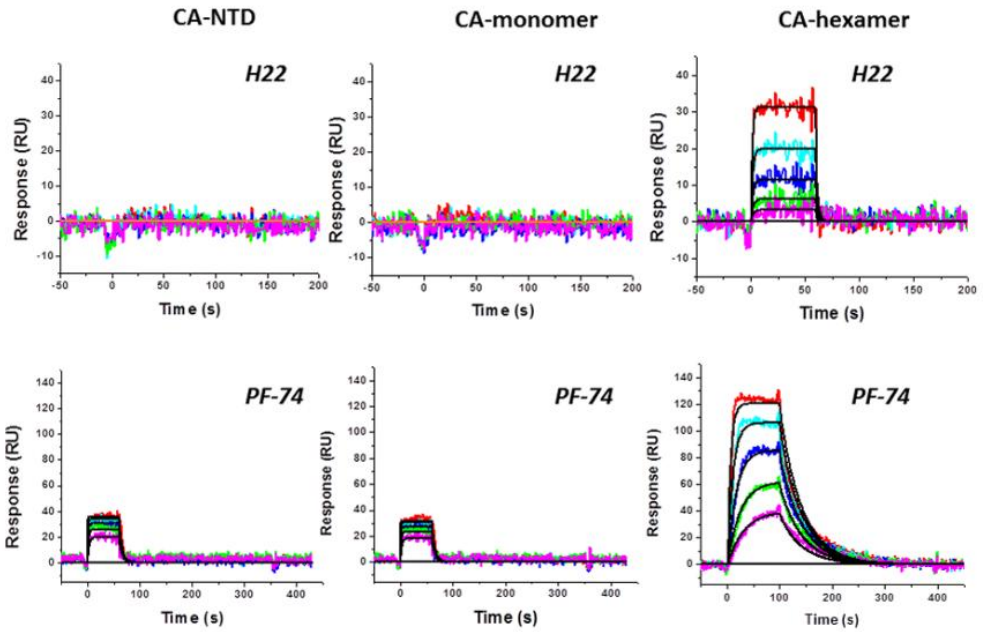
Figure 2: Single concentration (100 µM) screen of compounds identified from the Blaze procedure for activity against the infection of HIV-1. Red highlighted compounds denote those compounds that were chosen for target interaction analysis by SPR.

As can be seen in **Figure 2**, a number of compounds were identified that demonstrated modest activity. Therefore, those compounds with activity were then screened for a direct interaction with variants of the CA protein, specifically the CA NTD, monomeric CA, and hexameric CA, using surface plasmon resonance (SPR) as previously reported.[18, 23] From this analysis **compound 52** (designated **H22**) was demonstrated to interact solely with the hexameric CA (**Figure 3A**). The affinity of H22 derived from this analysis was 382±105 µM, which seemed to

us to be larger than what we predicted from the single concentration analysis. Therefore, we determined the affinity of PF74 using SPR and under the same conditions, and derived an affinity of 2.53±1.02 μM This is approximately 10-fold higher than the solution affinity obtained and published by Price et al.[25] and Bhattacharya et al.[26] using isothermal titration calorimetry. This may indicate that the inter-protomer pocket to which PF74 binds, and that we believe **H22** binds to also, maybe slightly deformed due to covalent attachment to the sensor chip. Applying the 10-fold discrepancy to the K_D of **H22** would yield an equilibrium dissociation constant of 38.2 μM which more accurately reflects the level of inhibition seen in the single concentration antiviral screen.

Having shown that **H22** directly interacts with the hexameric HIV-1 CA, we next sought to assess whether it affects the assembly of CA in a similar manner to PF74 and hence indicate that they share a common binding site. Soluble HIV-1 CA can be triggered to assemble into tubes similar in diameter and morphology to intact cores by dilution into high-ionic-strength buffer. The kinetics of assembly can be followed by monitoring the increase in turbidity using a spectrophotometer.[23] We therefore performed the assembly assay in the presence of **H22**, PF74 or DMSO control. As shown in **Figure 3B**, compound H22 accelerated the assembly of the CA *in vitro*, just as PF74, hinting towards a shared binding site and mechanism.

(A)



(B)

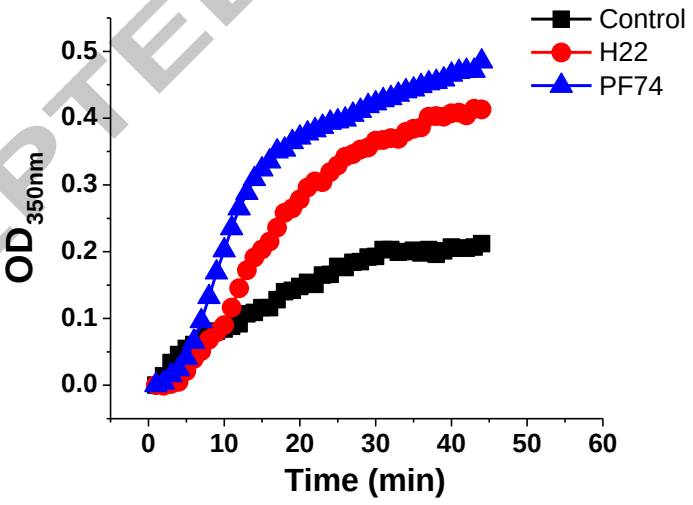


Figure 3: (A) Sensorgrams showing the interaction, or lack thereof, of H22 and PF74 with three different CA protein constructs. Colored lines represent the actual data, whereas black lines represent the fits to a simple 1:1 Langmuir binding isotherm model. **(B) Effect of H22 on assembly of HIV-1 CA *in vitro***. CA assembly as monitored by an increase in turbidity using a spectrophotometer at 350 nm. CA

was used at a final concentration of 30 µM, and H22 and PF74 were used at a final concentration of 100 µM.

We next sought to determine **H22**'s antiviral potency (half-maximal inhibitory concentration, IC_{50}) using the single-round infection assay in addition to its half-maximal cellular cytotoxicity (CC_{50}) value, using a water-soluble tetrazolium salt-based dual cell counting/cell toxicity kit (Dojindo Molecular Technologies, Inc.; data not shown). The inhibition profile of **H22** is shown in **Figure 4**. **H22** inhibits the infection of U87.CD4.CCR5 cells by HIV-1_{NL4-3} pseudotyped with the JR-FL envelope with an IC_{50} of 18.1 ± 2.4 µM. This value is close to the covalent attachment discrepancy corrected K_D. This inhibition is due to direct effects on the virus and not nonspecific toxicity as the **H22** half-maximal cell cytotoxic concentration (CC_{50}) for the U87.CD4.CCR5 cells was determined to be 526 ± 56 µM, giving **H22** a therapeutic index (TI = CC_{50}/IC_{50}) of > 29.

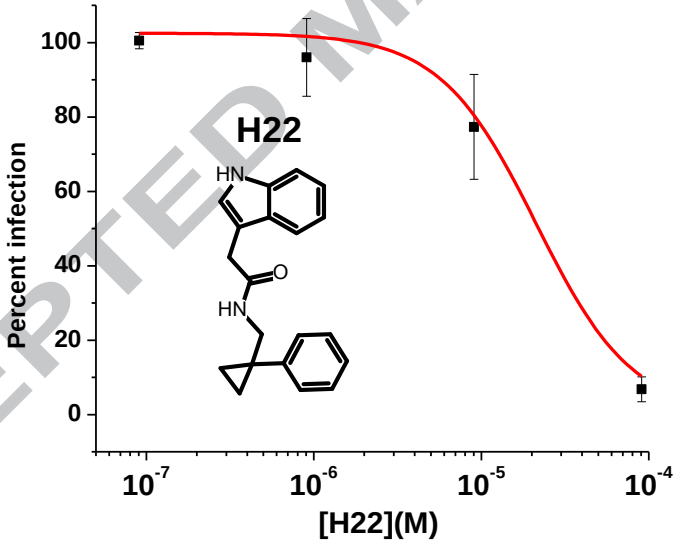


Figure 4: Effect of H22 on the infection of U87.CD4.CCR5 cells by HIV-1 single-round infective virus. H22 IC_{50} = 18.1 ± 2.4 µM. The structure of H22 is shown inset. The H22 half-maximal cell cytotoxic concentration (CC_{50}) for the U87.CD4.CCR5 cells was determined to be 526 ± 56 µM, giving H22 a therapeutic index (TI = CC_{50}/IC_{50}) of > 29.

H22 was identified based upon an in silico screen using the PF-74 pharmacophore, and as such should interact in the same inter-protomer binding pocket as PF-74. As mentioned previously, this pocket is the binding site for two host cell proteins, CPSF6 and NUP153.[25] As such, if **H22** interacts within this pocket it should inhibit the interaction of peptides derived from CPSF6 and NUP153. Therefore, we synthesized two peptides $CPSF6_{308–327}$ (DRPPPPVLFPGQPFGQPPLG) and a scrambled version of this peptide, both biotinylated at the N-terminus to allow capture on a streptavidin coated sensor chip. We also synthesized a non-biotinylated version of the CPSF6 peptide ($CPSF6_{313–327}$) as a control. Both CPSF6 peptides have been previously demonstrated to interact with hexameric CA.[25] We then implemented an SPR-based competition assay. CA hexamer at a concentration of 5μM, either alone or in combination with high concentrations of non-biotinylated peptide or compounds H22 or PF-74, were then passed over the experimental and control surface and the response recorded. As can be seen in **Figure 5**, the non-biotinylated $CPSF6_{313–327}$ drastically inhibited the interaction of the hexamer with the surface immobilized $CPSF6_{308–327.}$ In agreement with previously published results using ITC, PF-74 also inhibited, to a degree, the CA hexamer – CPSF6 peptide interaction.[25] Importantly, our newly discovered compound H22, also inhibited the CA hexamer – CPSF6 peptide interaction, indicating that it is binding in the inter-protomer

pocket as it was designed.

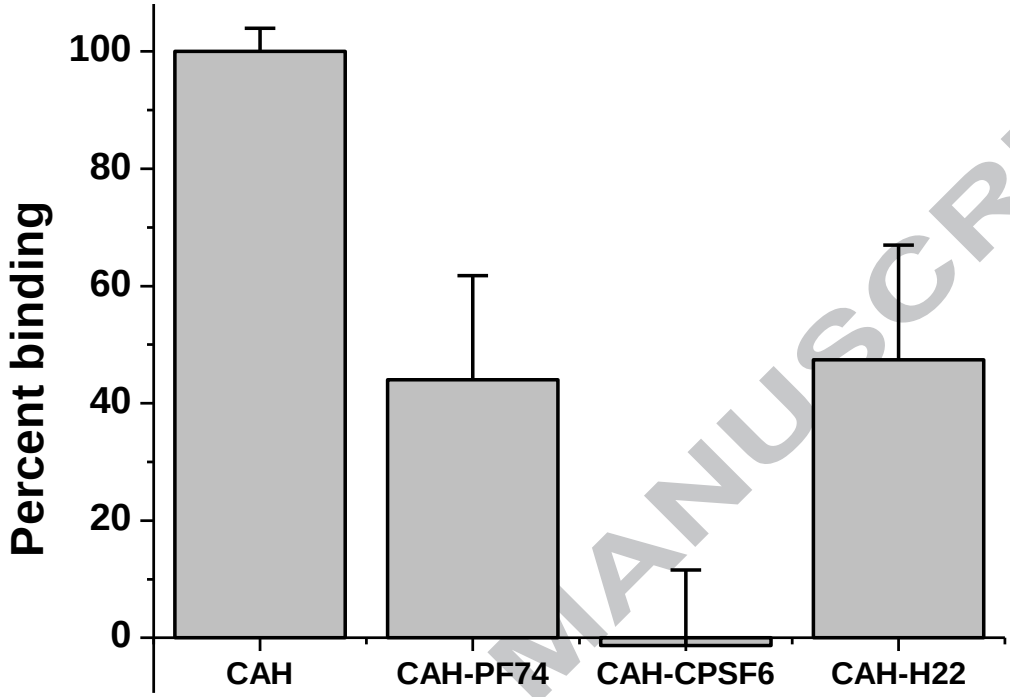


Figure 5: Effect of CPSF6$_{313-327}$, PF74, and H22 on the interaction between hexameric CA with sensor chip immobilized biotinylated CPSF6$_{308-327}$. CPSF6$_{313-327}$ was used at a concentration of 129µM (approximately 10-fold K_D), PF74 at 2.5µM (approximately 20-fold K_D), and H22 at 300µM (approximately 10-fold K_D) and a final concentration of 5µM CA hexamer (CAH).

Having demonstrated an interaction with the CA hexamer, an effect on CA assembly, and competition with a peptide derived from CPSF6, we sought to investigate a binding site model that could explain these results using molecular docking. Therefore, we performed molecular docking experiments with a dimeric unit taken from the recently described structure of the CA hexamer[10] using AutoDock Vina (through the web-based interface DockingServer; www.dockingserver.com). Binding poses were assessed for plausibility in light of the

experimental data. This analysis has revealed a plausible docking pose that could explain its interaction with only the CA hexamer, in addition to its effect upon CA assembly *in vitro*. This is shown in **Figure 6** and predicts hydrogen bond formation with amino acids Y169, N57, K70, R173, and Q63, and other interactions with L172, L56, L69, I73, K182, M66, and Y130 across the two protomers subunits. These residues are also involved in the interactions of CA with PF-74,[25] providing an explanation for our compounds similar effect upon assembly as PF74, and are also involved in interactions with CPSF6, providing explanation for the competition experiments.

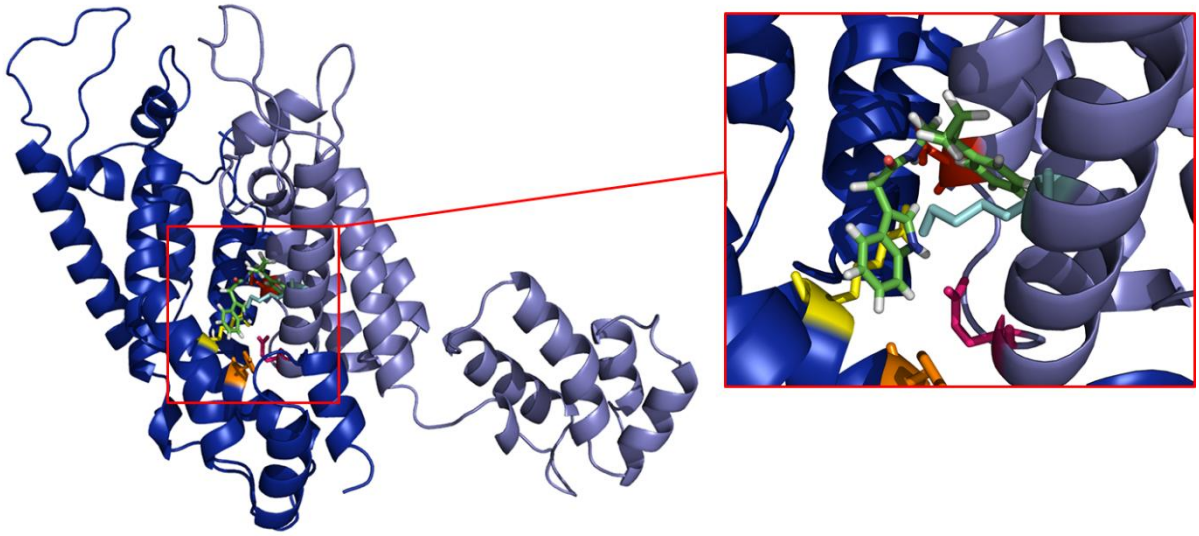


Figure 6: Proposed docking pose of H22 in the functionally relevant pocket between CA protomers in the hexameric configuration. Potential interacting residues are highlighted as follows: N57 = red; Q63 = hot pink; K70 = cyan; Y169 = orange; R173 = yellow.

In summary, we have used Field-based *in silico* screening to identify a new compound that inhibits HIV-1 infection through interaction with HIV-1 CA. The effect of this compound on CA assembly and upon CPSF6 interactions, in addition to the manner in which it was identified,

suggests that it shares a binding site with PF74 within a conserved inter-protomer pocket. A plausible binding model was proposed and work is ongoing in our group to substantiate this assertion, in addition to increasing the potency of this hit compound.

Acknowledgments

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