Let's get specific: the relationship between specificity and affinity

The factors that lead to high-affinity binding are a good fit between the surfaces of the two molecules in their ground state and charge complementarity. Exactly the same factors give high specificity for a target. We argue that selection for high-affinity binding automatically leads to highly specific binding. This principle can be used to simplify screening approaches aimed at generating useful drugs.

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In fields ranging from pharmacology to materials science, the affinity and specificity of binding interactions affect how well molecules serve their function. We have been led to consider the rules that govern affinity and specificity, and the relationship between them, by our work on oligonucleotide ligands discovered via the SELEX (systematic evolution of ligands by exponential enrichment) combinatorial chemistry protocol [1,2]. In its most straightforward application this protocol seeks oligonucleotides that bind to a particular target with high affinity, and yet high specificity for that target is frequently also found. We have come to the conclusion that a sufficiently high-affinity ligand can be confidently expected to be highly specific for its target. Here, we describe some observations on surprisingly specific target binding by SELEX-derived oligonucleotides, and the reasons for the correlation between affinity and specificity.

Sources of affinity and specificity

Electrostatic and hydrophobic effects dominate all attractive interactions between two molecules; hydrogen bonding is often particularly important, especially in nucleic acid recognition. Shape complementarity is also crucial. The highest possible affinity toward a target molecule would be obtained with a ligand having a perfect mirror image of the shape of the target surface, with a charge distribution perfectly complementing that of the desired target surface. Conformational flexibility can be a major factor limiting the affinity and specificity of interactions. Molecules that can easily undergo induced fit will be able to interact with a variety of targets, but will pay the price of decreased affinity due to a conformational energy penalty. On the other hand, molecules with well defined solution ground states that naturally complement the desired target will bind to fewer targets, but will do so with higher affinities. A truly ideal ligand will exhibit dynamic motions that are in tune with those of the target, so that both molecules can flex in concert, minimizing the loss of entropy on binding.

Chemists working on drug development have pursued several approaches to producing the ideal ligand, ranging from the traditional (and still the most successful) approach of natural product screening followed by systematic modification, to rational design, to combinatorial chemistry. The appeal of combinatorial chemistry is that, depending on the library size, a large array of interaction geometries can be explored simultaneously. A superior combinatorial drug discovery process would select for optimum interaction, electrostatic and hydrophobic, between the drug lead and the target, providing high affinity binding. As noted above, the process would ideally select molecules with a well defined ground state that do not have readily accessible tautomers or conformers; unless this is the case, the assay will frequently select ligands that require induced fit to bind to their targets, reducing their likely affinity and specificity. The ideal selection process must also allow the identification of very rare components of the library as tight-binding ligands, for example by allowing reiterative amplification and re-selection of components of the library. Our own focus, SELEX, is a reiterative combinatorial chemistry technology that meets all of these criteria.

Nucleic acids as drugs

The structural diversity of drugs is enormous. Approved drugs range from small, orally active, organic molecules to large proteins with molecular weights of up to 200 kD. The diversity of investigational drugs is even larger. What, then, defines a drug? One definition would be that a drug is any molecule that causes a desired alteration in metabolism or physiology by binding to a specific target molecule (or to a small number of targets). Any combinatorial system that routinely produces high-affinity ligands for target molecules therefore has a good chance of producing potential drug candidates.

Nucleic acids have turned out to be surprisingly good at generating high-affinity ligands for a variety of protein and small molecule targets. Ligands for over 100 protein targets have been developed using SELEX, generally with affinities in the nM range or better. One major reason for this success appears to be the relative rigidity of nucleic acids compared to peptides or other polymeric structures. Most of the functional groups that are involved in binding to target molecules are only one atom removed from the ring of a base or a sugar, with

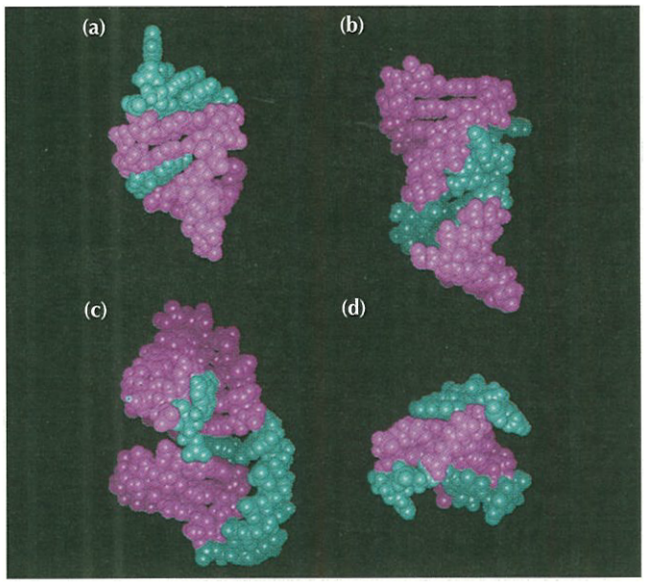

Fig. 1. Structural motifs for SELEX ligands. The four distinct motifs, **(a)** hairpin stem, **(b)** symmetric and asymmetric bulges flanked by helical regions, **(c)** pseudoknot, and **(d)** G quartet, are shown as three-dimensional models. The 'single-stranded' regions are colored light blue and the structural regions (helices and quadruplexes) are colored magenta.

the exception of the phosphodiester bond. The conformational flexibility of these groups is therefore very limited. Only the ribose ring, phosphodiester, and glycosidic bonds have torsional conformations, in contrast to proteins (or most polymeric materials) where, in addition to the backbone, the sides chains have significant torsional freedom and numerous conformational states. Base pairing further restricts the conformations of the monomers within oligonucleotides. These qualities make nucleic acids relatively rigid. For a nucleic acid ligand, a good fit is really good; a bad fit cannot be accommodated by 'induced fit' because of restricted conformational freedom, and therefore is selected against very early in the screening process. The ideas we describe here were developed from observations on nucleic acids, but we believe that they have broader relevance.

The ligand families found through SELEX to date primarily fall into four structural motifs (Fig. 1): hairpin stems and loops, symmetric and asymmetric bulges flanked by helical regions, pseudoknots, and G quartets [3]. The binding surfaces in these oligonucleotides always include some 'single-stranded' domains, probably because those domains provide the greatest variation in shapes and therefore have the best chance of generating a surface complementary to any target. Similarly, the binding sites of proteins are often formed from loop regions that do not have α-helix or β-sheet secondary structure. Only one detailed structure of a SELEX-derived oligonucleotide is available [4,5], although many such molecules are under study. We believe that the 'single-stranded' domains of these oligonucleotides will be found to be severely constrained through their fixed ends, standard and non-standard base pairing interactions, and tertiary interactions similar to those seen in the handful of oligonucleotide structures that have been determined [6–8]. Thus, the structured regions of these oligonucleotides are unlikely to reside solely in their helical regions.

The importance of being specific

Specificity is important both for drug discovery and for toxicity profiles. As more and more drugs are developed to inhibit particular molecular targets, it seems reasonable that extreme specificity of a potential drug for the intended target will be important. Many ideas about which target is the right one to use to treat a particular disease will be wrong, since our understanding of biology is far from perfect; to go forward from a right idea or to discard a wrong idea requires data that definitively show that a given compound, which inhibits the activity of its target completely and specifically, does or does not affect the course of a disease in an appropriate animal model (or in a human clinical trial). The overall process of drug discovery is thus facilitated by experiments with highly specific compounds. And, of course, a highly specific drug would be expected to have fewer side effects, simplifying the process of evaluating the results of a clinical trial.

How to achieve high specificity?

What are the strategies available for identifying molecules that bind to a target of interest with high specificity? Since we know relatively little about the environment in which the ligand will have to show highly specific binding, and what the molecules competing with the target for ligand binding may be, the best approach we can take is to select for optimum interaction, electrostatic and hydrophobic, between the drug candidate and the target, with good shape complementarity — in other words, to select for high affinity. We have used the SELEX protocol to screen for high-affinity ligands for a number of proteins and small molecules. The procedure is diagrammed in Figure 2; a pool of random oligonucleotide sequences are allowed to bind to a target, poor binders are discarded, and good binders are amplified for re-selection. Note that there is no element of selection for lack of binding to undesired targets in this strategy (although such counter-selection steps have been added in variations on this theme). Because it is possible to amplify selected pools directly in a nucleic acid library, the protocol allows one to search for compounds that are present at a very low level (perhaps at levels of 10^{-9}–10^{-13} [1]), which would be impossible to detect in a small-molecule or conventional peptide combinatorial library (amplifiable peptide libraries also exist, however; see [9]).

As noted above, it is possible to identify nucleic acid ligands for diverse protein targets using SELEX. We have also studied ~20 small molecule targets. The highest affinity small-molecule ligand reported thus far has a K_d of ~100 nM [1]. There are probably two reasons why ligands to small molecules typically have lower affinities

than to protein targets. First, many of the small molecule targets have been less rigid than the protein targets, and thus must themselves become conformationally fixed upon binding to the oligonucleotide ligand; thus, an entropic cost is paid as the target becomes more structured than it was before binding [10]. Second, the area over which interaction with a small molecule must take place is inevitably smaller than the area of interaction with a protein, reducing the number of functional groups available for binding interactions. High affinity can therefore be achieved only with exquisite shape and interaction complementarity over the few interaction sites available.

The SELEX-derived ligands do have remarkable specificity, however. Oligonucleotide ligands have been identified that can discriminate D versus L amino acids [11–13], reduced versus oxidized cofactors [14], theophylline versus caffeine [15], and ATP versus other purine nucleotides [16]. How does this high specificity arise from a selection process that is designed to identify high-affinity binders? How can relatively low-affinity binding lead to exquisitely specific binding?

Binding events and specificity

Specificity is a term used to describe a wide variety of processes in molecular biology, including enzyme kinetics, gene regulation, and drug molecule interactions. In this discussion, we will take specificity to refer to a functional discrimination between molecules competing for a common ligand. Such discrimination is usually the result of differences in the free energy of interaction for the molecules of interest, although kinetic discrimination can presumably exist for some systems. From a thermodynamic viewpoint, specificity can be defined by considering the coupled equilibria between species in solution. This analysis (after von Hippel and Berg, [17]) is shown in Box 1, from which it is easy to see that the specificity term, α_s, is the ratio of the product of K_d and concentration for the target protein P to the sum of the product of K_d and concentration for all other competing species. Thus, there is no relationship between specificity and the absolute affinity of L for target P. It is therefore possible to have high specificity with relatively low affinity provided that $K_L >> K_{Bi}$ for all B_i, when P and B_i are similar in concentration. The larger the aggregate concentration of B_i, the greater the difference between K_L and K_{Bi} required to give a desired level of specificity.

What is the effect on specificity of systematically increasing the affinity of a ligand for a particular target? Typically, this will result in higher specificity; the interactions that lead to high affinity of L for P will usually not lead to similar increase in binding affinity for all B_i. Increased affinity is achieved through increasingly exacting geometric dispositions of functional groups in the

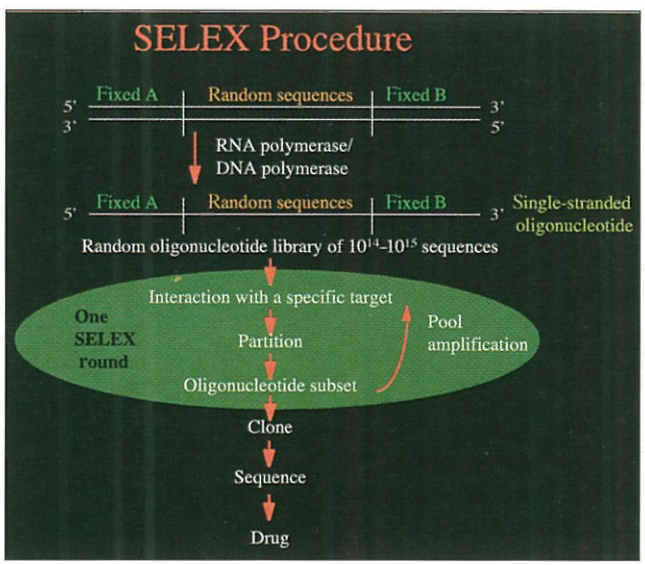

Fig. 2. Diagram of the SELEX procedure. A library composed of 10^{14} to 10^{15} sequences of single-stranded oligonucleotides is assembled. Each sequence contains a random region flanked by fixed sequences, denoted A and B here, that permit amplification and transcription. A round of SELEX begins by allowing the library to interact with the target, followed by a selection procedure, and finally pool amplification of the selected population. After several rounds the resulting population is cloned and sequenced. See [1,9] for details.

Box 1. The binding events that govern specificity.

Consider a protein target P that binds to its natural substrate S with an equilibrium binding constant K_S that relates the concentrations of the complexed and uncomplexed species, thus:

$$P + S \rightleftharpoons P{:}S\ ; \quad K_S = [P{:}S] / [P][S] \qquad (1)$$

where P:S is a non-covalent complex. Similarly, a second ligand, L, binds to P with a constant K_L and competes with S.

$$P + L \rightleftharpoons P{:}L\ ; \quad K_L = [P{:}L] / [P][L] \qquad (2)$$

L may be an inhibitor of P when P is an enzyme, or an antagonist or agonist of P when P is a signaling protein, for example. The relative affinities and concentrations of S and L for P will largely determine the biological response on P due to L; these considerations are most important when considering efficacy. But molecule L may interact with a variety of other proteins, B_is, in the system:

$$B_i + L \rightleftharpoons B_i{:}L; \quad K_{Bi} = [B_i{:}L]/[B_i][L] \qquad (3)$$

lowering its effective concentration and altering its efficacy.

We can define a measure (α_s) for the discrimination of L for its specific site P relative to the nonspecific sites (B_is) as

$$\alpha_s = [P{:}L] / \Sigma_i [B_i{:}L], \text{ or, } \alpha_s = K_L [P][L] / \Sigma_i K_{Bi}[B_i][L] \quad (4)$$

where α_s varies from 0 for poor specificity to infinity for high specificity. It is apparent by inspection that the specificity of binding does not depend on the concentration of L, thus

$$\alpha_s = K_L [P] / \Sigma_i K_{Bi}[B_i]. \qquad (5)$$

Thus, the specificity of binding is determined by the ratio of the product of the K_d and concentration for the desired binding event to the sum of the product of K_d and concentration for all other interfering binding events.

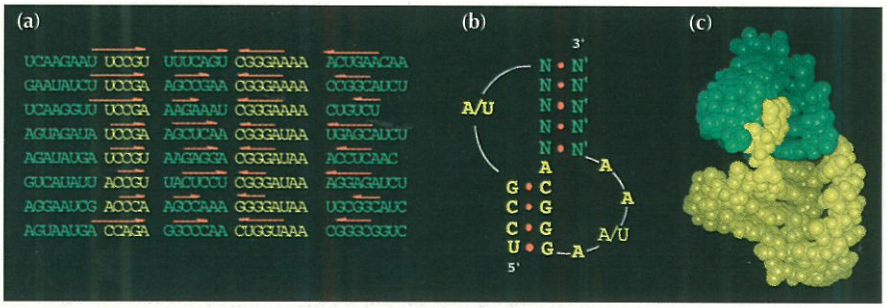

Fig. 3. SELEX ligands for HIV-1 RT. **(a)** Alignment of eight representative sequences reveals two regions of conserved sequence (highlighted in yellow). **(b)** The expected secondary structure. The two stems of the pseudoknot are separated by a variable length single-stranded region. **(c)** A model of the three-dimensional structure. Yellow, conserved sequence (presumably the binding site); green, variable regions.

ligand/target complex and these detailed geometries of functional groups are not shared by the background binders. Even if one background binder does show increased affinity, it is highly unlikely that all of them will; indeed, there must be at least an equal likelihood that changes that increase binding to P will decrease binding to one or more B_i (although decreases in the affinity with which the low-affinity competitors bind to the ligand will not be as important as changes in the affinity of the high-affinity competitors). On average, therefore, the term $\Sigma_i K_{Bi}[B_i]$ would be expected to increase less than the term $K_L[P]$.

For nucleic acid drugs, the class of molecules that can act as non-specific binders (B_i) may be particularly diverse. Many proteins bind all nucleic acids non-specifically, with affinities in the μM range. This suggests that diagnostic and therapeutic oligonucleotides should have nM affinities or better if they are to be useful; although this goal has been successfully achieved for protein targets, it appears to be harder to reach for small molecule targets.

HIV-1 reverse transcriptase

One example of surprisingly specific binding resulting from selection for high-affinity binding is the SELEX-derived RNA ligand for human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT) [18]. The winning family (see Fig. 3) of RNAs that bound to HIV-1 RT formed an obvious pseudoknot structure, based on the covariation of nucleotides that form base pairs. The winning RNA ligands bound to HIV-1 RT with K_ds in the nM range, as did ligands isolated from single-stranded DNA libraries [19]. Most importantly, and surprisingly, the HIV-1 RT ligands bound poorly to avian myeloblastosis virus (AMV), murine moloney leukemia virus (MMLV), and feline immunodeficiency virus (FIV) RTs [18,20]. This is particularly surprising since these ligands bind to the active site of HIV-1 RT, and the active sites of all of the viral RTs tested are expected to be very similar, since they all have the task of binding to similar primer–template complexes to initiate DNA synthesis. Indeed, ligands that were identified for AMV, MMLV or FIV RTs are also each exquisitely specific for their own target [20].

Selectivity for one member of a family

Finding a ligand that binds selectively to an enzyme from one virus and not to similar enzymes from related viruses may be surprising, but is not especially physiologically important. A more relevant story comes from experiments aimed at identifying high-affinity ligands for basic fibroblast growth factor (bFGF) [21]. This ligand binds in the heparin-binding site, a basic domain of the protein. The other members of the FGF protein family [22] also have a basic domain, and yet the selected ligand is not bound tightly by those proteins. Similarly, the ligand fails to bind tightly to other heparin-binding proteins. Comparable data have been obtained for the ligand that binds to vascular endothelial growth factor [23], for which even more highly specific ligands are reported in a paper in this issue [24], and also for the ligand that binds to keratinocyte growth factor (N. Pagratis *et al.*, personal communication).

The binding affinity of one of the bFGF ligands has been determined for five related proteins from the FGF family and for four heparin binding proteins and is presented in Table 1. The table also shows the specificity measure α_s defined in Box 1 for competition between the desired target and nonspecific targets (in this case, a single alternative binder) and the related measure f_s (the fraction of ligand bound to the desired target, calculated as $[P:L]/([L_{total}]-[L])$, or $f_s = (1+\alpha_s^{-1})^{-1}$. The ligand binds to the native bFGF protein with a K_d of 3.5×10^{-10} M, whereas the next best affinity, for FGF-5, is two orders of magnitude lower. The specificity descriptors were computed using a total protein concentration of 1.0×10^{-9} M for each species and a total ligand concentration of 1.0×10^{-8} M to ensure saturation of the specific site in the absence of

Table 1. Specificity of ligand for native bFGF. Relative binding affinity ($K_d^{bFGF}/K_d^{protein}$) of ligand for proteins from the FGF family of proteins and for several heparin-binding proteins.

Protein[a]	$K_d^{bFGF}/K_d^{protein}$ [b]	α_s	f_s
aFGF (FGF-1)	$3.6 \pm 0.4 \times 10^{-4}$	103.1	0.99
K-FGF (FGF-4)	$6.2 \pm 2.2 \times 10^{-4}$	60.3	0.98
FGF-5	$4.1 \pm 1.4 \times 10^{-2}$	1.9	0.65
FGF-6	$5.7 \pm 0.8 \times 10^{-4}$	65.5	0.98
KGF (FGF-7)	$7.8 \pm 0.9 \times 10^{-4}$	48.2	0.98
VEGF	$8.2 \pm 0.9 \times 10^{-4}$	45.9	0.98
PDGF AB	$2.5 \pm 0.3 \times 10^{-3}$	15.8	0.94
Antithrombin III	$8.2 \pm 1.2 \times 10^{-6}$	4483.5	1.00
Thrombin	$3.1 \pm 0.5 \times 10^{-5}$	1186.7	1.00

[a]Abbreviations: aFGF: acidic FGF; KGF: keratinocyte growth factor; VEGF: vascular endothelial growth factor; PDGF AB: platelet-derived growth factor, AB isoform.
[b]The K_d^{bFGF} value of $3.5 \pm 0.3 \times 10^{-10}$ M for ligand min24A/t (5'-GGUGUGUGGAAGACAGCGGGUGGUUC-3') is used for computing the relative affinities.

nonspecific binding. The individual values of α_s range from 1.9 to over 4500, while the fraction of specific sites occupied, f_s, varies from a low of 0.65 to a high of 1.0.

If all of the proteins listed in Table 1 were present together at the same concentration *in vivo*, α_s would be 1.5 and f_s 0.60. Only FGF-5 binds a significant amount of the ligand that was selected to have high affinity for bFGF; when 60% of the bFGF molecules in the mixture are bound to ligand, 35% of FGF-5 molecules will be bound as well.

The problem of closely-related targets

Our premise, as discussed above, is that selection for very high affinity binding will inevitably produce ligands with a high α_s. The example of the FGF growth factor family shows this, but also illustrates the main problem with an approach that selects only for high-affinity binding; an alternative target that is sufficiently closely related to the desired target will continue to be an effective competitor even at very high levels of affinity (in this case a K_d of 0.35 nM). Selecting for even tighter binding would, we believe, eventually give selective binding even when the competitor is closely related. If the competing target is known, however, it is usually more expedient to select against unwanted binding. A protocol called counter-SELEX has been developed for this purpose, and has been used to identify oligonucleotide ligands that selectively bind to theophylline, but not to caffeine. The two structures differ only at N7 where a hydrogen in theophylline is replaced by a methyl group in caffeine (Fig. 4); the ligand discriminates against caffeine by greater than 10 000-fold [15].

The effect of non-specific binding on specific binding

To consider the kind of competition that may occur *in vivo* in a more quantitative way, we have calculated the binding parameters for a ligand aimed at a target molecule present at 25 nM concentration, which is the average protein concentration for an organism with 100 000 different proteins of average mass 40 kD at a total concentration of 100 mg ml^{-1}. The ligand is assumed to have a K_d for the target protein of 10^{-10}. The fraction of the target protein bound — $[P:L]/P_{total}$ — is calculated as a function of ligand concentration (Fig. 5). The figure also shows the fraction of protein-bound ligand that is bound to the intended target, f_s. For the simplest case, with no

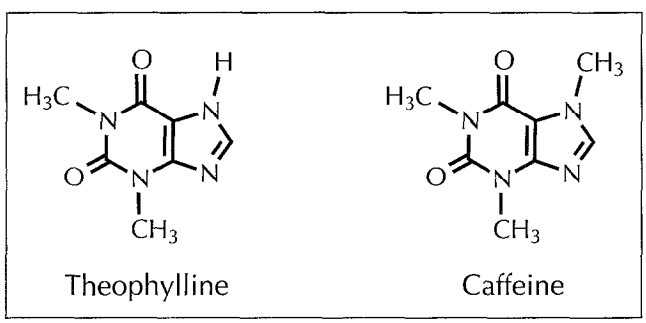

Fig. 4. The structures of theophylline and caffeine.

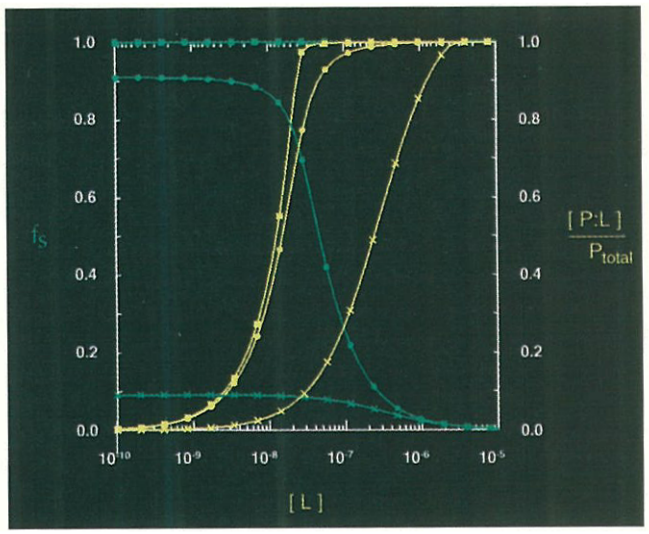

Fig. 5. The effects of nonspecific sites on specific binding. The yellow curves are the fraction of specific target bound by ligand, P:L, with 0.1 nM affinity, relative to P_{total} as a function of ligand concentration [L] for (squares) no nonspecific sites, (circles) 100 000 sites with 0.1 mM affinity and (crosses) 100 sites of 1nM affinity plus 100 000 sites at 0.1 mM affinity. The green lines are the specific fraction of L bound, f_s, as a function of [L] using the same marker coding as above.

other protein in the system, $f_s = 1$; all binding occurs to the specific target.

Let us now imagine that all 100 000 proteins, each present at 25 nM, have nonspecific binding at the same low affinity, having a K_d of 10^{-4}. (This is in the same range as the level of nonspecific binding shown for competitors such as thrombin, which are not FGF family members, in the bFGF example, above). In this case the binding of the ligand to the intended target occurs to nearly the same extent as when only the intended protein is in the system. Even when the target protein is 90 % saturated with ligand, roughly half of the ligand bound to protein is bound to the intended target. More importantly, the 100 000 proteins, each present at 25 nM, will themselves be bound with ligand to a negligible extent, since the ligand not bound to the intended target will distribute uniformly on all 100 000 proteins. In this situation there will be no effect on the activity of any of these 100 000 proteins.

In the final case, we have added 100 proteins with a K_d of 10^{-9} to the system, in addition to the 100 000 proteins with low affinity. This has two major effects. First, the amount of ligand needed to saturate the intended target is nearly 100-fold higher than in the other two cases. Second, and more importantly, the fraction of ligand that is bound to unintended proteins when the intended target is near saturation is very high. The ligand could therefore affect the activities of any of these proteins, assuming that it binds to a site that is important for the function of the protein. At 90 % saturation, 1.86 % of the bound ligand occupies the intended target and 47 % of each protein with nM affinity will be occupied. This

graphically illustrates the problem with unintended variables in an animal experiment or a human clinical trial. In the real situation, these 100 proteins will almost all be unidentified, and it is thus hard to imagine a strategy that could directly increase the specificity of a ligand by selecting against binding to the unintended targets. Fortunately, this model is probably not realistic; when a ligand reaches a K_d of 10^{-10}, it is likely to be highly specific for its intended target, and to cross-react significantly with only a few closely-related proteins (as is the case for the bFGF ligand described above). Thus, in most drug discovery efforts, aiming for very high affinity of binding may be sufficient to give a high enough selectivity for a compound to be clinically useful.

Although in this article we have focused on the potential of selection based on equilibrium thermodynamics, it should be noted that this is not the only strategy available. For example, it is possible to evolve ligands that irreversibly crosslink their targets with high specificity and affinity [25]. It is also possible to direct binding and crosslinking specifically to the active site of an enzyme [26]. For the purposes of SELEX-based drug development, it is encouraging that, so far, it has been possible to use SELEX to identify high-affinity ligands no matter what the target. That these high-affinity ligands are truly specific for their intended target can be shown by assaying them in the presence of whole blood or serum; the presence of multiple alternative protein targets does not interfere with the binding of these ligands to their intended target. It seems certain that nucleic acid based drugs will soon become a standard feature of the pharmacological landscape, both as diagnostics and as therapeutics.

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