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Non-peptide entry inhibitors of HIV-1 that target the gp41 coiled coil pocket

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ABSTRACT

The ectodomain of HIV-1 gp41 mediates the fusion of viral and host cellular membranes. The peptide-based drug Enfuvirtide[1] is precedent that antagonists of this fusion activity may act as anti HIV-agents. Here, NMR screening was used to discover non-peptide leads against this target and resulted in the discovery of a new benzamide **1** series. This series is non-peptide, low molecular weight, and analogs have activity in a cell fusion assay with EC50 values ranging 3–41 μM. Structural work on the gp41/benzamide **1** complex was determined by NMR spectroscopy using a designed model peptide system that mimics an open pocket of the fusogenic form of the protein.

The entry of HIV-1 into host cells requires the fusion of viral and cellular membranes.[2–6] The process is mediated by an envelope glycoprotein, gp41. The ectodomain of gp41 is a trimer, with its N-terminal helices forming a central parallel coiled coil.[7,6] The C-terminal helices of each monomer of gp41 can bind in an anti-parallel fashion into the hydrophobic grooves of the central trimeric coiled coil to form a trimer of helical hairpins that make up a six-helix bundle. Recent evidence suggests that the fusion active conformation of gp41 corresponds to this six-helix bundle.[8] In this model, fusion occurs when the C-terminal helices that are inserted into the viral membrane, are brought together with the three N-terminal helices in the host membrane to form the fusogenic six-helix bundle.[4,9,10]

Peptide mimics of the C-terminal peptide are known to block viral fusion during infection.[11,8,12] Enfuvirtide is an example of a peptide-based drug that uses this approach.[1] In principle, organic compounds that bind in this groove could also serve to block this process and may be the basis for discovery of a non-peptide drug.[1,13] We have recently reported on an engineered version of gp41 that has a N-helix/C-helix interface with a permanently unoccupied 'Trp-Trp-Ile pocket' that is suitable for ligand discovery.[14] This pocket is named for three highly conserved C-helix residues, W628, W631 and I635. This pocket has been proposed as a target for small molecule HIV-1 entry inhibitors.[15] Here, we report the use of this model gp41 protein (Protein-1) in the screening and discovery of compound **1** and determination of its bound structure. Analogs of **1** with improved affinity and more desirable physical properties were found and were used to delineate this series' SAR. The anti-HIV activity of compounds in this series were assessed using a cell assay of viral fusion and exhibited higher activity than the peptide-based benchmark inhibitor 'D-peptide' in this assay.[16,17]

A focused NMR based screen identified compound **1**, as a Protein-1 binder (Table 1). This analog is part of a chemical series previously reported to have ion-channel antagonist activity.[18] When compound **1** is titrated into a solution of Protein-1 the amide protons of G572, W571, and V570 and the methyl group resonances of L568, I573 and V570, as shown in Figure 1, are significantly perturbed. These same resonances shift when the D-peptide is added to Protein-1[14] indicating binding to the same pocket. These five amino acids are all located in the exposed coiled coil structure that Protein-1 was designed to mimic. Only resonances in this pocket were perturbed by ligand binding. Compound 2 which showed no shifts in the NMR assay has no observed (K_d > 5 mM) binding.

NMR structural studies were obtained with a twofold excess of compound **1** (Table 1) to Protein-1 monomer. An excess of compound **1** was used to increase occupancy under NMR sample

Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; 2D, two-dimensional; 3D, three-dimensional; HSQC, heteronuclear single quantum coherence.

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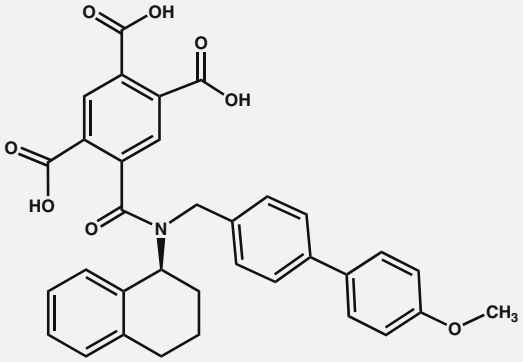
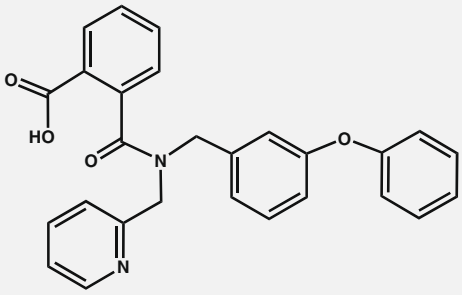
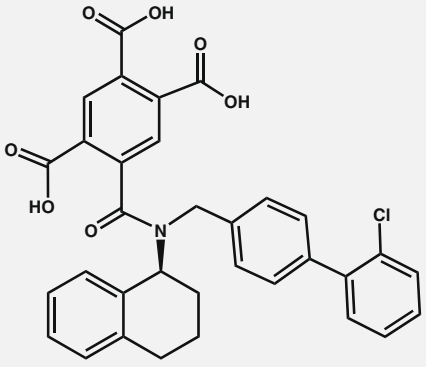
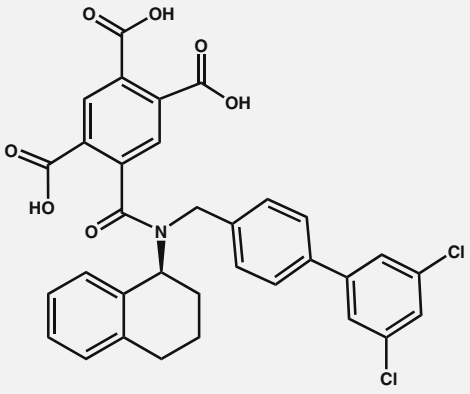
Table 1

Compd	Series	Structure	Cell–cell fusion EC_{50} (μM)
1[a]	Tri-acid		14
2	Mono-acid		Not tested (no binding by NMR)
3	Tri-acid		9
4	Tri-acid		4

(*continued on next page*)

Table 1 (*continued*)

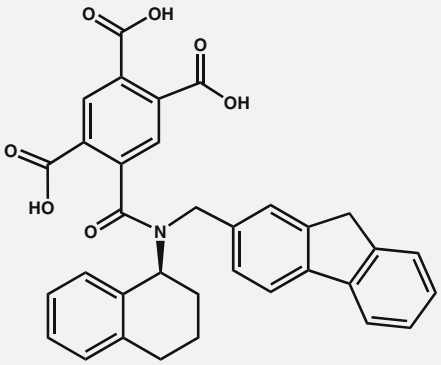
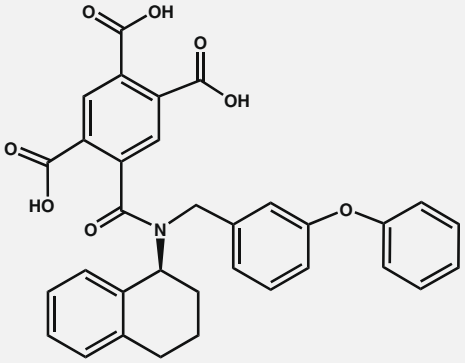
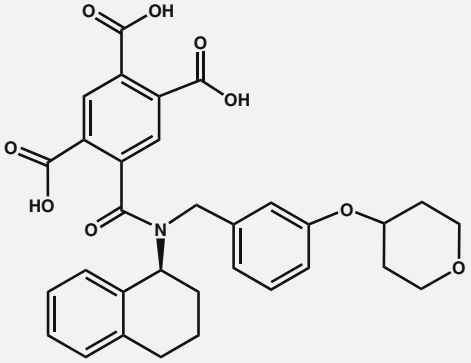
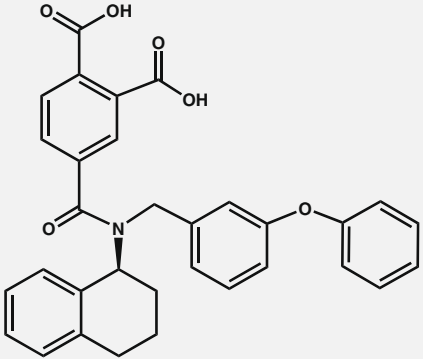
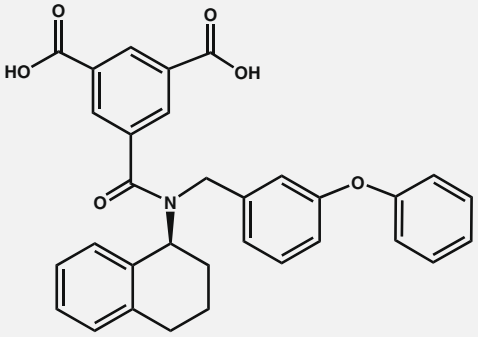
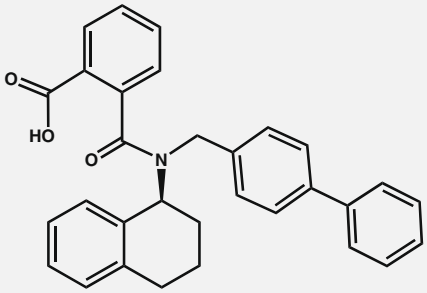
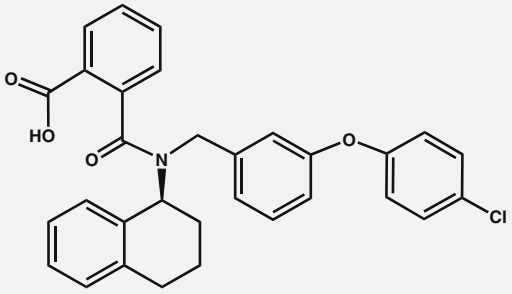
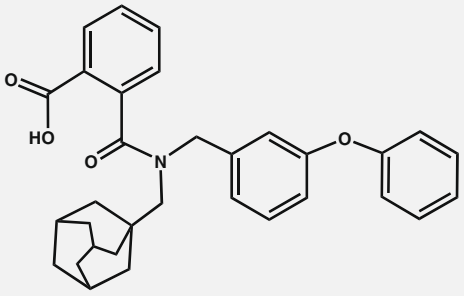
Compd	Series	Structure	Cell–cell fusion EC_{50} (μM)
5	Tri-acid		26
6	Tri-acid		13
7	Tri-acid		>250
8	Di-acid		34

Table 1 (*continued*)

Compd	Series	Structure	Cell–cell fusion EC_{50} (μM)
9	Di-acid		41
10	Mono-acid		16
11	Mono-acid		3
12	Mono-acid		7
—	C-peptide[23]	WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLEL	0.004
—	D-peptide[16]	Ac-KKGACELLGWEWAWLCAA-NH_2 (D-configuration, Cys–Cys disulfide)	19

[a] The synthesis of compounds **1** and **2** have been described previously.[25] Compounds **3**–**11** were prepared by analogous methods using anhydride ring opening or amide coupling reactions.

conditions where the K_d is ~100 μM. Under these conditions, one set of sharp peaks was observed for the protein and ligand in the complex. Our previous studies with cyclic peptide ligands suggested a binding stoichometry of 1:1 (1 ligand molecule for each peptide of the Protein-1 trimer) with equivalent ligand affinity at each site.[14] The same stoichometry likely extends to the compounds of this study. Resonances of the Protein-1 binding pocket were assigned in the complex using a combination of backbone triple resonance experiments[19], H(CC–CO)NH, (H)C(C–CO)NH and ^{13}C Tocsy/Noesy spectra.[20] The NMR data for Protein-1 showed HN–HN sequential NOEs, slow amide exchange rates and for the assigned backbone resonances angular values[21] expected for an all alpha helical protein. NOEs were also observed in the binding pocket that are consistent with cross monomer contacts found in the trimer of hairpins structure observed in the previously described X-ray structure of Protein-1.[14] The NMR results obtained

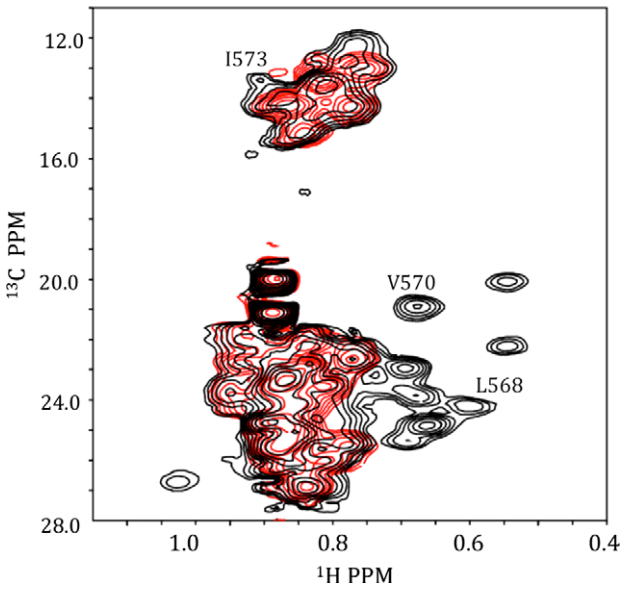


Figure 1. ^{1}H/^{13}C HSQC experiments of 0.1 mM Protein-1 in the presence of 0.6 mM ligand (black contours) and absence (red contours).

were consistent with the overall solution structure of Protein-1 being similar to what was found earlier in the apo crystal structure.[14] Based on this result we obtained a NMR based model for the complex by using a docking protocol driven by our ligand protein NOEs and using the apo X-ray crystal structure as our starting model. Sidechains were allowed to move but the backbone atoms were fixed in the docking protocol.

Ligand–protein NOEs were identified by 3D Filter Edited NOE spectra.[22] Protein resonances that had NOE contacts to compound **1** were on residues L565, L568, T569, V570, W571, G572, I573, K574, Q575, L576 and Q577 which is consistent with the compound **1** binding into the exposed hydrophobic pocket and making contacts to two adjacent monomers that form the sides of the hydrophobic pocket (see Supplementary data Fig. 1).

The ligand was docked into the X-ray structure of Protein-1 and refined using 38 protein–ligand, 3 ligand–ligand and 362 protein–protein NOE constraints to define the binding pocket. The Protein-1 backbone was kept fixed but sidechains were allowed to move in the docking calculation. In the final docked conformation there are no NOE violations greater than 0.4 . The ten lowest energy structures were submitted to a final energy minimization step. The average RMSD to the mean structure for the heavy atoms of the ligand and peptide in the pocket (Protein-1 565–577 chain A,B plus compound **1**) is 1.24 ± 0.09 for the five lowest energy structures.

In Figure 2a we show the lowest energy structure of the complex with selected sidechains from Protein-1 that have NOEs to the ligand rendered. Consistent with our titration studies the ligand is found to bind to the designed hydrophobic pocket of Protein-1. The ligand makes contacts to two adjacent monomer peptides of the Protein-1 homo trimer. Figure 2b shows a superposition of the residues W628, W631 and I635 of the native C-peptide[23] and compound **1**. Compound **1** has three substituent positions, a benzoate, a tetrahydro-napthalene and a biaryl, connected to the central amide bond. It can be seen that the *o*-methyl group and part of the attached biaryl ring of compound **1** is near the position corresponding to the indole ring of W628 in the C-peptide. The second aryl ring of the biaryl sits on top of but does not fill the pocket that W631 of the C-peptide occupies. This pocket is also not filled by W12 of the D-peptide but is instead only covered by it. The tetrahydro-napthalene aryl ring of compound **1** fills the I635 pocket but exposes much of the aliphatic ring of the group. The meta proton of the benzoate has NOEs to the aliphatic sidechain of K574. The position of K574 amine and the benzoic acids in **1** ring could not be well defined from the NMR based constraints. The low energy structure we selected for our comparison has the ortho-acid of the benzoate of compound **1**, in the vicinity of where D632 sits in the C-peptide. The acids on the phenyl group could also interact with the N-terminal amine of the peptide

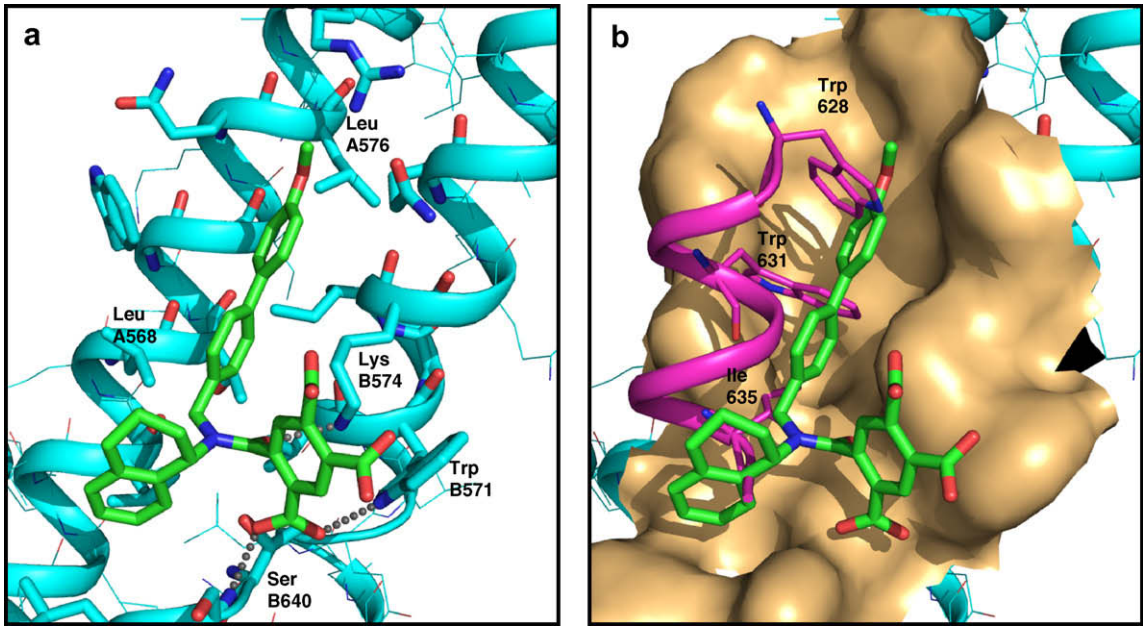


Figure 2. (a) NMR-determined binding mode of compound **1** (green) and two N-helices of Protein-1 (blue), with residues denoted Axxx and Bxxx for helix-a and helix-b, respectively. Helix-c is obscured behind helix-a and helix-b and is not shown. Three hydrogen bonds between the ligand and Lys B574 (sidechain), Trp B571 (sidechain), and Ser B640 (backbone NH) are indicated with gray dotted lines. Figure 2(b): Same orientation as in (a), but with surface of Trp-Trp-Ile binding site comprised of two N-helices shown (orange). C-helix is shown in thin lines (pink), with labeled Trp-Trp-Ile residues. Coordinates have been submitted to the protein data bank (pdb accession code 2kp8).

and/or the helix dipole of the peptide's shortened outer helix. The conformation of the inner coiled coil peptide residues that contact compound **1** are similar to what is found in the gp41 N36/C34 peptide core structure.[6]

Analogs of this initial lead were used to develop SAR for this series and selected compounds are shown in Table 1. Compounds in Table 1 show a tractable SAR typical for a series that has a common binding mode. Parent compound **1**, and its analogs were evaluated in a cell–cell fusion assay to determine their inhibitory effect on HIV-1 fusion in vitro. In this assay, HIV-1 glycoprotein-mediated fusion of Chinese CHO cells expressing HIV-1 envelope with SupT1 was monitored by the transfer of fluorescent dye.[24] Our initial lead compound, **1**, exhibited an EC_{50} value of 14 μM in this assay.

Replacing the methoxy group in **1** with more hydrophobic chloro-substituents in compounds in **3** and **4** yielded slightly more potent analogs, with values of 9 and 4 μM, respectively. Altering the aryl linkage in **1** with fluorene in analog **5** or phenoxyphenyl in analog **6** led to a slight loss of potency, 26 μM, or retention of potency, 13 μM, respectively. Replacing the hydrophobic phenyl of **6** with the polar tetrahydropyran ring of **7** gave a large loss in potency, with no anti-cell fusion activity observed up to 250 μM. We tested two di-acid analogs, **8** and **9**, which exhibited lower potency at 34 and 41 μM, respectively. Neither of these di-acids retained the carboxylate ortho to the amide linkage of **1**, which suggested an important role for the *ortho*-position acid. This hypothesis was confirmed with the mono-acid **10**, 16 μM, possessing the full potency of the initial tri-acid. The *p*-chlorophenoxyl analog in the mono-acid series, **11**, gave slightly improved potency at 3 μM. The final analog, **12**, which has an adamantyl group that replaces the phenyl ring of the tetrahydro-naphthalene of **1** shows slightly less activity at 7 μM. In summary, we determined from this analysis that a mono-acid, **11**, which is slightly more potent than the tri-acid screening lead **1**, is a viable lead compound. This compound has characteristics which are much more attractive as a lead structure for further SAR exploration. We also found small activity gains for the analogs of **1** and all showed low micromolar potency in the cell assay. Importantly, the small molecule organic inhibitor **11** is more potent than the peptide-based benchmark inhibitor 'D-peptide', which exhibits potency of 19 μM in this assay.[16,17] None of the compounds reported here are as potent as the 36-mer peptide 'C-peptide', which is a very close mimic of the natural C-terminal partner peptide for the timer of N-terminal peptides of native gp41 protein. C-peptide exhibits an EC_{50} value of 4 nM in the cell culture assay reported here.

All of the SAR reported above is consistent with the ligand binding model determined by NMR for compound **1** shown in Figure 2. The biaryl portion of **1** projects into the WW portion of the WWI-pocket (Trp-Trp-Ile pocket), and analogs with increased hydrophobic character, such as **3**, **4**, **10** or **11**, were equal or slightly improved in potency. The tri-acid portion of **1** is in position for the ortho-acid to accept hydrogen bonds from W571 and S640 of the protein, but the other two acids points to solvent. Importantly, mono-acids with this ortho-carboxylic acid group retained or improved potency, relative to **1**. The tetrahydro-napthalene also projects into a hydrophobic pocket with significant solvent exposure, and replacement by an adamantyl ring was permitted.

In this report, we identify a new chemical series for inhibition of the HIV-1 fusion process with micromolar potency. Structural studies indicate that the compounds bind to the 'Trp-Trp-Ile pocket' of a model fusogenic form of the gp41 protein. Compounds such as **11** found in this study may be amenable to additional optimization by traditional small molecule drug design efforts and lead to an alternative to peptide-based inhibitors.

Supplementary data

Supplementary data (expanded experimental methods section. Figure summarizing NOE contacts between Compound **1** and Protein-1 trimer) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.076.

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