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Structure-based design of substituted biphenyl ethylene ethers as ligands binding in the hydrophobic pocket of gp41 and blocking the helical bundle formation

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ABSTRACT

A series of substituted biphenyl ethylene ether compounds has been designed to target the gp41 N-trimer in order to inhibit formation of the six-helical bundle that represents the end state of gp41-mediated viral fusion. A size exclusion HPLC based helical bundle formation (HBF) assay was developed to evaluate in vitro inhibitory affinity of the inhibitors. The most potent compound **1** had an IC_{50} of 31 μM. The binding of compound **1** to the proposed hydrophobic pocket of gp41 was further validated by site-directed peptide mutagenesis experiments.

The process by which HIV attaches to and enters cells provides several potential targets for drug discovery.[1] HIV viral fusion is initiated by interaction between the homotrimeric viral glycoprotein gp120 and the CD4+ receptor of the host cell. This interaction is followed by a conformational change in gp120 and then an additional interaction with a cell surface chemokine coreceptor (CXCR4 or CCR5). These events result in the exposure of a transmembrane glycoprotein, namely gp41, which inserts its fusion peptide into the cell membrane and generates a pre-hairpin intermediate. It is then followed by the collapse of the HR-2 portion of gp41 onto the HR-1 region to form a coiled-coil helical bundle of hairpins, which brings the virus and host cell membrane into fusion proximity.[2] Based on this model, inhibition of the gp41 six-helix bundle formation process, the final step of HIV-1 membrane fusion, will stop the viral entry. Enfuvirtide (Fuzeon), an FDA approved peptide therapeutic that has proven effective in reducing the viral load of patients, is an example of this newly validated treatment modality. Several other peptides have been reported to inhibit viral fusion in a similar way.[3]

The X-ray crystal structure of the post-fusion gp41 six-helix bundle has been obtained using two peptides, N36 (a fragment of HR-1) and C34 (a fragment of HR-2).[4] In this structure, the inner core parallel N-trimer is surrounded by three antiparallel C-peptides (Fig. 1a). Evidence suggests that the cavity formed by the inner core trimer, which is occupied by C-peptide residues Trp628, Trp631, and Ile635, plays a crucial role in the stability of the hexamer (Fig. 1b). It has been proposed that small molecules which bind with high affinity into this pocket could inhibit six-helix bundle formation and, therefore, exhibit an antiviral effect.

Two lines of structural evidence support this fusion inhibition mechanism. HIV-1 neutralization by a gp41 fusion intermediate-directed antibody demonstrated that both binding and neutralization events depend on residues in the antibody loop region that protrude into the conserved gp41 hydrophobic pocket, as well as a large pocket in the region surrounding core gp41 residues.[5] Acyclic peptides, such as C34 and Enfuvirtide, and cyclic peptides, such as 2 K-PIE1,[6] which specifically bind to the conserved pocket have been shown to block HIV-1 entry. Extensive efforts have been made to identify small molecule compounds as gp41 inhibitors.[7,8] However, there remain significant barriers to further optimization and development of these initial lead compounds in order to overcome low potency and toxicity issues.

Using Locus' computational fragment-based discovery technology,[9] we applied a de novo ligand design process starting from the gp41 N36/C34 six-helix bundle crystal structure.[4] Briefly, C34-peptides were computationally removed from the crystal structure and binding of privileged fragments to the N36-peptide trimer core protein was calculated and rank-ordered. A high affinity binding site that centers around Lys574 (residue in green, Fig. 2) was identified from the computational fragment data. The fragment cluster wraps over the top of the lysine and down into a deep hydrophobic groove. It then continues through a narrow gap toward Arg579 (residue in red, Fig. 2).

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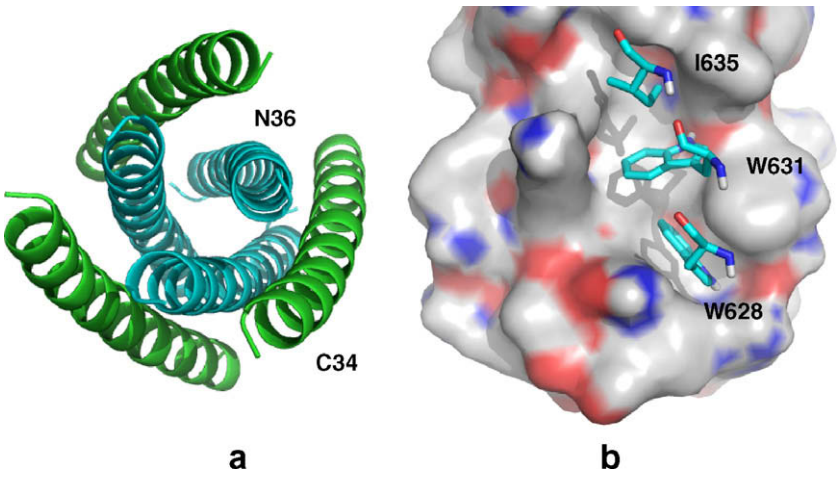


Figure 1. (a) The structure of the six-helix bundle. (b) The conserved hydrophobic binding pocket on the inner core trimer. The amino acid residues from C-peptides that occupy the pocket are also shown.

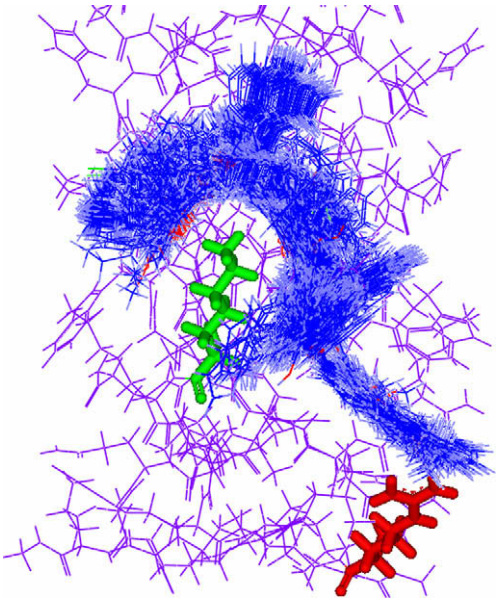


Figure 2. Predicted fragment cluster map (blue) on the N36-peptide trimer core of HIV-1 gp41 (purple) with key residues Lys574 (green) and Arg579 (red).

In the binding area near Lys574, a cluster of fragments with high affinity to the positively charged amino group has been identified. In the hydrophobic binding pocket to the right of Lys574, fragments such as substituted benzene, biphenyl and biphenyl ether showed good computed affinity. Continuing past the hydrophobic pocket, the simulation predicts a narrow groove defined by Gln577, Trp571 and Arg579, where aromatic moieties such as biphenyl ether, biphenyl, substituted benzene, naphthalene and indole, are predicted to interact. Based on this fragment map, several series of target molecules were designed and synthesized. Among them, a series of small molecules represented by compound **1** (Fig. 3) demonstrated a reasonable inhibitory effect against gp41 helical bundle formation. In addition, the interaction of this series of compounds with gp41 was further validated by a peptide mutagenesis study. Herein, we report the synthesis, possible binding mode, and biochemical and biophysical study of this series of compounds represented by compound **1**.

The synthesis of biphenyl ether compound **1** is shown in Scheme 1. Briefly, 1,4-dibromonaphthalene was first converted to

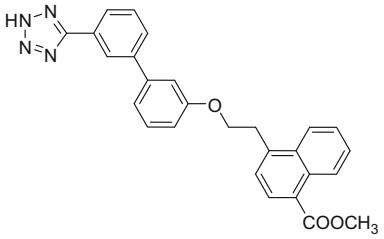
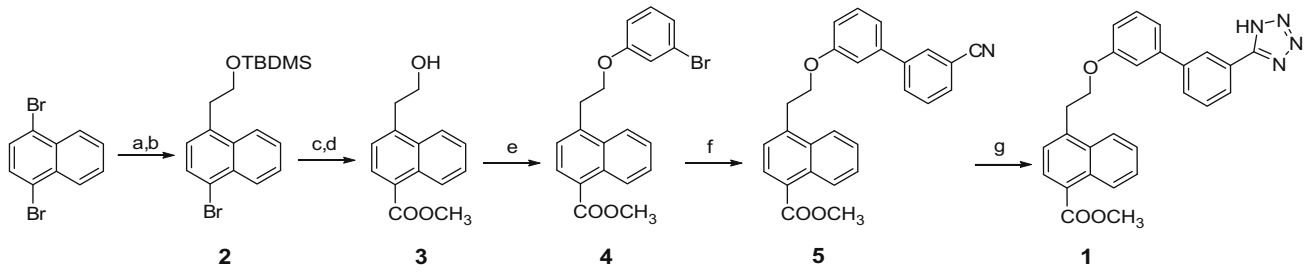


Figure 3. Structure of compound **1**.

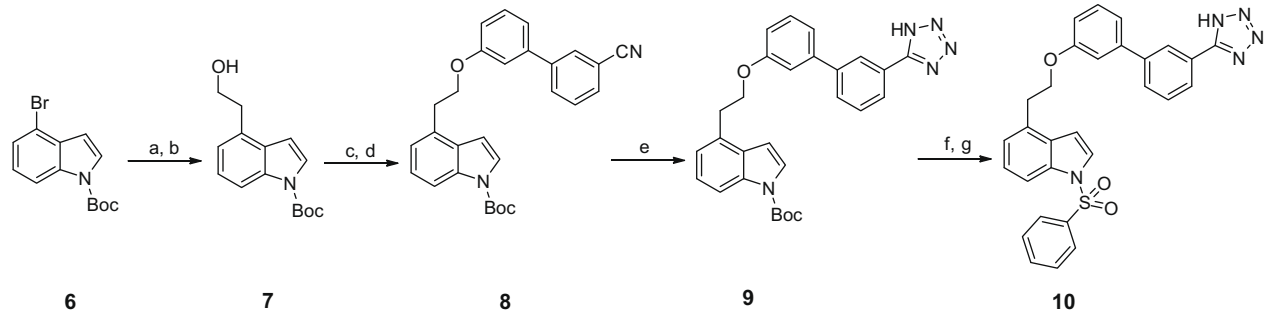
2-(4-bromonaphthalen-1-yl)-ethanol and the hydroxyl group was protected with TBDMS to give intermediate **2**. Pd-mediated carboxylation followed by deprotection afforded compound **3**. A subsequent Mitsunobu reaction generated intermediate **4**. Suzuki coupling between **4** and 3-cyanophenylboronic acid afforded compound **5**, which was then treated with azidotributylstannane to give the targeted molecule **1**.

In addition to naphthalene compounds, indole analogs of compound **1** proposed by the simulation were also synthesized (Scheme 2). To this end, 4-bromoindole-1-carboxylic acid *tert*-butyl ester **6** was converted to *tert*-butyl 4-(2-hydroxyethyl)-1*H*-indole-1-carboxylate **7** via a Stille coupling and hydroboration sequence. As for the synthesis of **1**, Mitsunobu reaction followed by Suzuki coupling gave compound **8**, which was subsequently converted to tetrazole **9**. Removal of the Boc protecting group and subsequent treatment with toluene sulfonyl chloride yielded compound **10**.

In order to evaluate the in vitro binding affinity of small molecules to the gp41 inner core trimer and their ability to disrupt hexamer formation, we developed a gp41 binding assay. This assay is based on size exclusion chromatography (SEC) separation of the six-member bundle formed by mixing of the HR-1 derived N36-peptide with the HR-2 derived C34-peptide. This six-helix bundle formation mimics the function of HIV gp41 in the biological setting which is required for the HIV virus to enter into the host cell. The basic experimental design and typical results are illustrated in Figure 4. In the first chromatogram (green), the N36-peptide is mixed with C34-peptide, and the hexamer bundle is identified by SEC. In the second chromatogram (magenta), the N36-peptide is first mixed with a helix bundle inhibitor, such as compound **1**. After a 30 min pre-incubation period, C34-peptide is added and the reaction mixture is applied on the HPLC size exclusion column.



Scheme 1. Reagents and conditions: (a) BuLi, hexane/ether, −78 °C, then Oxirane, THF, 0 °C; (b) TBDMSCl, imidazole, THF; (c) $Pd(OAc)_2$, dppp, CO, DMSO/MeOH, 80 °C; (d) TBAF, THF, rt; (e) 3-bromophenol, DIAD, PPh_3, THF, rt; (f) 3-cyanophenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME, 80 °C; (g) nBu_3SnN_3, 80 °C.



Scheme 2. Reagents and conditions: (a) tributylvinylstannane, $Pd(PPh_3)_4$, DMF, 85 °C; (b) 9-BBN, THF, rt, then H_2O_2, NaOH, −20 °C; (c) 3-bromophenol, DIAD, PPh_3, THF, rt; (d) 3-cyanophenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, DME, 80 °C; (e) nBu_3SnN_3, 80 °C; (f) TBAF, THF, reflux; (g) TosCl, TEA, DCM.

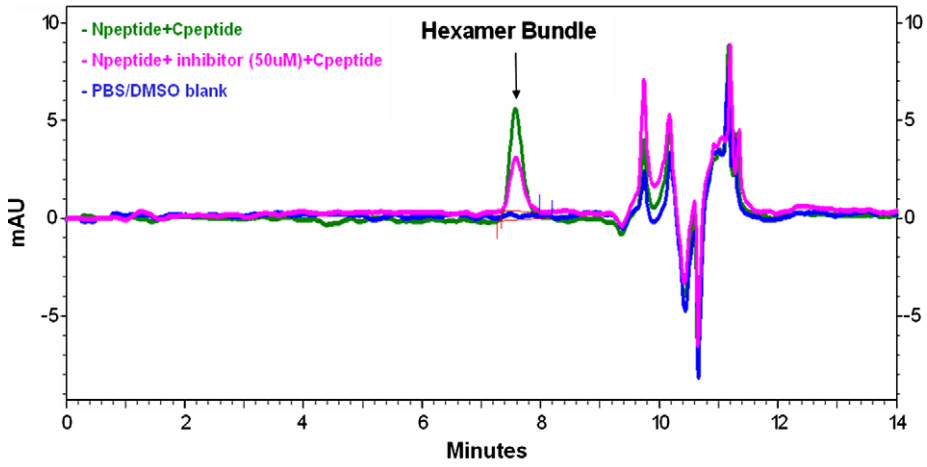


Figure 4. An overlay of three chromatograms illustrating the hexamer bundle formation (HBF) assay by SEC–HPLC (see text for description).

The peak size of the hexamer bundle is diminished presumably due to the binding of compound **1** to inhibit the formation of helical bundle. In the control experiment without N36- or C34-peptides, as shown in the last chromatogram (blue), no hexamer peak at the corresponding retention time is observed. The hexamer bundle formation (HBF) assay was cross-validated by comparing the results with those of an ELISA method reported by Jiang and co-workers.[10] In the ELISA assay, an antibody that recognizes the intact six-helical bundle was developed. A porphyrin compound (mesotetra (4-carboxyphenyl) porphine, MTCPP) that is known to prevent helical bundle formation was reported to have an IC_{50} of ca. 1 μM in this ELISA sandwich assay.[10] In our HPLC–SEC assay, the same compound disrupts gp41 bundle hexamer formation with an IC_{50} of 0.9 μM and hence serves as a positive control in all of our assays.

Unlike published circular dichroism (CD) methods, this assay method is compatible with dimethyl sulfoxide (DMSO) solvent that is used to solubilize most small organic compounds for drug discovery. The major limitation is the narrow range of useful concentration of the N36-peptide (1–6 μM). We found that below 1 μM, N36 does not efficiently form a six-helix bundle with C34, even when C34 is in excess. Above 6 μM, the N36-peptide begins to aggregate and six-helix bundle formation is maximally saturated, that is, addition of more N36 and C34 does not result in more hexamer bundle formation. The assay range could be extended by creation of N36-peptide derivatives with better physical properties, such as increased trimer stability and solubility. A subsequent dose–response experiment was used to determine IC_{50}'s, the in vitro binding of compounds **1**, **9** and **10** were tested for their ability to disrupt the hexamer bundle and the results are listed

Table 1
Inhibition of listed compounds in SEC–HBF assay

Compound	% Inhibition screening at 50 μM	IC_{50}, μM
1	95	31.0
9	100	na
10	100	32.9

in Table 1. Compounds **1**, **9** and **10** all showed ca. 100% inhibition at the screening concentration of 50 μM. The IC_{50}'s of compounds **1** and **10** were estimated to be 31 μM and 32.9 μM, respectively.

Computationally predicted interactions of compound **1** with the inner trimer core of gp41 are shown in Figure 5. In this binding mode, the tetrazole moiety interacts with Lys574, and the biphenyl groups occupy the neighboring hydrophobic pocket. The naphthylene ester projects into the narrow pocket defined by Trp571 and Arg579. Ideally, a co-crystal structure of compound **1** with gp41 N-trimer constructs would validate the predicted binding. However, due to the nature of the target protein, its binding pocket, and compound properties, attempts to obtain an X-ray co-crystal structure of compound **1** with several gp41 N-trimer constructs were unsuccessful.[11]

In lieu of a co-crystal structure, we conducted site-directed mutagenesis studies to assess gp41 interactions with compound **1** and gain some insight into the mode of binding. In this study, each of the three amino acid residues that were proposed to interact with compound **1** in N36 were mutated to Ala one at a time (Fig. 6). The effect of the mutation on hexamer formation and on the inhibition potency of compound **1** was then measured in the HBF assay. Consistent with the predicted binding mode of the biphenyl compounds, the results demonstrated that interaction of biphenyl compound **1** with N-trimer is highly dependent on gp41 residues Trp571 and Arg579. Although Trp571Ala and Arg579Ala mutations did not affect the formation of the hexameric bundle, the mutation of these residues to Ala reduces compound **1** inhibitory activity by 20% and 60%, respectively. Another important residue is Lys574, which is predicted to provide a key interaction point with the tetrazole moiety of compound **1**. However, we found that in Lys574Ala mutant, N36 was incapable of forming a hexameric bundle with C34, so it was not possible to measure the effect of this mutation on inhibitor binding.

Additionally, the binding of compound **1** to the gp41 hydrophobic pocket was further evaluated in a fluorescence assay developed to quantify small molecule binding in the hydrophobic pocket.[12] Compound **1** showed significant binding to the designed pocket in this assay.[13]

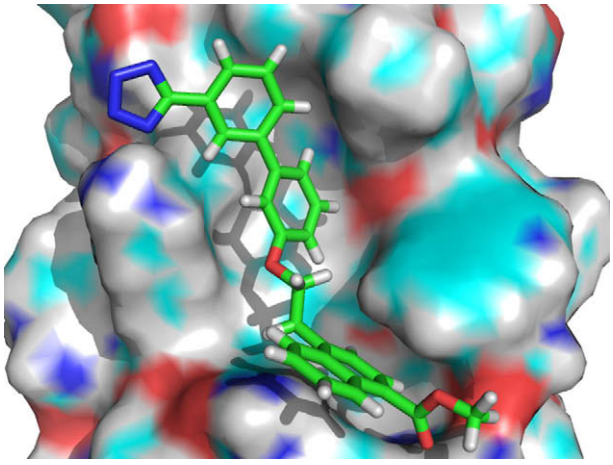


Figure 5. Predicted binding of biphenyl compound **1** to gp41 N36 trimer surface.

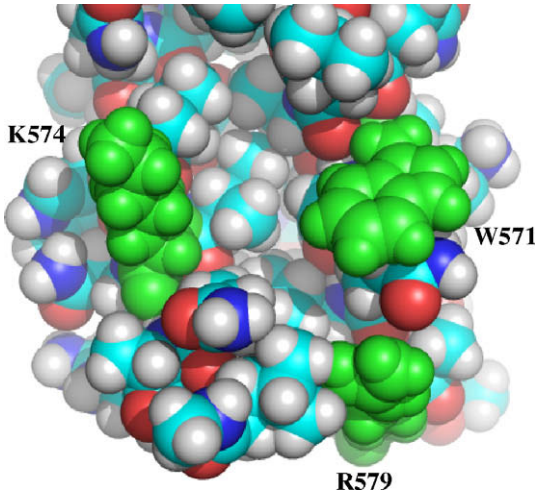


Figure 6. Position of gp41 amino acid residues Trp571, Lys574 and Arg579 (green) in the mutation study.

Despite the inhibition of the helical bundle formation, compound **1** did not show cell fusion inhibition activity up to 70 μM in cell–cell fusion inhibitory assay.[14] The disconnection between the inhibition of helical bundle formation observed for this series of compounds and the in-activity in cell–cell fusion and cytoprotection assays is not clearly understood at this time, but it may be due to the poor physical properties of the synthetic ligand.

In summary, we have applied de novo design to synthesize a series of substituted biphenyl ethylene ether compounds targeting the gp41 inner core trimer in order to disrupt the formation of the helical bundle that facilitates viral fusion. The compounds showed reasonable binding affinity to the gp41 inner core and prevented the helical bundle formation. The interaction between the small molecules and gp41 was also validated by targeted mutagenesis of the gp41 peptide. The current study provides an important example for structure-based drug design for protein–protein interaction targets.

Acknowledgments

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13. This experiment was conducted by the Miriam Gochin lab using that lab's previously reported FRET-based assay method, which measures the ability of a test compound to disrupt six-helix bundle formation. When tested in a screening experiment at 25 μM, compound **1** produced 75% response relative to the response produced by 25 μM control peptide C18Aib (K_d = 1 μM) and 67% response relative to 25 μM control compound ADS-J1 (K_d = 0.4 μM). The K_d of compound **1** was not determined in this assay. For assay details and control compounds, see Ref. 12.
14. Cell–cell fusion test was conducted at ImQuest, Inc.