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Thermodynamics-based drug design: strategies for inhibiting protein–protein interactions

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Abstract

The inhibition of protein–protein interactions and their ensuing signaling processes play an increasingly important role in modern medicine. Small molecular-weight inhibitors that can be administered orally are the preferred approach but efficient strategies for developing them are not yet generally available. Due to the large size difference between the protein–protein interface and the small molecule, inhibitor interactions are expected to extend to only a small region of the interface. If this is the case, classical competitive inhibition may be hard to achieve. In addition, competitive inhibition wastes binding energy that can be effectively used to inhibit signaling. The best and most energy-efficient approach would be the development of small molecules that bind at the protein–protein interface and inhibit the signaling process without displacing the protein ligand. This approach seems feasible knowing that the binding energy is not evenly distributed within the binding interface but concentrated in discrete hotspots, and that the initiation of signaling may not overlap with those hotspots. We outline a general protein–protein inhibition model that extends from competitive to noncompetitive scenarios and apply it to the development of HIV-1 gp120-CD4 inhibitors. This rigorous model can be easily applied to the analysis of protein–protein inhibition data and used as a tool in the optimization of inhibitor molecules.

Protein–protein interactions play a critical role in biological signaling. Their inhibition defines a major target for drug development against different pathological conditions including cancer, inflammation, autoimmune diseases, diabetes, osteoporosis and infection. The number of targets of interest is continuously increasing and range from a vast number of cell-surface receptors, such as EGFR, TNFR and IGFR, to other proteins involved in signaling and regulation [1,2]. Until now, biologics, that is, monoclonal antibodies or recombinant versions of ligand proteins and/or soluble regions of the receptors, define the therapeutic arsenal aimed at targeting those interactions. Biologics, however, have certain problems; they are not orally bioavailable, they can trigger inflammatory processes at the site of injection, severe immunological responses and opportunistic infections during treatment [3]. Their large size also places a limit on their ability to cross the blood–brain barrier or to penetrate deep tissues such as dense tumors [4]. The ideal drug would be a small-nonpeptidic compound that can be orally administered. However, is that possible? And if so, what is the best approach to develop small-molecule inhibitors of the signaling process triggered by protein–protein interactions?

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The therapeutic goal of inhibiting protein–protein interactions, such as protein ligands and cell-surface receptors, is not the inhibition of binding *per se* but the inhibition of the signaling cascade that is initiated by their binding. A survey of protein ligand–receptor interactions indicates that they bind with affinities on the nanomolar and high picomolar level [5–15]. Examples of subnanomolar interactions are the binding of IL-4 and erythropoietin to their respective receptors with K_d values of 0.2 nM [7,9]. Equally important as the affinity, is the physiological concentration of the protein ligands. The ability of a competitive inhibitor to efficiently disrupt the interactions between two proteins depends on both the binding affinity of the inhibitor and the concentration and affinity of the natural ligand. For example, IGF-1, and IL-2 bind to their receptors with similar affinities of 5 nM [16] and 10.5 nM [8], respectively; however, their physiological concentrations are very different. The concentration of IGF-1 varies between 1 and 130 nM [17], whereas the local concentration of IL-2 has been estimated to be as high as 1–100 mM [18].

The surfaces involved in the interactions between two proteins are normally large and of the order of 1500–4000 Å² [13,14,19,20]. The interactions between the two proteins are not evenly distributed within the interaction surfaces, however, and favorable interactions within much smaller regions, so-called **binding hotspots**, contribute most of the binding energy [1,21]. Small molecular-weight inhibitors will only cover a small fraction of the entire interaction surface and can be expected to effectively target one or a few hotspots. Recently, the idea that the binding hotspots do not necessarily overlap with the initiation points for **allosteric signaling** has been advanced [22]. This idea is also supported by the existence of single point protein mutants that maintain their binding affinity but are unable to trigger the signaling response [6]. If this is the case, the possibility of identifying small molecules that bind at the protein–protein interface and inhibit signaling without completely inhibiting binding becomes feasible.

The binding of the cell-surface receptor, CD4, to the HIV-1 envelope glycoprotein, gp120, is critical for HIV-1 infection [23,24]. Accordingly, the development of CD4–gp120 inhibitors has been a very active area of research [25,26]. These efforts have resulted in the discovery of small-molecule inhibitors of gp120 that have been considered either competitive or noncompetitive with CD4 [27–34]. In this paper, the competitive or noncompetitive character of those inhibitors has been examined experimentally. It is found that those inhibitors are neither competitive nor noncompetitive. A generalized protein–protein inhibition model is presented to account for those results. This model provides important quantitative guidelines for the development and optimization of inhibitors of protein–protein interactions.

A general protein–protein inhibition model

Competitive inhibition is classically defined as an all-or-none process: either the inhibitor or the natural ligand/substrate is bound but not both. This idea originated in the early days of enzyme inhibition and is based on the premise that the inhibitor is able to completely block the active site, thus preventing binding of the substrate. The situation is different if one considers the inhibition of protein–protein interactions by small molecular-weight compounds. In this case, the protein–protein interaction surface is much larger than a small molecule (~500g/mol) allowing for the possibility of simultaneous binding of protein and inhibitor. Since the binding energy between two proteins is not evenly distributed throughout the interacting interface but concentrated in discrete hotspots [1,21], the small molecule will interact with only a fraction of them. Depending on the exact location and/or interactions of the small molecule within a particular location, it may affect to a different extent the binding of the protein ligand. In fact, given the size difference, one may ask if there are truly competitive (i.e., all-or-none) small-molecule inhibitors of protein–protein

interactions. A small molecule may significantly lower the affinity between the two proteins; however, a truly competitive inhibitor should lower the affinity to zero; that is, either the inhibitor or the protein ligand is bound but not the two of them simultaneously. For the protein ligand to bind, the truly competitive inhibitor needs to be displaced. The protein ligand cannot bind if the inhibitor is bound. Under those circumstances, binding of the protein ligand to the protein target only occurs by displacement of the inhibitor, a process that reduces the apparent affinity of the protein ligand by an amount that depends on the concentration and affinity of the inhibitor.

The generalized protein–protein inhibition model in which the simultaneous binding of protein ligand and inhibitor is allowed, is illustrated in Figure 1. According to this model, the two proteins (e.g., protein ligand and protein receptor) bind with an affinity constant, K , the small-molecule inhibitor binds to the protein receptor with affinity, K_I , and the protein ligand binds to the inhibitor-bound protein receptor with affinity βK , where β represents the degree by which the inhibitor affects protein binding. For classic all-or-none competitive inhibition, $\beta = 0$ (and, therefore, the affinity βK is also zero), whereas for the situation in which the inhibitor does not affect the binding affinity between the two proteins, $\beta = 1$. The model also includes the rare situation in which the inhibitor enhances the affinity between the two proteins, where $\beta > 1$.

The important quantity is the fraction of receptor protein that is bound to inhibitor, F_b , which is given by:

$$F_b = \frac{K_I [I] (1 + \beta K [L])}{1 + K_I [I] (1 + \beta K [L]) + K [L]} \quad \text{Equation 1}$$

where $[I]$ and $[L]$ are the free concentrations of the inhibitor and protein ligand, respectively.

For a classical competitive inhibitor ($\beta = 0$), Equation 1 reduces to the standard competition equation:

$$F_b = \frac{K_I [I]}{1 + K_I [I] + K [L]} \quad \text{Equation 2}$$

And for the situation in which the inhibitor does not affect protein binding ($\beta = 1$), Equation 1 reduces to:

$$F_b = \frac{K_I [I]}{1 + K_I [I]} \quad \text{Equation 3}$$

which is equivalent to the situation in which the protein ligand is absent. A purely noncompetitive inhibitor will follow Equation 3. It must be noted that, from a mathematical point of view and the validity of the model, the exact location of the inhibitor binding site is immaterial.

Figure 2 illustrates the expected situation for a range of β values between zero and one. In general, the fraction of protein receptor bound to inhibitor depends on the affinity and concentration of the protein ligand $K[L]$. $K[L]$ is equal to the ratio $[L]/K_d$, if written in terms of the protein ligand dissociation constant $K_d = 1/K$. For any given set of conditions, the fraction of inhibitor-bound protein increases as β tends to one, that is, as the inhibitor becomes less competitive. The drug-design goal is to make the fraction of protein bound to

the inhibitor equal to the fraction of protein that is inhibited, that is, every inhibitor that binds should fully inhibit the protein. This goal is maximized by targeting the sites that serve as the initiation for allosteric signaling. If this is not the case, a proportionality constant (i.e., $F_{inhibited} = \alpha F_{bound}$) should be introduced (for simplicity in this paper we will assume that $\alpha = 1$, i.e., the allosteric signaling of all protein receptor that is bound to inhibitor is inhibited). It is clear that the best situation is achieved when the inhibitor does not compete with the protein ligand but is able to suppress the signaling cascade. From a practical drug-design point of view, the identification of initiation points for allosteric signaling with an β value close to 1 and low degree of competitiveness ($\beta \sim 1$) can be achieved by scanning mutagenesis of the protein–protein interface [6].

Of particular importance is the inhibitor concentration, $[I]_{F_x}$, at which a particular degree of inhibition, F_x , is achieved. This quantity is given by the equation:

$$[I]_{F_x} = \frac{F_x}{(1 - F_x)} \times \frac{(1 + K[L])}{K_i(1 + \beta K[L])} \quad \text{Equation 4}$$

Figure 3 illustrates different situations as a function of the $[L]/K_d$ ratio. Again, this figure demonstrates that the most energy-efficient situation occurs when the inhibitor does not need to displace the natural protein ligand in order to inhibit the protein receptor. Since $[L]/K_d$ is a biological system variable, that is, the ratio of the natural ligand concentration to its affinity to the receptor, it needs to be explicitly considered to set appropriate design goals, as it varies from target to target.

HIV-1 cell-entry inhibitors

With few exceptions, very little is known regarding the degree of competitiveness of small molecular-weight protein–protein inhibitors. One possible exception is given by the inhibitors of HIV-1 cell entry, where precise calorimetric binding data have been reported [35]. The first step in HIV-1 infection is the binding of the virus envelope glycoprotein, gp120, to the human CD4 receptor. The binding of CD4 to gp120 triggers a large conformational change in the viral protein allowing it to bind to the chemokine coreceptor (usually CCR5 or CXCR4). Binding of gp120 to the coreceptor leads to the activation of gp41 and the subsequent fusion of the viral and human host cell membranes [23,24]. The inhibition of CD4–gp120 binding or the ensuing signaling cascade has been recognized as a valid strategy to block viral entry. In fact, several small molecular-weight inhibitors that target gp120 have been developed and tested in binding and cell-inhibition assays [27–34], and very recently in humans [36,37]. As of yet, however, the mechanistic character of these inhibitors is only partially known. Are these inhibitors competitive, noncompetitive or somewhere in between?

NBD-556 and its analogs (Figure 4a & b), have been considered as competitive inhibitors of CD4 [30,35]. They are small molecules (MW <500 g/mol) that bind to a deep pocket within the CD4 binding site in gp120, which was previously demonstrated experimentally and by docking NBD-556 into the structure of gp120 [30]. An unpublished structure has also recently been determined with gp120 in complex with NBD-557 [34] and the Fab fragment of the monoclonal antibody 48d [Kwon, Young D, Kwong PD, Unpublished Data]. Figure 5 shows gp120 bound to CD4 and with NBD-556 docked into the structure. This figure clearly illustrates the large size difference between the protein–protein interaction surface and the small-molecule inhibitor. The original compound (NBD-556) binds gp120 with an affinity of 3.7 μM and the analog considered in this paper (compound 14) [28] binds to the same site with a similar affinity (3.0 μM). The binding of the soluble form of CD4 (sCD4) to gp120 was determined by **isothermal titration calorimetry** in the absence and presence of 200

μM of each inhibitor. The association constant, K_{app} , measured in the presence of the inhibitor is given by:

$$K_{app} = \frac{K(1 + \beta K_I [I])}{1 + K_I [I]} \quad \text{Equation 5}$$

where K is the binding constant of sCD4 to gp120 in the absence of inhibitor, K_I the association constant of the inhibitor and $[I]$ the inhibitor concentration in the experiment. Equation 5 permits experimental determination of the parameter β , since all the other parameters are experimentally known. This experimental approach allows the researcher to establish structure–activity relationships with β , and optimize the noncompetitive character of the inhibitor. Similar experiments were performed with the inhibitor BMS-806 (MW = 406 g/mol) (Figure 4c) [27,29], which has been considered to be either competitive [27] or noncompetitive with CD4 [38]. Table 1 summarizes the results. It is clear that none of the inhibitors is either truly competitive or noncompetitive with CD4. NBD-556 is the most competitive and lowers the affinity of sCD4 by a factor of 18.5. Surprisingly, compound 14, an analog of NBD-556, is the least competitive, despite its close structural similarity. Presumably, the higher flexibility of compound 14 combined with the lack of the bulky piperidine moiety allows for better accommodation within the cavity and lower interference with sCD4 binding. Compound 14 lowers the affinity of sCD4 by only a factor of three. These results indicate that small variations in the chemical structure of potential inhibitors may affect their degree of competitiveness and, consequently, their inhibitory potency, independently of their binding affinity. On the other hand BMS-806, which belongs to a different chemical class of inhibitors, lowers the affinity of sCD4 by a factor of seven, indicating that it is also not purely competitive or noncompetitive. Although the exact location of the binding site of BMS-806 in gp120 is not known, mutational analysis suggest that it binds somewhere between the CD4 and coreceptor sites, and that binding and antiviral potency is affected by changes in the variable loops [39]. In any case, it is clear that the inhibition of protein signaling, which is the goal in drug design, can be accomplished by either blocking the binding of the protein ligand or by allosterically blocking the propagation of binding effects. Since small-molecule inhibitors that bind at the protein–protein interface do not necessarily displace the protein ligand, allosteric inhibitors of signaling do not need to bind outside the protein binding interface.

Figure 6 graphically illustrates the required inhibitor concentration for 99% binding to gp120 as a function of the $[\text{sCD4}]/K_d$ ratio. It is evident that the inhibitor concentration required to saturate gp120 is a strong function of the degree of competitiveness of the inhibitor, as defined by the β parameter. The more competitive, the higher the amount of inhibitor required to achieve the same degree of binding. Inhibitors that do not need to compete with the protein ligand may effectively utilize their binding energy in inhibition of the protein signal rather than in the displacement of the protein ligand.

Effects of protein variability

The amino acid sequences of gp120 from the HIV-1 subtypes A and B isolates used in these studies (GenBank accession numbers, L34667 and M93258, respectively) show differences at 108 locations out of 510 amino acids. Although the majority of the polymorphisms are located away from the conserved CD4 binding site, CD4 binds to gp120-A with an affinity that is approximately ten times weaker than for gp120-B [40]. Two particularly important variations, D474N and E429R, are located at the CD4–gp120 binding interface and lie within 5 Å of NBD-556 as indicated in Figure 5 [30,40]. The competitiveness of the inhibitors is also affected by those amino acid variations as shown in Table 1. The binding

affinity of sCD4 to gp120-A is lowered by a factor of 1.5 by NBD-556, compared with 18.5 for gp120-B. A similar effect is observed for BMS-806. In this case the affinity of sCD4 to gp120-A is lowered by a factor of 1.9 compared with a factor of 7 for gp120-B. The effects are shown in Figure 7. These results demonstrate that small mutations in the protein are also able to affect the degree of competitiveness of the inhibitors. Presumably, mutations that weaken a binding hotspot (sCD4 binds to gp120-A with a ten-fold lower affinity than gp120-B) will also lower the degree of competitiveness of an inhibitor that binds in that region. Coincidentally, the ratio of the β values for NBD-556 to gp120-B and gp120-A is also close to ten.

Conclusion

The results obtained for the small-molecule inhibitors of sCD4/gp120 considered in this paper indicate that compounds that were believed to be competitive [30,35] or noncompetitive [38], were neither. It must be noted that in this paper a rigorous definition derived from a general model and precise competition experiments, rather than an operational definition, is utilized. Nevertheless, this rigorous definition provides an accurate way of experimentally evaluating the degree of competitiveness of a protein–protein inhibitor. This experimental approach can be utilized to establish structure–activity relationships aimed at guiding inhibitor optimization. In the development of these inhibitors as therapeutic drugs, the goal in structure–activity relationships should be the design of high-affinity protein–protein inhibitors in which the value of the α and β parameters approaches 1.

Another important result is that small chemical variations in the compounds or amino acid variations in the protein may have a profound impact on the degree of competitiveness. Retrospectively, those effects should not have come as a surprise. Within the binding interface only few spots contribute more than 1.5 kcal/mol to binding [21,41]. The β value measured for NBD-556 in gp120-B corresponds to a decrease of 1.7 kcal/mol in binding free energy, which is within the range expected for blocking a binding hotspot with a small molecule. The differences observed between gp120-B and gp120-A also support the idea that the decrease in binding affinity induced by the small molecular-weight inhibitor is of the same order to that observed by disrupting the particular binding spot to where it binds.

The results obtained from these studies indicate that the more energy-efficient inhibitors are those that are less competitive, provided that they effectively block protein signaling ($\alpha = 1$; $\beta = 1$). Due to the nature of the protein–protein interface, inhibitors with a low degree of competitiveness need not be located at distal regions from the protein binding site as demonstrated by NBD-556 and compound 14. By considering subsites within the interacting surface as target sites for small-molecule inhibitors several advantages are apparent. High-resolution structures of the protein complexes are usually available and different techniques, such as alanine scanning mutagenesis, can be utilized to identify binding hotspots and initiation signaling sites. On the other hand, the identification of potential target sites at distal regions from the protein–protein interface is not straightforward from structure.

The results presented here also delineate a clear strategy for the identification and optimization of small molecular-weight inhibitors of protein–protein interactions. First, it is evident that targeting binding soft spots (resulting in larger β values) is more advantageous than targeting high-affinity hotspots (resulting in β values approaching zero). As the inhibitor does not need to displace the protein ligand, a larger fraction of bound inhibitor is obtained under any set of conditions for larger β values. Ideally, one would like to identify a binding soft spot that is implicated in the initiation of the signaling cascade in order to approach the ideal combination, $\alpha = 1$ and $\beta = 1$. In addition to the situation with HIV-1

gp120–CD4 mentioned in this paper [22], the occurrence of non-overlapping binding hotspots and initiation signaling sites have been experimentally demonstrated for TNF- α binding to TNFR1 [6]. The mutation of a single residue in TNF- α [6] has been shown to affect allosteric signaling but not binding affinity, indicating that binding hotspots and non-overlapping initiation signaling sites can be identified by systematic mutagenesis within the protein–protein interaction surface.

Future perspective

The development of small molecular-weight inhibitors of protein–protein interactions and signaling will most likely succeed within the next decade. As a result, we will see a progressive replacement of biologics by small molecules.

Executive summary

- The inhibition of protein–protein interactions is a major target for drug development for different pathological conditions.
- The therapeutic goal of inhibiting protein–protein interactions is not the inhibition of binding but the inhibition of the signaling cascade that is initiated by binding.
- Results from HIV-1 entry inhibitors show that compounds that were believed to be competitive or noncompetitive inhibitors were neither.
- The more energy-efficient inhibitors are those that are less competitive, provided that they effectively block protein signaling.
- A general protein–protein inhibition model that extends from competitive to noncompetitive scenarios has been developed. The model provides rigorous design guidelines to achieve maximal inhibitory potency.
- A design goal in drug development is the engineering of inhibitors with high α and β values.

Key Terms

Binding hotspots	Although the surfaces involved in protein–protein interactions are large, relatively few amino acids within discrete regions contribute most of the binding energy. These regions are called binding hotspots.
Allosteric signaling	Interactions that take place in one region of a molecule and have functional effects in a distal region as a result of long-range interactions. The biological effect that originates from the binding of a protein ligand to a protein receptor provides a typical example of allosteric signaling.
Competitive inhibition	Pure competitive inhibition occurs when the natural ligand is displaced upon binding of the inhibitor molecule to the same site. Either the ligand or the inhibitor binds, but not both simultaneously.
Isothermal titration calorimetry	Technique that measures the heat released or absorbed when one reactant is added stepwise to a stirred solution containing the target molecule. The affinity and its components, the enthalpy and entropy changes, are obtained from a single titration experiment.

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Bibliography

1. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. *Nature*. 2007; 450(7172):1001–1009. [PubMed: 18075579]
2. Zinzalla G, Thurston DE. Targeting protein–protein interactions for therapeutic intervention: a challenge for the future. *Future Med. Chem.* 2009; 1(1):65–93. [PubMed: 21426071]
3. Callen JP. Complications and adverse reactions in the use of newer biologic agents. *Semin. Cutan. Med. Surg.* 2007; 26(1):6–14. [PubMed: 17349557]
4. Thurber GM, Schmidt MM, Wittrup KD. Factors determining antibody distribution in tumors. *Trends Pharmacol. Sci.* 2008; 29(2):57–61. [PubMed: 18179828]
5. Banner DW, D'arcy A, Janes W, et al. Crystal structure of the soluble human 55 kd TNF receptor–human TNF beta complex: implications for TNF receptor activation. *Cell*. 1993; 73(3):431–445. [PubMed: 8387891]
6. Shibata H, Yoshioka Y, Ohkawa A, et al. Creation and x-ray structure analysis of the tumor necrosis factor receptor-1-selective mutant of a tumor necrosis factor-alpha antagonist. *J. Biol. Chem.* 2008; 283(2):998–1007. [PubMed: 18003610]
7. Hage T, Sebald W, Reinemer P. Crystal structure of the interleukin-4/receptor alpha chain complex reveals a mosaic binding interface. *Cell*. 1999; 97(2):271–281. [PubMed: 10219247]
8. Rickert M, Wang X, Boulanger MJ, Goriatcheva N, Garcia KC. The structure of interleukin-2 complexed with its alpha receptor. *Science*. 2005; 308(5727):1477–1480. [PubMed: 15933202]
9. Philo JS, Aoki KH, Arakawa T, Narhi LO, Wen J. Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: one high-affinity and one low-affinity interaction. *Biochemistry*. 1996; 35(5):1681–1691. [PubMed: 8634300]
10. Syed RS, Reid SW, Li C, et al. Efficiency of signalling through cytokine receptors depends critically on receptor orientation. *Nature*. 1998; 395(6701):511–516. [PubMed: 9774108]
11. Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB. Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol. Cell Biol.* 1997; 17(12):7040–7046. [PubMed: 9372935]
12. Kussie PH, Gorina S, Marechal V, et al. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*. 1996; 274(5289):948–953. [PubMed: 8875929]
13. Sundstrom M, Lundqvist T, Rodin J, Giebel LB, Milligan D, Norstedt G. Crystal structure of an antagonist mutant of human growth hormone, G120R, in complex with its receptor at 2.9 Å resolution. *J. Biol. Chem.* 1996; 271(50):32197–32203. [PubMed: 8943276]
14. Walsh ST, Jevitts LM, Sylvester JE, Kossiakoff AA. Site2 binding energetics of the regulatory step of growth hormone-induced receptor homodimerization. *Protein Sci.* 2003; 12(9):1960–1970. [PubMed: 12930995]
15. Abbate EA, Berger JM, Botchan MR. The x-ray structure of the papillomavirus helicase in complex with its molecular matchmaker E2. *Genes Dev.* 2004; 18(16):1981–1996. [PubMed: 15289463]
16. Jansson M, Hallen D, Koho H, et al. Characterization of ligand binding of a soluble human insulin-like growth factor I receptor variant suggests a ligand-induced conformational change. *J. Biol. Chem.* 1997; 272(13):8189–8197. [PubMed: 9079636]
17. Chi MM, Schlein AL, Moley KH. High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology*. 2000; 141(12):4784–4792. [PubMed: 11108294]
18. Kaplan D. Autocrine secretion and the physiological concentration of cytokines. *Immunol. Today*. 1996; 17(7):303–304. [PubMed: 8763813]
19. Jones S, Thornton JM. Principles of protein–protein interactions. *Proc. Natl Acad. Sci. USA*. 1996; 93(1):13–20. [PubMed: 8552589]

20. Lo Conte L, Chothia C, Janin J. The atomic structure of protein–protein recognition sites. *J. Mol. Biol.* 1999; 285(5):2177–2198. [PubMed: 9925793]
21. Clackson T, Wells JA. A hot spot of binding energy in a hormone-receptor interface. *Science.* 1995; 267(5196):383–386. [PubMed: 7529940]
22. Schön A, Madani N, Smith AB, Lalonde JM, Freire E. Some binding-related drug properties are dependent on thermodynamic signature. *Chem. Biol. Drug Des.* 2011; 77(3):161–165. [PubMed: 21288305]
23. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature.* 1984; 312(5996):763–767. [PubMed: 6096719]
24. Klatzmann D, Champagne E, Chamaret S, et al. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature.* 1984; 312(5996):767–768. [PubMed: 6083454]
25. Kadow J, Wang H-G, Lin P-F. Small-molecule HIV-1 gp120 inhibitors to prevent HIV-1 entry: an emerging opportunity for drug development. *Curr. Opin. Investig. Drugs.* 2006; 7(8):721–726.
26. Repik A, Clapham PR. Plugging gp120s cavity. *Structure.* 2008; 16(11):1603–1604. [PubMed: 19000812]
27. Guo Q, Ho HT, Dicker I, et al. Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions. *J. Virol.* 2003; 77(19):10528–10536. [PubMed: 12970437]
28. Lalonde JM, Elban MA, Courter JR, et al. Design, synthesis and biological evaluation of small molecule inhibitors of CD4–gp120 binding based on virtual screening. *Bioorg. Med. Chem.* 2011; 19(1):91–101. [PubMed: 21169023]
29. Lin PF, Blair W, Wang T, et al. A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proc. Natl Acad. Sci. USA.* 2003; 100(19):11013–11018. [PubMed: 12930892]
30. Madani N, Schon A, Princiotto AM, et al. Small-molecule CD4 mimics interact with a highly conserved pocket on HIV-1 gp120. *Structure.* 2008; 16(11):1689–1701. [PubMed: 19000821]
31. Meanwell NA, Wallace OB, Fang H, et al. Inhibitors of HIV-1 attachment. Part 2: an initial survey of indole substitution patterns. *Bioorg. Med. Chem. Lett.* 2009; 19(7):1977–1981. [PubMed: 19251416]
32. Meanwell NA, Wallace OB, Wang H, et al. Inhibitors of HIV-1 attachment. Part 3: a preliminary survey of the effect of structural variation of the benzamide moiety on antiviral activity. *Bioorg. Med. Chem. Lett.* 2009; 19(17):5136–5139. [PubMed: 19632112]
33. Wang T, Kadow JF, Zhang Z, et al. Inhibitors of HIV-1 attachment. Part 4: a study of the effect of piperazine substitution patterns on antiviral potency in the context of indole-based derivatives. *Bioorg. Med. Chem. Lett.* 2009; 19(17):5140–5145. [PubMed: 19664921]
34. Zhao Q, Ma L, Jiang S, et al. Identification of *N*-phenyl-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamides as a new class of HIV-1 entry inhibitors that prevent gp120 binding to CD4. *Virology.* 2005; 339(2):213–225. [PubMed: 15996703]
35. Schon A, Madani N, Klein JC, et al. Thermodynamics of binding of a low-molecular-weight CD4 mimetic to HIV-1 gp120. *Biochemistry.* 2006; 45(36):10973–10980. [PubMed: 16953583]
36. Hanna GJ, Lalezari J, Hellinger JA, et al. Antiviral activity, pharmacokinetics, and safety of BMS-488043, a novel oral small-molecule HIV-1 attachment inhibitor, in HIV-1-infected subjects. *Antimicrob. Agents Chemother.* 2011; 55(2):722–728. [PubMed: 21078951]
37. Lu RJ, Tucker JA, Pickens J, et al. Heterobiaryl human immunodeficiency virus entry inhibitors. *J. Med. Chem.* 2009; 52(14):4481–4487. [PubMed: 19534463]
38. Si Z, Madani N, Cox JM, et al. Small-molecule inhibitors of HIV-1 entry block receptor-induced conformational changes in the viral envelope glycoproteins. *Proc. Natl Acad. Sci. USA.* 2004; 101(14):5036–5041. [PubMed: 15051887]
39. Madani N, Perdigo AL, Srinivasan K, et al. Localized changes in the gp120 envelope glycoprotein confer resistance to human immunodeficiency virus entry inhibitors BMS-806 and #155. *J. Virol.* 2004; 78(7):3742–3752. [PubMed: 15016894]

40. Brower ET, Schon A, Freire E. Naturally occurring variability in the envelope glycoprotein of HIV-1 and development of cell entry inhibitors. *Biochemistry*. 2010; 49(11):2359–2367. [PubMed: 20166763]
41. Delano WL. Unraveling hot spots in binding interfaces: progress and challenges. *Curr. Opin. Struct. Biol.* 2002; 12(1):14–20. [PubMed: 11839484]

Figure 1. Continuum model for the inhibition of protein-protein interactions

The binding energy between two proteins is not evenly distributed throughout the binding interface. Since the protein-binding surface is larger than the surface of a small molecular-weight (500-Da) compound, the possibility exists that it will only perturb a binding spot and still allow the two proteins to bind, albeit with lower affinity. Depending on the exact binding location and the interactions of the small molecule, a variable effect may be obtained. The effect of the small molecule is given by the parameter β . If $\beta = 0$ the classical all-or-none competitive situation is obtained. If $\beta = 1$ the inhibitor does not affect the protein-binding affinity. Intermediate effects are obtained by different β values.

Figure 2. Fraction of inhibitor bound for different degrees of competitiveness

This shows the fraction of a protein receptor bound to inhibitor as function of inhibitor concentration for β values of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0. The curves were calculated by using a binding affinity for the inhibitor of 100 nM and at constant $[L]/K_d$ ratio of 10. To facilitate reader visualization, the inhibitor concentration is expressed in $\mu\text{g/mL}$ by assuming a molecular weight of 500 g/mol.

Figure 3. The inhibitor concentration at which 95% protein saturation is obtained for different β values

The values have been plotted for a wide range of $[L]/K_d$ values. For these simulations, the inhibitor affinity has been assumed to be 10 nM and its molecular weight 500 g/mol.

Figure 4. Chemical structures of some HIV-1 cell entry inhibitors: (A) NBD-556, (B) Compound 14 and (C) BMS-806

Figure 5. The structure of gp120 (blue) in complex with CD4 (green) and with the inhibitor NBD-556 (red) docked into the structure of gp120 (PDB entry 1G9M) NBD-556 binds within the large interaction surface. The figure clearly illustrates the size difference between the CD4 binding footprint and the small-molecule inhibitor. The arrows indicate the location of important amino acid polymorphisms between gp120 from subtype B and subtype A.

Figure 6. The concentration of NBD-556, compound 14 and BMS-806 required for 99% saturation, $[I]_{99}$, of subtype B gp120 as a function of the $[sCD4]/K_d$ ratio. NBD-556 and compound 14 have similar affinities, however, NBD-556 is the most competitive and the concentration required for saturation of gp120 is much higher for NBD-556 than for compound 14.

Figure 7. Comparison of the $[I]_{99}$ values for inhibition of gp120 from subtypes A and B with NBD-556 and BMS-806

NBD-556 is a much less competitive inhibitor of CD4 binding to subtype A than subtype B gp120 and saturation is reached at lower concentration despite the somewhat weaker affinity.

Table 1

Inhibition of CD4 binding to gp120 from subtypes A and B.

sCD4	NBD-556		Compound 14		BMS-806	
Protein K_d (μ M)	K_d (μ M)	β	K_d (μ M)	β	K_d (μ M)	β
gp120-B 0.014 [†]	3.7 [‡]	0.054	3.0 [‡]	0.32	0.7 [§]	0.14
gp120-A 0.1 [¶]	5.5 [¶]	0.67	ND	ND	0.7	0.53

K_d values were determined at 25°C by isothermal titration calorimetry as described previously [35]. gp120 from the A and B subtypes were expressed and produced as described elsewhere [35,40]. β -values were determined by measuring CD4 binding to gp120 in the absence and presence of 200 μ M inhibitor. Compounds were kindly provided by A Smith, University of Pennsylvania, Philadelphia, PA, USA.

[†] Data from [35].

[‡] Data from [28].

[§] Data from [38].

[¶] Data from [40].

ND: Not determined.