Title

Biophysical studies of HIV-1 glycoprotein-41 interactions with peptides and small molecules – Effect of lipids and detergents

Journal

Biochimica et Biophysica Acta (BBA) - General Subjects, 1864(12)

ISSN

0304-4165

Authors

Zhou, Guangyan
Chu, Shidong
Kohli, Aditya
et al.

Publication Date

2020-12-01

DOI

10.1016/j.bbagen.2020.129724

Peer reviewed

HHS Public Access

Author manuscript

Biochim Biophys Acta Gen Subj. Author manuscript; available in PMC 2021 December 01.

Published in final edited form as:

Biochim Biophys Acta Gen Subj. 2020 December ; 1864(12): 129724. doi:10.1016/j.bbagen.2020.129724.

Biophysical studies of HIV-1 glycoprotein-41 interactions with peptides and small molecules – Effect of lipids and detergents

Guangyan Zhou[1], **Shidong Chu**[1], **Aditya Kohli**[2], **Francis C. Szoka**[2,3], **Miriam Gochin**[1,3]

[1]Department of Basic Sciences, College of Osteopathic Medicine, Touro University California, Vallejo CA 94592

[2]Department of Bioengineering and Therapeutic Sciences, UCSF School of Pharmacy, San Francisco, CA 94143

[3]Department of Pharmaceutical Chemistry, UCSF School of Pharmacy, San Francisco, CA 94143

Abstract

Background—The hydrophobic pocket (HP) of HIV-1 glycoprotein-41 ectodomain is defined by two chains of the N-heptad repeat trimer, within the protein-protein interface that mediates 6HB formation. It is a potential target for inhibitors of viral fusion, but its hydrophobic nature and proximity to membrane in situ has precluded ready analysis of inhibitor interactions.

Methods—We evaluated the sensitivity of ^{19}F NMR and fluorescence for detecting peptide and small molecule binding to the HP and explored the effect of non-denaturing detergent or phospholipid as cosolvents and potential mimics of the membrane environment surrounding gp41.

Results—Chemical shifts of aromatic fluorines were found to be sensitive to changes in the hydrogen bonding network that occurred when inhibitors transitioned from solvent into the HP or into ordered detergent micelles. Fluorescence intensities and emission maxima of autofluorescent compounds responded to changes in the local environment.

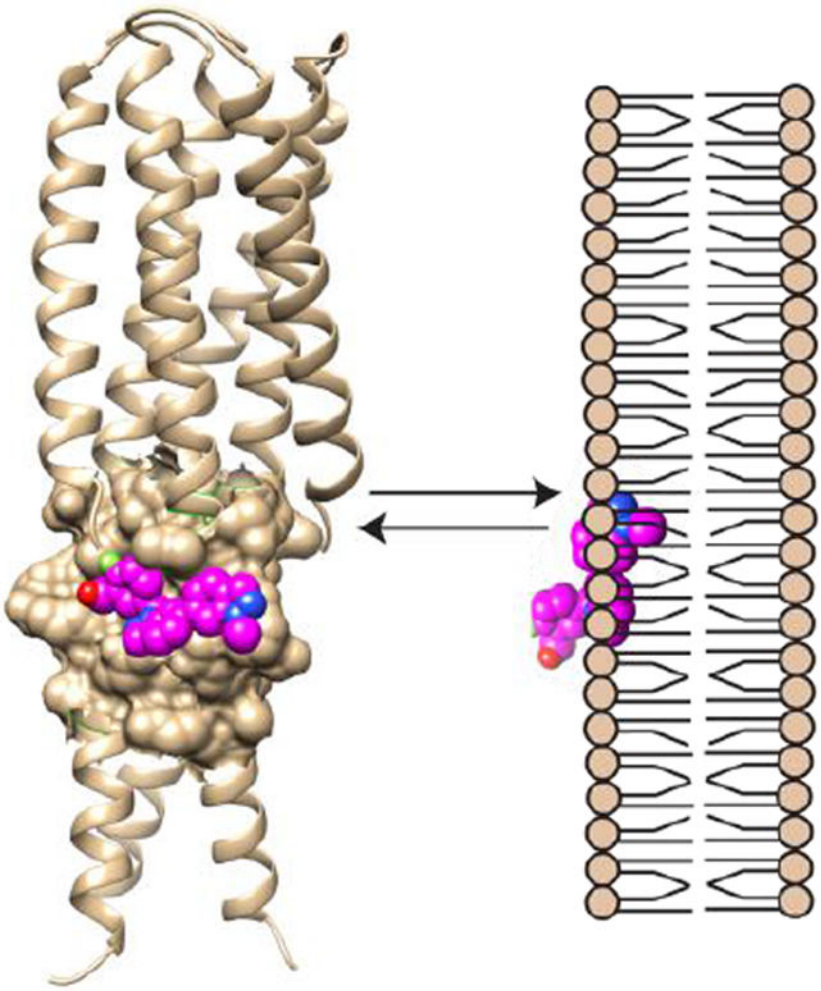
Conclusions—Gp41 - ligand binding occurred under all conditions, but was diminished in the presence of detergents. NMR and fluorescence studies revealed that dodecylphosphocholine (DPC) was a poor substitute for membrane in this system, while liposomes could mimic the membrane surroundings.

CRediT author statement
G. Zhou: Methodology, Investigation
S. Chu: Methodology, Investigation
A. Kohli: Investigation
F.C. Szoka: Methodology, Resources, Writing – Review and Editing
M. Gochin: Conceptualization, Resources, Investigation, Writing – Original Draft, Visualization, Supervision

Declaration of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

General Significance—Our findings suggest that development of high potency small molecule binders to the HP may be frustrated by competition between binding to the HP and binding to the bilayer membrane.

Graphical Abstract



Keywords

HIV-1 gp41; 19F NMR; Fluorescence; Detergents; Liposomes; Hydrophobic pocket interactions

1. Introduction

HIV-1 glycoprotein-41 (gp41) is a transmembrane protein that forms part of the spike on the viral surface and that is involved in fusion between HIV-1 and host cells. It consists of an ectodomain, transmembrane domain and cytoplasmic domain,[1, 2] and exists in a metastable state prior to fusion. Fusion is triggered by viral attachment and release of glycoprotein-120 (gp120), followed by collapse of trimeric N-heptad repeat (NHR) and C-heptad repeat (CHR) helices of the ectodomain into a six helix bundle (6HB).[1, 3-6]

Inhibitors targeting gp41 have been studied for their ability to prevent entry and infection. A hydrophobic pocket (HP) defined by two chains of the NHR trimer is contained within the protein-protein interface that mediates 6HB formation.[7] The pocket is considered to be a potential target for low molecular weight fusion inhibitors.[8-13] Nevertheless, small molecule fusion inhibitors with drug-like potency have not been found. The most effective (nM or better) fusion inhibitors discovered to date are peptides based on the CHR domain of gp41,[14] including T20 (enfuvirtide), an FDA approved fusion inhibitor. They act in a dominant-negative fashion by preventing the conformational changes in gp41 required for fusion.

It has proved difficult to study gp41 – small molecule interactions by biophysical methods as an aid to structure based drug design. Isolated gp41 ectodomain adopts the collapsed 6HB form in which the hydrophobic pocket is obscured (Figure 1A).[15, 16] In order to study binding in the pocket, we designed reverse hairpin proteins in which a truncated CHR preceded the NHR in sequence with a short connecting loop (Figure 1B).[17, 18] C28(L4)N50, named according to the length of the domains, has an exposed HP in solution. We also prepared CHR peptide containing the HP binding domain (HPbd) attached to the small globular domain GB1, allowing for straightforward bacterial synthesis and labeling for NMR. The resulting ^{15}N-labeled constructs CP^{i635} (shown in Figure 1B) was shown by HSQC to bind to C28(L4)N50.[17]

While C28(L4)N50 proved to be trimeric and highly helical,[18] it gave broad low quality HSQC spectra (Supplementary Material Figure S1), evidence of significant dynamics on the NMR chemical shift time scale. Furthermore ligands are hydrophobic and complexes with protein are poorly soluble at concentrations required for structural studies in aqueous solution. Therefore protein-detected ligand binding and structural analysis by NMR was not feasible for this system.

We observed that non-denaturing detergents helped to solubilize some of the more hydrophobic inhibitors and their mixtures with protein. We reasoned that lipids or membrane mimetic components could contribute to the mechanism of action in the milieu of the in situ fusion reaction. Here we examined this possibility using ^{19}F NMR and fluorescence experiments. ^{19}F NMR is a useful probe of molecular interactions due to the sensitivity of ^{19}F-resonances to changes in local environment,[19] as is deuterium NMR,[20] and the low concentrations required for measurement. Additionally, ^{19}F NMR is readily applicable to the study of proteins in the presence of detergents, lipids or stabilizing agents, because of the absence of background signals from these compounds. We examined both peptide and low molecular weight inhibitor binding to the HP, as well as the effect of phospholipid detergent dodecylphosphocholine (DPC) and Tween-20. Tween-20 is a mild non-ionic non-denaturing detergent that is used to solubilize membrane proteins without disrupting structure,[21, 22] DPC is a zwitterionic detergent that has been used in NMR studies of membrane-associated peptides and proteins as a membrane mimetic. The small size of DPC micelles together with the availability of DPC in perdeuterated form have favored its use for solution state proton NMR studies,[23] with the caveat that functional activity should be checked on a case by case basis.[24] Previous studies of peptides and constructs of the 6HB and associated domains have resulted in disparate interpretations of their interactions with detergent

micelles and lipid bilayers[2, 25, 26]. Some studies have indicated that DPC can alter the monomer-trimer equilibrium in gp41, which would disrupt the HP binding site.[27-29] Other studies have suggested that DPC is a suitable membrane mimetic in which to evaluate structure and fusion-inhibitory agents targeting gp41 domains.[30-32] Interpretation is likely compounded by the difficulties inherent in biophysical studies of the hydrophobic peptides, making the outcomes sensitive to exact composition, concentration and conditions. Overall, it appears that the 6HB, stable in aqueous solution, is destabilized or more dynamic in DPC micelles, while the structure is retained in zwitterionic lipid bilayers representative of the outer leaflet of the plasma membrane[33, 34]. Isolated NHR domain but not 6HB has also been shown to disrupt lipid ordering.[25, 26]

Since our ligands contain indole or indazole groups with intrinsic fluorescence, we additionally studied binding by examining changes in fluorescence spectra. Fluorescence studies were also conducted in the presence of liposomes that could more accurately represent the bilayer interface, giving us the opportunity to examine whether DPC micelles were adequate mimetics for this protein – lipid – small molecule system.

2. Material and methods

Experimental Procedures are described in Sections S1 - S3 of the Supplementary Material. These include synthetic routes of synthesis of ligands, binding and antiviral assays, NMR and fluorescence experiments and preparation of liposomes.

3. Results and Discussion

3.1 C-heptad repeat domain binding in the hydrophobic pocket is readily detected by ^{19}F NMR

We first established the sensitivity of ^{19}F NMR to the binding interaction in the HP by studying the validated CP^{i635} – C28(L4)N50 interaction (Figure 1).[17] CP^{i635} and C28(L4)N50 were labeled with 5-F-tryptophan by addition of 5-F-indole to the growth media.[19]. Tryptophan residues predominate in the CHR-NHR interaction in the HP. CP^{i635} contains three tryptophan residues, two in the HPbd and one within GB1. The NHR receptor C28(L4)N50 contains a single tryptophan residue in the HP. Fluorine substitution at the 5-position on Trp indole was shown to have negligible effect on protein structure.[19, 35] The results of the ^{19}F study are shown in Figure 2. The Kd of the interaction is 270 μM. Two of the three Trp residues in CP^{i635} undergo significant chemical shift and linewidth changes upon titration of C28(L4)N50, allowing for identification of the HPbd Trp resonances. The GB1 Trp residue at −123.45ppm was insensitive to binding, as expected. Previously we observed no change in ^{15}N or ^{1}H chemical shifts of GB1 residues with binding.[17] The shifts accompanying pocket sequestering of the W628 and W631 in the HPbd were extrapolated to 73.8 Hz and 51.9 Hz at 100% bound, indicating the sensitivity of ^{19}F chemical shifts to pocket binding, as well as the expected downfield shift associated with increased hydrophobicity of the surroundings. A 70 Hz increase in linewidth occurred. Crystal structures of NHR – CHR complexes show that W628 and W631 interpolate edgewise into the pocket,[36, 37] forming hydrophobic contacts with Leu, Ile, Gln residues

and in the case of W631, the edge of the pocket Trp571. In another structure, a stacking interaction with the pocket Trp was observed.[15]

In contrast, W571 in ^{19}F-labeled C28(L4)N50 shifted upfield after an initial downfield shift. Upfield shifts are consistent with **π-π** stacking interactions with HPbd Trp, but the reason for the initial downfield shift is unclear. It may be related to conformational change of W571 or to a decrease in aggregation of C28(L4)N50 upon C-peptide binding. We were able to sample less than 20% fraction bound in this experiment due to prohibitive line broadening and did not pursue the analysis further.

3.2 ^{19}F resonances of fluorine-labeled small molecules shift downfield upon addition of protein or detergent, indicating an increase in hydrophobic environment

We examined a series of small molecules with varying hydrophobicity and affinity, consisting of an indole or indazole ring substituted at the N_1 position with benzoic acid, and at the C6-position with different ring structures (Table 1). A fluorine atom was substituted at the meta position of the benzoic acid in **1**, **3**, **4** and **5** and at the ortho position in **2**; in 5 an -OCF_3 group replaced an OCH_3 group. Compound **6**, included in Table 1, has no fluorine atoms, but is similar to **5**, has good biological activity and is included in fluorescence studies (below). The K_I's for each of these compounds was determined using a previously described competitive inhibition assay.[38] Antifusion activity increased in the order **1** < **2** < **3** < **4** < **5** ≤ **6**, commensurate with K_I and the degree of hydrophobicity. Because of weak, broad or missing signals for the more hydrophobic compounds in buffer especially upon addition of protein, spectra were also obtained in the presence of solubilizing detergents DPC and Tween-20. These detergents reduced the affinity of C-peptide for the HP on the NHR trimer by a factor of ≥ 25 (DPC) or 5 (Tween-20) (Supplementary Material Figure S2, Table S1). The reduction in affinity was shown to depend on the characteristics of the C-peptide, suggesting that C-peptide partitioning into detergent micelles was significant. The data did not rule out interaction of the NHR trimer with DPC with a resulting equilibrium between solution and micelle bound forms. This is in line with previous studies demonstrating unfolding of a gp41 hairpin model in DPC and small unilamellar vesicles (SUV) into monomeric counterparts[28, 29]; the earlier data were interpreted in terms of a fusion mechanism involving NHR and CHR segments melting into the membrane surface prior to 6HB formation.

^{19}F NMR offered an opportunity to examine whether a similar effect occurs with small molecule binding, i.e. that hydrophobic small molecules could partition into a membrane-mimetic environment with reduced affinity for the HP. By extension, such behavior could infer a possible role of membrane in gp41 – small molecule interactions in situ.

An overlay of ^{19}F NMR spectra of the small molecules and chemical shifts under various conditions is shown in Figure 3. Peaks of the *m*-F benzyl group occurred at ~−113 ppm relative to external TFA at −75.597 ppm, while *o*-F substituted **2** resonated at ~−117 ppm. The resonance of the –OCF_3 group in **5** occurred at ~ −58 ppm.

3.2.1 Addition of protein in buffer: Addition of protein in buffer caused ^{19}F peaks to shift downfield (less negative) by the same order of magnitude as was observed for C-

peptide interactions. This is consistent with the increase in hydrophobicity expected with binding to the hydrophobic pocket. The size of the shift increased in the order **1** < **3** < **4** < **2**, with no observable peak for **5**. The small chemical shift change for **1** reflected its low binding affinity, with < 25% bound under the conditions of the experiment. The largest chemical shift change occurred for *o*-F substituted **2**, which is 50% bound to protein, while **3** and **4** are > 70% bound. Upon addition of protein, significant broadening was observed for the more hydrophobic and potent compounds **3** and **4**. The absence of an observable peak for **5** bound to protein is due to line broadening and / or insolubility of the complex. The peak could be observed by adding detergent (see below).

3.2.2 Addition of detergents—Addition of detergent caused larger downfield ^{19}F chemical shifts relative to buffer than addition of protein, with the largest shift occurring upon addition of DPC. Changes also occurred in the proton spectra upon addition of detergent (e.g. see Supplementary Material Figure S3). This reflects strong partitioning of the compounds into micelles. For **1**, **3**, **4** (all *m*-F substituted), the size of the shift matched the calculated logP, as would be expected. However, **5** and **2** were outliers. Compound **2** was an interesting case, since it was the most sensitive to changes in buffer conditions between aqueous, Tween and DPC as well as to addition of protein in buffer or Tween. Changes as large as 1 ppm occurred. It has fluorine substituted ortho to the carboxylate group on the benzyl ring, and as a result is significantly more sensitive to changes in the ionization state and H-bonding network of the carboxylate group. Fluorine substitution ortho to the carboxylic acid on a benzene ring is known to perturb the pKa more than meta-fluorine substitution.[39] Compound 5 showed small shifts upon addition of detergent, despite having the highest logP in the dataset. Noting the weak intensity of the ^{19}F signal of **5** in buffer, it is conceivable that **5** is aggregated, affecting the observed “free” chemical shift. The -OCF_3 resonance of **5** was particularly insensitive to addition of either detergent or protein or both. It appears that the ^{19}F chemical shift may be more sensitive to changes in ionization state than to changes in hydrophobicity of the surroundings.

3.2.3 Addition of protein in detergents—In the presence of detergents, resonance shifts were observed for all the compounds upon adding protein. For the more polar compounds **1** and **2**, the size and direction of the shift incurred upon adding protein was similar in Tween-20 and in buffer but was dampened in DPC. For the more hydrophobic compounds **3** and **4**, a significant dampening of the chemical shift caused by adding protein was observed in Tween compared to buffer. In DPC, a change in direction of the shift of the aromatic fluorine resonance of **2**, **3**, **4** and **5** occurred upon adding protein, towards that observed in buffer. The compounds are expected to move to a more polar environment upon moving from DPC micelles to protein bound in solution. The reversed shift direction was also observed for **5** in Tween-20.

Sensitivity of ^{19}F spectra of small molecules to addition of protein in the presence of Tween or DPC showed that the interaction was not abolished by these additives. The fact that the observed chemical shifts of the complexes in buffer were not reproduced when detergents were present indicated that an equilibrium between micelle and aqueous phase of the components or complexes was occurring, This could be due to partitioning of protein, ligand

or protein – ligand complex within the micelles. We confirmed that the interaction was retained by observation of signal in a WaterLOGSY experiments on a solution of 300 μM compound **1** or **3** and 10 μM C28(L4)N50 in perdeuterated 2 mM DPC. Signals were somewhat smaller than in the absence of DPC. In part this is due to line broadening in DPC (Figures S3A, S3B, Supplementary Material).

3.3 Compound partitioning into DPC micelles was confirmed by fluorescence

Many of the compounds in Table 1 have intrinsic fluorescence, owing to the presence of indole and indazole groups. Fluorescence wavelengths and amplitudes of indoles are known to be sensitive to local environment,[40] so can complement the NMR studies. The effect of partitioning into an ordered lipid environment was to cause a large increase in fluorescence quantum yield.[41] This is apparent for compounds **2**, **3** and **5** (Figure 4), where fluorescence intensity increased by a factor of 1.18, 2.54 and 2.98 respectively, proportionate to the degree of hydrophobicity. A shift of fluorescence maximum also occurred. The extent of the fluorescence signal change upon adding DPC exceeded that observed when adding protein, analogous to a similar observation with ^{19}F NMR chemical shifts. Addition of protein in buffer caused fluorescence intensity changes of a factor of 1.04, 1.21 and 0.65 for **2**, **3** and **5** respectively, with the amplitude of the change mirroring their relative affinities for the HP (Figure 4A). The reduction in intensity of **5** with addition of protein suggested conversion from an aggregated to a protein bound state, in agreement with our assessment of ^{19}F chemical shift changes for 5 in buffer. We observed that DPC significantly dampened the protein – ligand interaction changes in the fluorescence spectra, leading to small intensity changes in the presence of DPC (Figure 4B). Thus fluorescence was not as sensitive as ^{19}F NMR in detecting residual protein binding.

3.4 DPC is not a good membrane mimetic for studying interactions in the HP

The ^{19}F NMR results do not indicate definitively whether gp41 unfolds in the presence of DPC micelles, as was found in the earlier study.[28] In that study, unfolding also occurred in small unilamellar vesicles (SUV's). To examine possible denaturation in our reverse hairpin system, we compared changes in fluorescence spectra of compound upon addition of C28(L4)N50 in DPC micelles versus SUV's. SUV's containing lipid bilayers are generally considered to be relevant models for membrane studies, but are too large for typical NMR solution state experiments. We used liposomes composed of a 60:40 ratio of POPC : cholesterol, selected both for in vitro stability[44] and as a balance between the composition of lipids in cell membranes[42] and in the cholesterol-rich viral envelope.[43] We utilized compound 6 for this study, as a good model compound with high hydrophobicity, affinity and antiviral activity. Figure 5 shows the fluorescence spectra of an equimolar mixture of **6** and C28(L4)N50 in buffer, 2mM DPC, and 0.1 mM liposomes The spectrum of the mixture is compared to the sum of the individual component spectra, with a difference indicating an interaction between **6** and the protein. Partitioning into the liposomes or DPC micelles generated a large blue shift associated with reduced H-bonding with solvent, as well as a large intensity enhancement associated with the ordered environment, compared to compound fluorescence in buffer. However, while the interaction between protein and **6** was readily observed by fluorescence in buffer and in liposomes, it caused only subtle changes in the spectrum with DPC. In liposomes, the dampening of the intensity enhancement upon

protein binding is indicative of repartitioning of ligand into the protein pocket. This is reminiscent of the NMR observation of a reversal of the direction of the chemical shift change upon adding protein in the presence of micelles. The fluorescence result suggested that DPC is an imperfect medium in which to consider the behavior of gp41 and small ligand interactions. The results also showed that unlike the previous study on a different gp41 model, denaturation of C28(L4)N50 did not occur in liposomes.

4. Conclusions

This study demonstrated that HPbd peptide binding and small molecule binding in the hydrophobic pocket were readily detected by ^{19}F NMR and fluorescence. ^{19}F resonances of fluorine-labeled peptides and small molecules shifted downfield upon addition of protein and / or detergent, indicating an increase in hydrophobic environment. In peptides, ^{19}F spectra of 5-F-Trp were very sensitive to binding. However, in the small molecules, sensitivity of ^{19}F chemical shifts was highest for ^{19}F atoms that sampled changes in the hydrogen bonding network around the ligands, rather than in hydrophobic interactions. For example, the largest shifts were observed for ^{19}F substituted ortho to the carboxylic acid group on the aromatic ring, lesser shifts were observed for m-F substitution, and almost no shifts for ^{19}F in a - OCF3 group. This is true both for protein binding and for association with detergent micelles. The ^{19}F chemical shift changes were not necessarily a reflection of binding strength or environment. This result might be caused by lack of a specific interaction of the –OCF_3 group with protein or detergent, or by a non-specific interaction of the whole ligand with protein or micelles. The study also showed that ^{19}F chemical shifts and fluorescence intensity changes gave clues about the aggregation state of compound **5**.

Ligands partitioned into Tween-20 and DPC micelles, with a significant downfield shift in the ^{19}F NMR and a blue shift and intensity enhancement in fluorescence spectra. Binding to the protein, as detected by ^{19}F NMR, still occurred in detergent containing mixtures, although it was abrogated, especially in DPC micelles. The effect was emphasized by the observation of exceedingly small changes in fluorescence spectra upon addition of protein to ligand in the presence of DPC. Fluorescence spectra demonstrated that DPC is a poor substitute for a bilayer membrane in biophysical studies of the gp41 – small molecule interaction. In the presence of SUV’s, the interaction between protein and ligand remained strong.

The abrogation of binding in DPC appeared to be accompanied by partitioning of either C-peptide or small molecules into the micelles, rather than unraveling of the reverse hairpin structure in DPC. Earlier CD studies in DPC showed no effect on helical content compared to buffer and a high melting point (> 70°C) for C28(L4)N50.[18] In the fluorescence binding experiment, the effect of DPC on the interaction between peptide and receptor depended on the fluorophore used to label the C-peptide, with a factor of 25 observed for Lucifer Yellow labeled C-peptide and 180 for the more hydrophobic fluorescein labeled C-peptide. The effect was also much smaller in the presence of Tween, about a factor of 7. Similarly, abrogation of binding was more severe for more hydrophobic small molecules with a likely higher partition coefficient into the micelles. Free ligand was not observed in NMR spectra of protein – ligand complexes in the presence of DPC, even for compounds with apparent

Kd's in the low μM, despite the fact that observation of free ligand would be anticipated based on the expected exchange rate. It is likely that the Kd's are significantly higher in DPC.

It is interesting to consider the effect of lipid partitioning on the efficacy of low molecular weight inhibitors in cell-based antiviral experiments. We have observed correlations between anti-fusion activity and K_I determined in the absence of lipids or detergent (Table 1, and references [13, 45, 46]). An example is shown in the Supplementary Material Section S7 and Figure S4 for a previous compound set[13]. Furthermore, higher activity molecules are invariably more hydrophobic with a higher likelihood of partitioning into a non-aqueous phase. When we recalculated the K_I's using the solution and lipid bound fraction of each ligand, determined from experimental partition coefficients,(Zhou et al, Data in Brief, 2020) we obtained a better correlation of antiviral activity to the solution fraction (Supplementary Material Figure S4). These factors suggest that lipid partitioning is less significant for small molecule antiviral activity than binding affinity to the HP. They also imply that a trimeric gp41 intermediate with an intact HP must be available at some point during the in situ fusion reaction, and that it is not embedded in the membrane. Nevertheless, the competition between lipid and protein binding may explain why it is difficult to get potent small molecule fusion inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH grants GM087998 and AI122847 to MG.

References

[1]. Naider F, Anglister J, Peptides in the treatment of AIDS, Curr. Opin. Struct. Biol 19 (2009) 473–482. [PubMed: 19632107]

[2]. Cai L, Gochin M, Liu K, Biochemistry and biophysics of HIV-1 gp41 - membrane interactions and implications for HIV-1 envelope protein mediated viral-cell fusion and fusion inhibitor design, Curr Top Med Chem 11 (2011) 2959–2984. [PubMed: 22044229]

[3]. Markosyan RM, Cohen FS, Melikyan GB, HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation, Mol. Biol. Cell 14 (2003) 926–938. [PubMed: 12631714]

[4]. Melikyan GB, Markosyan RM, Hemmati H, Delmedico MK, Lambert DM, Cohen FS, Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. [Comment In: J Cell Biol. 2000 Oct 16;151(2):F9-14], J. Cell Biol 151 (2000) 413–423. [PubMed: 11038187]

[5]. Chen Y-H, Xiao Y, Dierich MP, HIV-1 gp41: role in HIV entry and prevention, Immunobiology 201 (2000) 308–316. [PubMed: 10776787]

[6]. Chen J, Skehel JJ, Wiley DC, N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil, Proc. Natl. Acad. Sci. U. S. A 96 (1999) 8967–8972. [PubMed: 10430879]

[7]. Chan DC, Chutkowski CT, Kim PS, Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target, Proc. Natl. Acad. Sci. USA 95 (1998) 15613–15617. [PubMed: 9861018]

[8]. Eckert DM, Malashkevich VN, Hong LH, Carr PA, Kim PS, Inhibiting HIV-1 entry: discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket, Cell 99 (1999) 103–115. [PubMed: 10520998]

[9]. Welch BD, Francis JN, Redman JS, Paul S, Weinstock MT, Reeves JD, Lie YS, Whitby FG, Eckert DM, Hill CP, Root MJ, Kay MS, Design of a potent D-peptide HIV-1 entry inhibitor with a strong barrier to resistance, J. Virol 84 (2010) 11235–11244. [PubMed: 20719956]

[10]. Yu F, Lu L, Du L, Zhu X, Debnath AK, Jiang S, Approaches for identification of HIV-1 entry inhibitors targeting gp41 pocket, Viruses 5 (2013) 127–149. [PubMed: 23344560]

[11]. Luftig MA, Mattu M, Di Giovine P, Geleziunas R, Hrin R, Barbato G, Bianchi E, Miller MD, Pessi A, Carfi A, Structural basis for HIV-1 neutralization by a gp41 fusion intermediate-directed antibody, Nat Struct Mol Biol 13 (2006) 740–747. [PubMed: 16862157]

[12]. Pan C, Liu S, Jiang S, HIV-1 gp41 fusion intermediate: a target for HIV therapeutics, J. Formos. Med. Assoc 109 (2010) 94–105. [PubMed: 20206833]

[13]. Zhou G, Sofiyev V, Kaur H, Snyder BA, Mankowski MK, Hogan PA, Ptak RG, Gochin M, Structure-Activity Relationship Studies of Indole-Based Compounds as Small Molecule HIV-1 Fusion Inhibitors Targeting Glycoprotein 41, J. Med. Chem 57 (2014) 5270–5281. [PubMed: 24856833]

[14]. Zhang D, Li W, Jiang S, Peptide fusion inhibitors targeting the HIV-1 gp41: a patent review (2009 - 2014), Expert Opin Ther Pat 25 (2015) 159–173. [PubMed: 25428639]

[15]. Caffrey M, Cai M, Kaufman J, Stahl SJ, Wingfield PT, Covell DG, Gronenborn AM, Clore GM, Three-dimensional solution structure of the 44 kDa ectodomain of SIV gp41, EMBO J. 17 (1998) 4572–4584. [PubMed: 9707417]

[16]. Root MJ, Kay MS, Kim PS, Protein design of an HIV-1 entry inhibitor, Science 291 (2001) 884–888. [PubMed: 11229405]

[17]. Walsh JD, Chu S, Zhang SQ, Gochin M, Design and characterization of swapped-domain constructs of HIV-1 glycoprotein-41 as receptors for drug discovery, Protein Eng Des Sel 28 (2015) 107–116. [PubMed: 25792539]

[18]. Chu S, Kaur H, Nemati A, Walsh JD, Partida V, Zhang SQ, Gochin M, Swapped-Domain Constructs of the Glycoprotein-41 Ectodomain Are Potent Inhibitors of HIV Infection, ACS Chem Biol 10 (2015) 1247–1257. [PubMed: 25646644]

[19]. Campos-Olivas R, Aziz R, Helms GL, Evans JN, Gronenborn AM, Placement of 19F into the center of GB1: effects on structure and stability, FEBS Lett. 517 (2002) 55–60. [PubMed: 12062409]

[20]. Shaghaghi M, Keyvanloo A, Huang Z, Szoka FC, Thewalt JL, Constrained Versus Free Cholesterol in DPPC Membranes: A Comparison of Chain Ordering Ability Using Deuterium NMR, Langmuir 33 (2017) 14405–14413. [PubMed: 29120186]

[21]. Arachea BT, Sun Z, Potente N, Malik R, Isailovic D, Viola RE, Detergent selection for enhanced extraction of membrane proteins, Protein Expression Purif. 86 (2012) 12–20.

[22]. Arnold T, Linke D, Phase separation in the isolation and purification of membrane proteins, BioTechniques 43 (2007) 427–430, 432, 434 passim. [PubMed: 18019333]

[23]. Puthenveetil R, Vinogradova O, Solution NMR: A powerful tool for structural and functional studies of membrane proteins in reconstituted environments, J. Biol. Chem 294 (2019) 15914–15931. [PubMed: 31551353]

[24]. Chipot C, Dehez F, Schnell JR, Zitzmann N, Pebay-Peyroula E, Catoire LJ, Miroux B, Kunji ERS, Veglia G, Cross TA, Schanda P, Perturbations of Native Membrane Protein Structure in Alkyl Phosphocholine Detergents: A Critical Assessment of NMR and Biophysical Studies, Chem. Rev 118 (2018) 3559–3607. [PubMed: 29488756]

[25]. Klug YA, Rotem E, Schwarzer R, Shai Y, Mapping out the intricate relationship of the HIV envelope protein and the membrane environment, Biochim Biophys Acta Biomembr 1859 (2017) 550–560. [PubMed: 27793589]

[26]. Aisenbrey C, Bechinger B, Structure, interactions and membrane topology of HIV gp41 ectodomain sequences, Biochim Biophys Acta Biomembr 1862 (2020) 183274. [PubMed: 32197992]

[27]. Lakomek NA, Kaufman JD, Stahl SJ, Louis JM, Grishaev A, Wingfield PT, Bax A, Internal dynamics of the homotrimeric HIV-1 viral coat protein gp41 on multiple time scales, Angew. Chem. Int. Ed. Engl 52 (2013) 3911–3915. [PubMed: 23450638]

[28]. Roche J, Louis JM, Grishaev A, Ying J, Bax A, Dissociation of the trimeric gp41 ectodomain at the lipid-water interface suggests an active role in HIV-1 Env-mediated membrane fusion, Proc. Natl. Acad. Sci. U. S. A 111 (2014) 3425–3430. [PubMed: 24550514]

[29]. Roche J, Louis JM, Aniana A, Ghirlando R, Bax A, Complete dissociation of the HIV-1 gp41 ectodomain and membrane proximal regions upon phospholipid binding, J. Biomol. NMR 61 (2015) 235–248. [PubMed: 25631354]

[30]. Perez Y, Gomara MJ, Yuste E, Gomez-Gutierrez P, Perez JJ, Haro I, Structural Study of a New HIV-1 Entry Inhibitor and Interaction with the HIV-1 Fusion Peptide in Dodecylphosphocholine Micelles, Chemistry 23 (2017) 11703–11713. [PubMed: 28677862]

[31]. Li Y, Tamm LK, Structure and plasticity of the human immunodeficiency virus gp41 fusion domain in lipid micelles and bilayers, Biophys. J 93 (2007) 876–885. [PubMed: 17513369]

[32]. Murphy RE, Samal AB, Vlach J, Saad JS, Solution Structure and Membrane Interaction of the Cytoplasmic Tail of HIV-1 gp41 Protein, Structure 25 (2017) 1708–1718 e1705. [PubMed: 29056482]

[33]. Sackett K, Nethercott MJ, Shai Y, Weliky DP, Hairpin folding of HIV gp41 abrogates lipid mixing function at physiologic pH and inhibits lipid mixing by exposed gp41 constructs, Biochemistry (Mosc). 48 (2009) 2714–2722.

[34]. Lev N, Fridmann-Sirkis Y, Blank L, Bitler A, Epand RF, Epand RM, Shai Y, Conformational stability and membrane interaction of the full-length ectodomain of HIV-1 gp41: implication for mode of action, Biochemistry (Mosc). 48 (2009) 3166–3175.

[35]. Liu L, Byeon IJ, Bahar I, Gronenborn AM, Domain swapping proceeds via complete unfolding: a 19F- and 1H-NMR study of the Cyanovirin-N protein, J. Am. Chem. Soc 134 (2012) 4229–4235. [PubMed: 22296296]

[36]. Chan DC, Fass D, Berger JM, Kim PS, Core structure of gp41 from the HIV envelope glycoprotein, Cell 89 (1997) 263–273. [PubMed: 9108481]

[37]. Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC, Atomic structure of the ectodomain from HIV-1 gp41 [see comments], Nature 387 (1997) 426–430. [PubMed: 9163431]

[38]. Cai L, Gochin M, A Novel Fluorescence Intensity Screening Assay Identifies New Low Molecular Weight Inhibitors of the gp41 Coiled Coil Domain of HIV-1, Antimicrob. Agents Chemother 51 (2007) 2388–2395. [PubMed: 17452484]

[39]. Stewart R, Granger MR, The Basicities of Ortho-Substituted Benzoic Acids, Can. J. Chem 39 (1961) 2508–2515.

[40]. Van Duuren BL, Solvent Effects in the Fluorescence of Indole and Substituted Indoles, J. Org. Chem 26 (1960) 2954–2960.

[41]. Lakowicz JR, Keating S, Binding of an indole derivative to micelles as quantified by phase-sensitive detection of fluorescence, J. Biol. Chem 258 (1983) 5519–5524. [PubMed: 6853531]

[42]. van Meer G, Voelker DR, Feigenson GW, Membrane lipids: where they are and how they behave, Nat Rev Mol Cell Biol 9 (2008) 112–124. [PubMed: 18216768]

[43]. Carravilla P, Nieva JL, Eggeling C, Fluorescence Microscopy of the HIV-1 Envelope, Viruses 12 (2020).

[44]. Veatch SL, Keller SL, Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol, Biophys. J 85 (2003) 3074–3083. [PubMed: 14581208]

[45]. Zhou G, Wu D, Snyder B, Ptak RG, Kaur H, Gochin M, Development of indole compounds as small molecule inhibitors of HIV-1 gp41, J. Med. Chem 54 (2011) 7220–7231. [PubMed: 21928824]

[46]. Whitby LR, Boyle KE, Cai L, Yu X, Gochin M, Boger DL, Discovery of HIV fusion inhibitors targeting gp41 using a comprehensive alpha-helix mimetic library, Bioorg. Med. Chem. Lett 22 (2012) 2861–2865. [PubMed: 22424973]

Highlights

- ^{19}F NMR and fluorescence are sensitive measures of ligand binding to HIV-1 gp41
- DPC is a poor substitute for membrane when studying interactions of gp41
- The gp41 N-heptad repeat does not unfold in liposomes composed of POPC and cholesterol
- Lipid binding competes with protein binding for small molecule hydrophobic pocket inhibitors
- Observed antiviral activity is most closely correlated with the aqueous fraction of the inhibitors.

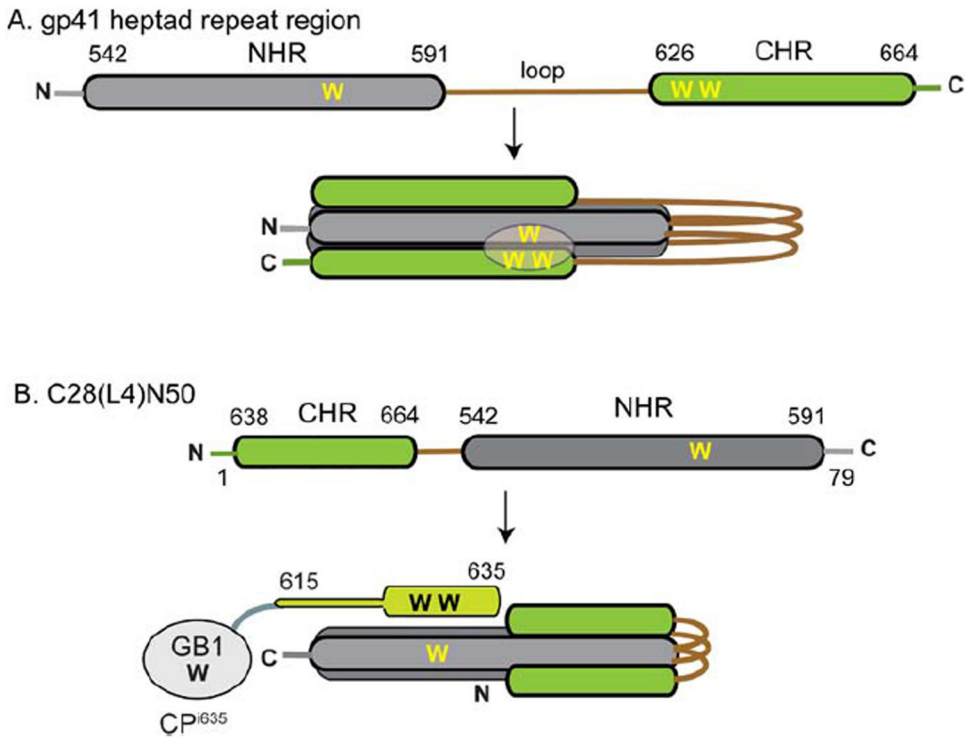


Figure 1.

Protein constructs for studying HP binding. A. gp41 heptad repeat: linear arrangement of NHR and CHR domains and schematic of folded trimeric hairpin. B. Linear and folded depiction of 79 residue construct C28(L4)N50. The protein CP^{i635} containing HPbd residues connected to GB1 is shown associated with C28(L4)N50. Three Trp residues involved in the HP - HPbd interaction are indicated with W. There is one Trp residue in GB1.

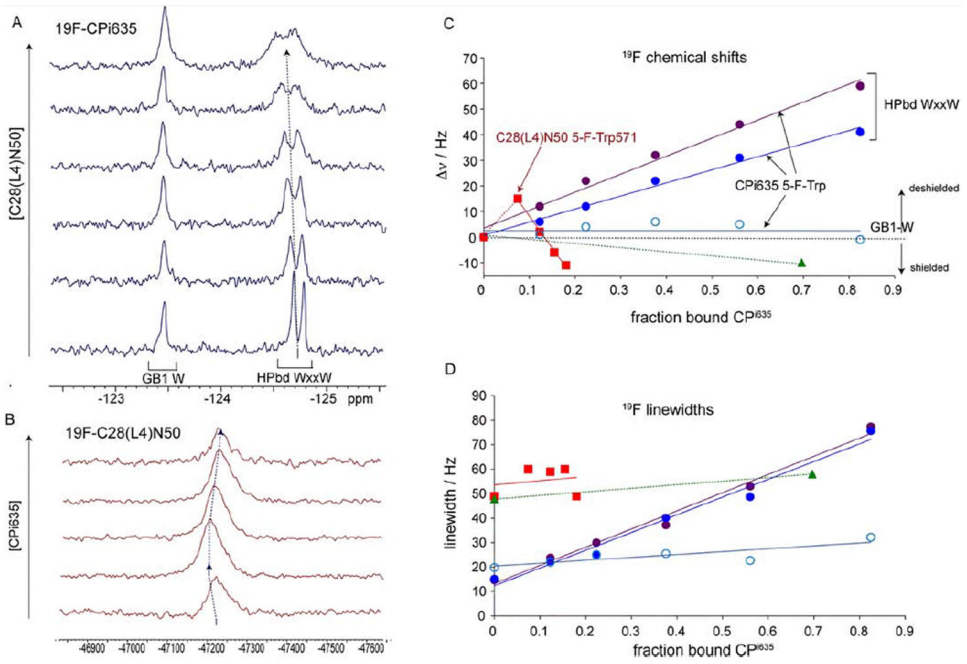


Figure 2.

5-F-Tryptophan as a probe of peptide binding in the hydrophobic pocket. A. Titration of C28(L4)N50 into ^{19}F-CPi635. C28(L4)N50 concentrations were 0, 50, 100, 200, 400 and 1350 μM. [CP^{i635}] was held constant at 100 μM. B. Titration of CP^{i635} into ^{19}F-C28(L4)N50. Concentrations of C28(L4)N50 were varied from 69 to 32.5 μM, and of CPi635 from 0 to 65 μM. C. Plot of the chemical shift changes as a function of fraction bound. D. Plot of line width changes as a function of fraction bound.

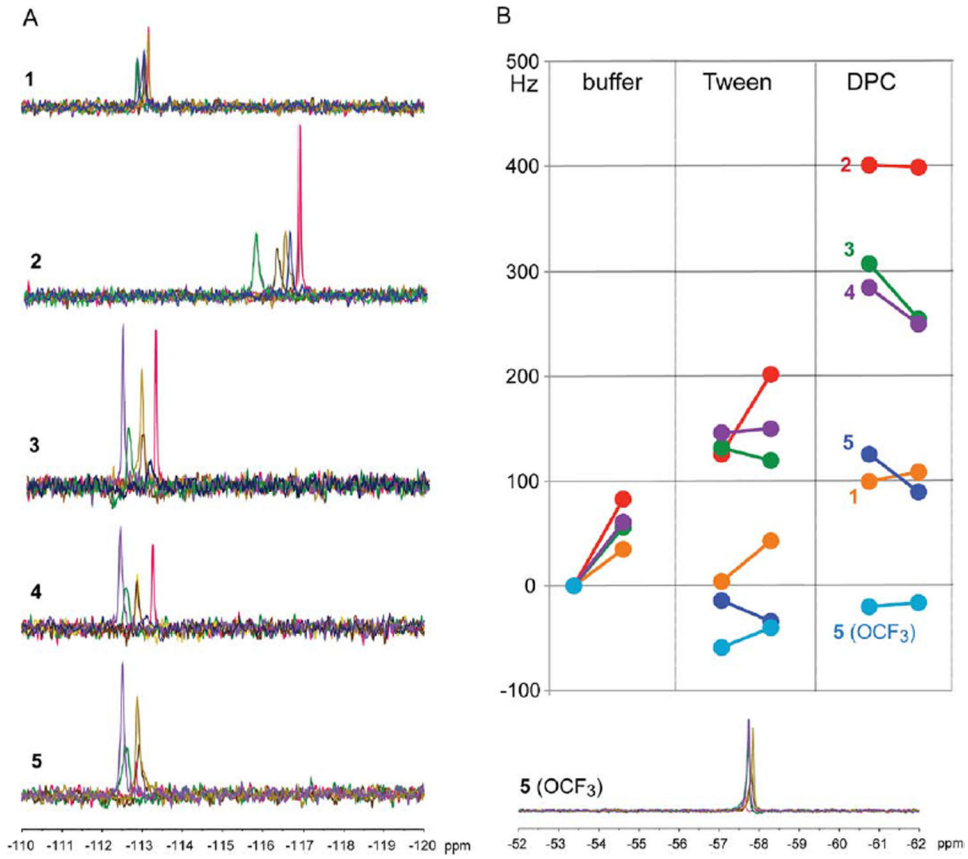


Figure 3.

Overlay of ^{19}F spectra of the fluorinated compounds. Compound concentration was 40μM, and chemical shifts were measured relative to external TFA at −75.597 ppm. A. ^{19}F spectra ± detergent ± protein. **5** has two fluorine signals, that due to the –OCF*3* group is shown offset to the right. Free in buffer (pink); +40μM C28(L4)N50 (blue); in 0.05% Tween-20 (yellow); in 0.05% Tween-20 +40μM C28(L4)N50 (brown); in 2% DPC (purple); in 2% DPC + 40μM C28(L4)N50 (green). B. Change of chemical shift relative to that for compound in buffer for each condition before (left symbol) and after (right symbol) adding 40μM C28(L4)N50.

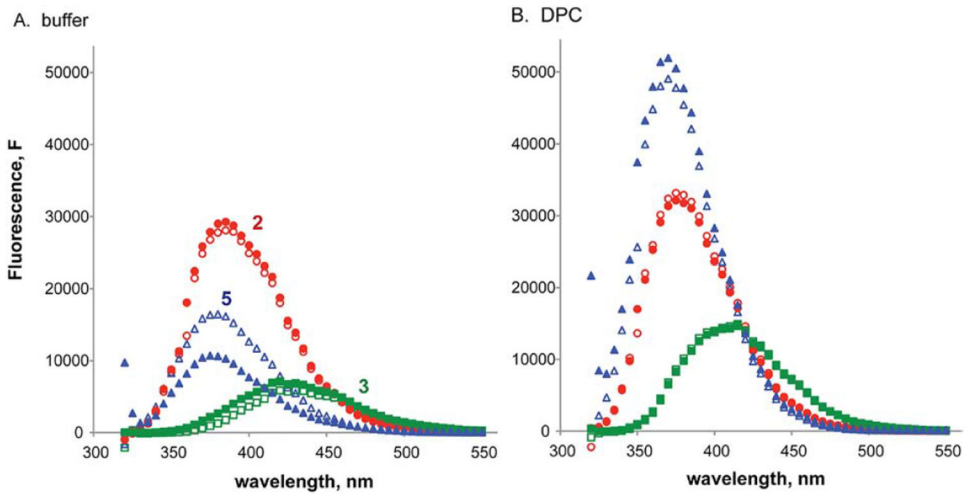


Figure 4.

Fluorescence spectra of 5 μM **2**, **3** and **5** in A. buffer and B. 2 mM DPC. Excitation was at 300 nm. Curves are color coded as indicated, and were measured in the absence (open symbols) and presence (closed symbols) of 5 μM C28(L4)N50.

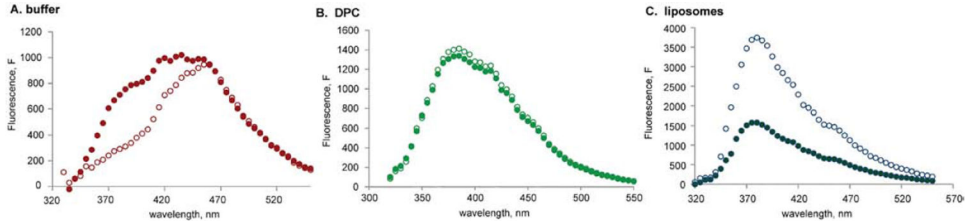
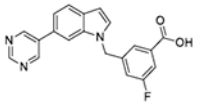
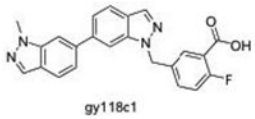
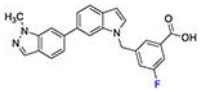
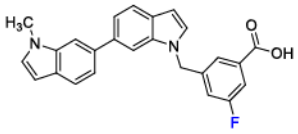
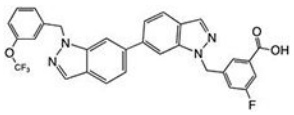
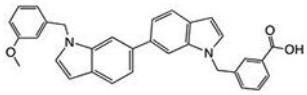


Figure 5.

Fluorescence spectra of an equimolar mixture of **6** and C28(L4)N50 under different conditions, comparing the spectrum of the sum of the components(open symbols) with those of the mixture (closed symbols). Excitation was at 300nm. Spectra were recorded in A. buffer; B. 2mM DPC, 3. 0.1mM POPC: cholesterol SUV (containing 40% cholesterol).

Table 1.

Small molecules used in the study

Name	Chemical structure	MWt	ClogP *	K_I (HP) buffer (μM)	IC_{50} CCF § (μM)	IC_{50} VCF Ba-L § (μM)
1		347.3	3.53	100	> 20	n.d.
2 [a]	 gy118c1	400.4	4.61	19	> 25	> 20
3 [a]		399.4	5.49	4	7	10
4 [a]		398.4	6.44	2.5	4	8
5		560.1	6.85	0.5	2	n.d.
6 [a]		486.6	7.64	1	0.8	0.9

*calculated log water-octanol partition coefficient using Bio-Loom

[a]previously published

§Selectivity Index (SI) > 20 in all cases, where SI = CC_{50}/IC_{50}. CC_{50} is the 50% cytotoxic concentration, IC_{50} is the half maximal inhibitory concentration.