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■ INTRODUCTION

Appropriate tuning of binding selectivity is a primary objective in the discovery and optimization of a compound on the path toward developing a drug. The environment in which drugs act is complex, with many potential interaction partners. Proteins, DNA, RNA, lipids, sugars, metabolites, and other small molecules all have the potential to interact with a drug, and in many cases these unexpected interactions lead to undesired and often severe side effects. Conversely, the ability to interact with multiple targets or drug resistance mutants can be advantageous in certain contexts. Designing a drug with the appropriate balance of avoidance of undesirable targets (narrow selectivity) and coverage of one or more targets of interest (broad selectivity, also referred to as promiscuity) is a continual drug development challenge. In many cases this objective is attained through trial and error, but there are rational approaches that can guide the tuning of selectivity, and examples have been published that illustrate a number of generalizable strategies. In this review, we discuss fundamental principles that account for selectivity and highlight examples where selectivity has been attained through rational design. An understanding of the general principles that drive selectivity should allow for more efficient design of compounds with desirable selectivity profiles.[1−3]

Traditionally, drug design has been pursued with the primary objective of finding a compound that binds with high affinity to a target of interest.[4] Recently, considerable effort has been expended measuring off-target interactions with partners such as ion channels (including the Kv11.1 potassium ion channel hERG),[5,6] cytochrome P450s (CYPs),[7,8] and other proteins that can lead to adverse side effects. Other considerations, such as family or subtype selectivity have gained considerable attention for targets with homologues that bind to the same or similar native substrates. A common example is the kinase family (i.e., phosphotransferases), for which each family member binds ATP in the process of transferring a phosphate group to a substrate.[9] From a drug discovery perspective, the aim is to hit only one or a subset of kinases along the biochemical pathway of interest while avoiding other kinases for which inhibition may result in adverse effects.[10] In practice, absolute selectivity for a single kinase may be unattainable, but modulating the selectivity profile can lead to improved drug properties and in many cases hitting multiple kinases can be beneficial.[11]

While it is most common to design away from interactions with undesirable proteins, in other cases it is desirable to hit a panel of targets.[12,13] An example of this type of broad coverage involves designing a drug that is not sensitive to resistance mutations, which requires a molecule that binds to drug-resistant variants as well as to the wild-type target. This type of promiscuous, broad coverage is particularly important for rapidly mutating targets, such as those that occur in infectious disease (with HIV being a prototypical example) and cancer. This aspect of drug discovery is of growing importance, as witnessed by the evolution of resistance to existing anti-cancer[14−16] and antimicrobial agents (antibiotics,[17] antivirals,[18] antifungals,[19] and antimalarials[20]). Similarly, when multiple pathways are accessible for a given signaling cascade, it may be desirable to hit at least one member of each parallel pathway in order to successfully block the downstream signal. Recently, the idea of deliberately using promiscuous drugs has gained credence.[11] However, this promiscuity must itself be selective for a given subset of targets, and nonspecific binding is always undesirable. In general, there is a fine balance in designing the appropriate level of narrow and broad selectivity, and one must determine the design criteria for selectivity based on the relevant biological processes.

The importance of gaining selectivity has been appreciated for many years, and there are a number of experimental approaches to screen for off-target interactions.[21−23] While performing an exhaustive selectivity screen against all possible interaction partners is still intractable, it is possible to construct selectivity screening panels that can be used to gain insights and find more selective compounds.[21]

Conceptually, the problem of designing for a particular selectivity profile is significantly more complex than designing for high affinity to a single target. This is true whether purely experimental approaches are being undertaken or whether computational analysis and design are involved. The underlying problem is challenging because it is necessary to evaluate energy differences for each ligand binding to a panel of targets and decoys rather than to a single desirable target. Computational methods are of limited accuracy when predicting affinities of individual complexes; these difficulties are compounded when multiple relative affinities are required to accurately design appropriate specificities. From a computational perspective, structure-based design methods typically are developed to yield low false-positive rates (i.e., to maximize the
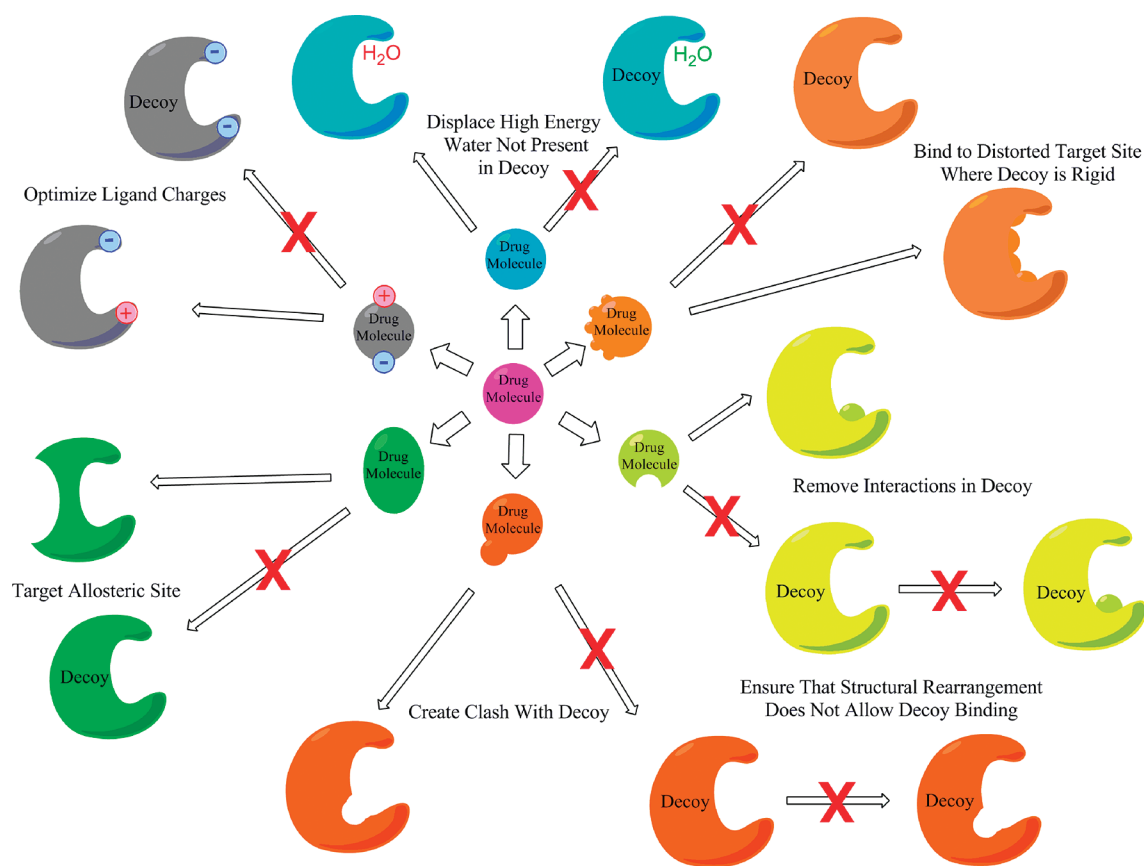
Figure 1. Selectivity Strategies. This cartoon illustrates six design strategies based on five principles (shape, electrostatics, flexibility, hydration, and allostery) that can be employed to gain binding selectivity for a given target: (A) optimization of ligand charges specifically for the target and against the decoy; (B) displacement of a high-energy water molecule in the target that is not present in the decoy; (C) binding to an allosteric pocket in the target that is not present in the decoy; (D) creating a clash with the decoy receptor but not the target receptor, where the decoy is unable to alleviate the clash by structural rearrangement; (E) binding to a receptor conformation that is accessible in the target but inaccessible in the decoy; (F) creating an interaction with the target receptor but not the decoy receptor, where the decoy is unable to form the interaction by structural rearrangement. Note that (D) and (F) are different manifestations of the same underlying principle (shape complementarity), with (D) decreasing binding to the decoy through the introduction of a clash and (F) increasing binding to the target through the introduction of a favorable contact.

chance that predictions of tight binders are in fact tight binders) at the expense of higher false-negative rates (tight binders that are not predicted to be so by the computational method). Accurate selectivity prediction and design require reducing the false-negative rate without increasing the false-positive one. This is a difficult search problem and can require very fine sampling of conformational space, including protein and ligand intramolecular degrees of freedom, as well as intermolecular ("pose") degrees of freedom. This problem becomes increasingly more difficult if the proteins and/or ligands have significant flexibility, as the size of the search space increases enormously. Essentially, designing for selectivity is significantly more complex than designing for affinity for two reasons: first, because of the multifactorial nature of the task and, second, because of the inherent difficulty of considering all modes of relaxation with sufficient accuracy, particularly when ligands bind decoy receptors.

In this review we highlight some recent examples of successful approaches to achieving changes in selectivity. We present cases where the goal required narrowing the binding profile to one or a small number of targets and increasing the relative binding affinity to targets over decoys, and we present cases where the goal required broadening the binding profile to increase the number of targets bound and flattening the relative affinity across the panel of targets. We have deliberately elected to organize the discussion around a set of principles that have

proven enabling in realizing selectivity goals. In very simple yet still useful terms, achieving broad selectivity involves recognizing and exploiting similarities in binding capabilities across a collection of targets, and narrow selectivity involves identifying and exploiting differences between targets and decoys. Most of the review examines five aspects of binding and complementarity that have proven useful handles that we have grouped together as structure-based approaches. These five features (shape, electrostatics, flexibility, hydration, and allostery) have been utilized because they differ, whether subtly or substantially, across sets of target and decoy molecules sufficiently to realize the affinity changes necessary for selectivity. The principles of exploiting the features listed above are schematically represented in Figure 1, and we will describe and discuss each in detail. The review continues by discussing other approaches that involve higher-level concepts beyond taking advantage of structural similarities and differences, although ultimately they can often be achieved through structure-based approaches. We describe a substrate-mimetic approach to developing broad inhibition across a population of rapidly mutating enzyme targets (called the substrate envelope hypothesis), and we also describe methods for leveraging differences in cellular environments to achieve selectivity goals. We have necessarily chosen a limited number of examples from the recent literature to review and illustrate the narrative that
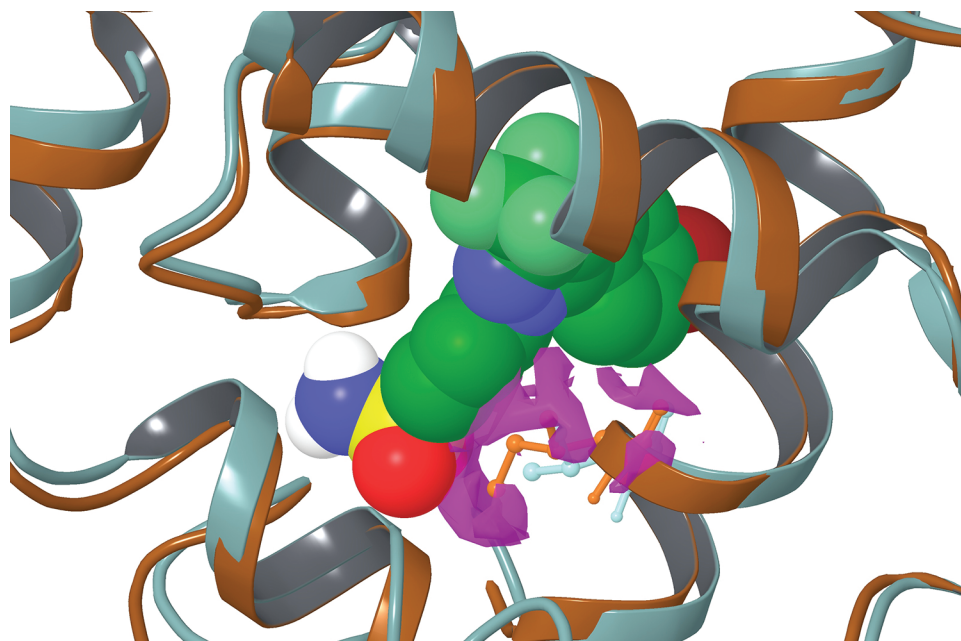
Figure 2. Shape complementarity in specific COX-2 inhibition. The crystal structure of COX-2 complex from PDB entry 6COX[32] overlaid with the apo crystal structure of COX-1 from PDB entry 3N8V.[181] The ligand is displayed in atom colored space filling. The proteins are displayed as colored ribbons, and residues V523 from COX-2 and I523 from COX-1 are displayed as colored balls and sticks. The difference between the molecular surfaces of COX-2 residue V523 and COX-1 residue I523 is displayed in magenta.

we have set forward. We apologize in advance for necessary omissions and any inadvertent oversights that kept us from including all of the truly wonderful advances in this field. We also note that reviews on related topics have appeared that will also be of use to the interested reader.[24−28]

■ STRUCTURE-BASED SELECTIVITY DESIGN CONSIDERATIONS

Shape Complementarity. Shape complementarity between ligands and receptors is a fundamental aspect of molecular recognition,[29] and there are numerous cases where selectivity for natural substrates is attributable to the specific shape of the binding site.[30,31] Unsurprisingly, molecular shape has proven to be important in the rational design of selective inhibitors. For example, narrow selectivity is essential for effective COX-2 inhibitors to control pain and inflammation while lowering the risk of peptic ulcers and renal failure associated with nonselective COX inhibitors. Structural analysis by Kurumbail et al. highlighted a selectivity pocket that is accessible in COX-2 but not in COX-1 because of the V523I substitution.[32] Other than this small change, the binding site residues are identical within 3.5 Å of the ligand in the COX-2 structure from PDB entry 6COX,[32] and the only other changes in the binding site are Arg to His and Ala to Ser in a flexible loop adjacent to the ligand. Over the years, this V523I difference has been exploited to design inhibitors with exquisite selectivity of over 13000-fold for COX-2 relative to COX-1.[33] The single extra methylene group of Ile523 in COX-1 is enough to induce a significant clash with COX-2-specific ligands, as seen in Figure 2. This example illustrates how small changes in protein shape can be used to gain substantial selectivity. However, it is important to note that otherwise unfavorable interactions can be accommodated in some contexts because of molecular plasticity and the resulting rearrangement of the protein target. In the case presented above, COX-1 is not able to alleviate the clash with the ligand

through protein rearrangement. However, to predictively exploit this effect, accurate assessments of the potential for relieving unfavorable interactions must be made.

In the case of COX-1/2, selectivity has been achieved by designing compounds that fit within and bind tightly to the larger site of COX-2 but clash with the smaller site of COX-1. That is, over 13000-fold selectivity against the *smaller* binding site is achievable. Given this finding, it is reasonable to ask whether similar selectivity is achievable against a *larger* site by shape complementarity alone. In cases where shape complementarity is the only mechanism operating, selectivity against a smaller site primarily takes advantage of the strongly repulsive van der Waals potential at short distances, whereas the energetic driver for selectivity against a larger site is the loss of favorable van der Waals and other interactions. The nature of van der Waals interactions suggests that removing favorable interactions will be a much weaker effect than introducing clashes. Similarly, other interactions, such as $\pi-\pi$ and cation$-\pi$, are unlikely to exhibit as pronounced an effect on binding as the repulsive van der Waals potential.

In support of this notion, a number of examples can be found in HIV-1 protease involving binding of inhibitors to wild type and to mutants that increase the size of the binding site, such as the I84V mutation. Darunavir binds to wild-type protease with an affinity of 0.22 nM but to the I84V mutant with an affinity of 1.1 nM.[34] Structural analysis suggests that the smaller valine residue has less favorable van der Waals interactions with the ligand.[35] Apparently, neither the ligand nor the protease has enough flexibility to restore the lost favorable interactions, thereby resulting in a loss of potency. The change elicits a modest selectivity of 5-fold in this case, which is far from the 13000-fold change observed in the case of COX-1/2, where a clash was introduced. Other HIV-1 protease mutants suggest that binding to a smaller site can yield 50-fold selectivity,[36] but we find no evidence of a larger effect. These examples are not ideal, however, because the goal of drug design in these cases

was to optimize for broad binding to wild type and mutants rather than optimization for narrow selectivity, which would need to be done to address how large a selectivity effect could be achieved against a *larger* site by shape complementarity alone.

Small differences in shape have also been exploited to gain selectivity in the ATP binding pocket of kinases. Several isoquinoline and pyridine derivatives have exhibited selectivity toward Rho-kinases, such as ROCK-1, with a lower affinity for other kinases such as PKA, PRK2, MSK1, and S6K1.[37] This selectivity was attributed to five key residues in the ATP binding pocket of ROCK-1 (Met123, Ala142, Asp158, Ile186, and Phe327). Residue Phe327 is part of a C-terminal strand that has only been found in a small subset of kinases, including PKA, PKB, ROCK-1, and ROCK-2. For the other four residues, sequence alignment of 491 kinases indicated that they were relatively common, with frequencies of 25.1% (Met123), 28.9% (Ala142), 32.2% (Asp158), and 37.9% (Ile186).[38] However, the specific combination of these residues found in ROCK-1 is rare and thus generates a uniquely shaped inhibitor binding pocket. This allows for selective binding to ROCK-1 even though no single residue is unique compared with other kinases.

While it is possible that modifications introduced to clash with one conformation of a decoy can potentially be alleviated by reorganization of the decoy structure,[39] in many cases, as has been shown here, the binding pocket is rigid enough to avoid this problem. As another example of kinase selectivity arising from shape changes, a series of pyridinylimidazole p38 MAPK inhibitors from Vertex Pharmaceuticals[40] was shown to attain selectivity through specific interactions with a single residue (Thr106), which is different in other MAP kinases such as JNK1 (methionine) or ERK1 (glutamine). Treatment with one of the pyridinylimidazole derivatives reduced the p38 kinase activity to approximately 20% at 30 μM, whereas the p38 mutant T106M showed approximately 80% kinase activity at the same ligand concentration, highlighting the direct effect of this single residue.

Molecular shape can be accounted for in a number of ways using computational methods. Ligand-based methods that use shape overlap, such as ROCS[41] or Phase Shape,[42] operate by superimposing molecules onto the shape of a known active molecule in its actual or putative bioactive conformation. This general approach is attractive because it can retrieve molecules that are able to adopt a similar three-dimensional structure to active molecules that are known to fit into the target binding site of interest. ROCS has been applied successfully to a number of drug-design projects, including the design of small molecule inhibitors of the ZipA−FtsZ protein−protein interaction, an antibacterial drug target.[43] While we have been unable to find a publication highlighting shape-based screening tools being applied directly to selectivity, it is possible that the approach could be used to design for either narrow or broad selectivity by requiring a high degree of shape complementarity with the target(s) of interest while not matching the shapes of undesirable decoy targets. For example, a screening protocol could be developed where compounds are screened against an ensemble of desirable target shapes and undesirable decoy shapes. These shapes could be derived from active molecules for the desirable and undesirable targets. An objective function could then be developed to tune the level of selectivity, where a baseline level of similarity is desired for the target shapes while ensuring that there is a relatively low level of similarity to the decoys shapes. More sophisticated objective

functions could be developed that look at specific regions of the shapes around areas that are known or hypothesized to be associated with narrow selectivity, since an agnostic approach to the shapes may result in designing differences in solvent exposed regions that might not significantly impact selectivity.

In summary, shape complementarity is a vital aspect of molecular recognition. Identifying differences in shape, even small differences, can be a powerful approach to gain selectivity across a series of related proteins. The examples of COX-2 and COX-1 above highlight that very large gains in selectivity can be realized by binding to a site or subsite that is larger in the target of interest than in the decoys, suggesting that differences of this type should be one of the first things to consider when designing for selectivity. In the case of HIV-1 protease, it was shown that selectivity could be gained in the context of binding to a smaller subsite, although the changes were less pronounced because of the asymmetry of the van der Waals potential.

While modeling of shape complementarity may at first seem to be trivial, the negative design aspect effectively requires a rigorous consideration of protein flexibility, since induced-fit effects will always act to lower the binding affinity for the true bound decoy structure compared to the rigid decoy structure. Understanding the subtleties and challenges of receptor flexibility is an essential part of selectivity design and will be discussed in more detail in the section entitled Conformational Selection and Flexibility. In addition to protein flexibility, ligand flexibility could also be a determinant of shape-based selectivity. To achieve this, ligand modifications could be made to lock a molecule into a conformation that can be better accommodated by one target than another. This has proven to be a useful strategy in gaining binding affinity, but the literature does not appear to contain any direct applications to selectivity design. It is clear that leveraging differences in shape complementarity can be an effective strategy in selectivity design, although the outcomes will be context dependent and difficult to predict from a simple analysis of rigid shapes because of the ability of proteins to relax in order to alleviate unfavorable interactions.

Electrostatic Complementarity. Electrostatics encompasses interactions among charged groups, neutral polar groups, and solvent. Electrostatic complementarity is necessarily a more complex concept than shape complementarity because interfacial polar and charged groups generally pay a desolvation penalty when moving from an aqueous environment in the unbound state to a partially or fully desolvated one in the bound state. In favorable circumstances, the desolvation penalty is outweighed by the complementary new interactions formed between charged or polar groups across the interface, thereby resulting in a net gain in binding affinity. In less opportune situations, the favorable interactions are outweighed by the unfavorable desolvation and a net loss in binding affinity is observed. Because charged and neutral polar groups have significantly different desolvation penalties and improving binding affinity involves a fine balance between maximizing favorable interactions while minimizing the unfavorable desolvation penalty, deciding the most complementary group for a particular site is nontrivial. So-called electrostatic charge optimization theory provides both a useful definition and a method of computing electrostatic complementarity.[44,45]

Electrostatic complementarity, while conceptually more complex than shape complementarity, is often easier to apply as a tool to design selective compounds. This is consistent with the longstanding view that salt bridges and electrostatic interactions can be used to explain and design specificity in

protein folding and molecular recognition.[46−48] Whereas small changes in protein conformation can relieve a shape clash introduced to disfavor binding to a decoy, such changes in protein conformation cannot as easily relieve an electrostatic repulsion introduced to achieve the same goal. This is due to the longer-range nature of electrostatic interactions compared to excluded-volume repulsion. In each case, the target must tolerate the interaction introduced to negatively affect the decoy. There are numerous examples where this objective has been achieved for the binding of naturally occurring protein binding partners.[49,50] The general notion that electrostatic selectivity can be sought by identifying differences and similarities in polar and charged environments in binding sites across the set of targets and decoys is largely applicable, subject to the caveats above as well as the limited range of charge distributions obtained through available chemistries and geometric constraints.

Continuum electrostatic theory has been used to systematically explore the relationship between the distribution of polarity within a molecule and the relative promiscuity of its binding interactions.[51] The results suggest that polar and charged molecules will tend to have narrower binding selectivity compared to less polar molecules, which will tend to be more promiscuous. This is due to the strong orientational dependence of electrostatic interactions, making polar and charged molecules more sensitive to molecular shape than less polar molecules. It is also due to the nature of chemical space that, on average, provides more partners for less polar molecules. One might imagine that increased molecular flexibility would lead to greater selectivity because a molecule can reconform to bind different partners. Interestingly, this study found the opposite for polar and charged molecules: increased flexibility allowed the attainment of especially favorable electrostatic interactions with a small number of binding partners, leading to narrowed selectivity compared to less polar molecules with the same shape and conformational degrees of freedom.[51]

Positive and Negative Design with Electrostatic Interactions. Differences in the pattern of hydrophobic, polar, and charged groups across potential binding partners can be exploited through positive design (the introduction of groups that make especially good interactions with targets) and negative design (groups that make especially unfavorable interactions with decoys but are tolerated by targets). As illustrations of these concepts, examples from blood clotting factors and signaling kinases are discussed here. Each requires narrow selectivity, to some extent, because of the large number of related enzymes: serine proteases for the case of clotting and kinases for the case of signaling.

Clot formation is induced through one mechanism by a cascade of at least 20 interactivating proteins, including thrombin and factors V (Va), VIII (VIIIa), IX (IXa), and X (Xa).[52] Many cardiovascular patients are on long-term anticoagulation therapy,[53] which has proven difficult to develop for robust implementation across a broad patient population without careful monitoring, although recently approved entities promise improvement.[54−56] Comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) have been used to identify electrostatic differences among the binding sites of serine protease blood clotting factors thrombin, factor Xa, and the structurally related trypsin.[57] They identify a region in which increasing the negative electrostatic potential would enhance selectivity toward trypsin. An inhibitor placing an electronegative ester into this

region shows increased selectivity, binding to trypsin (pK_i = 7.10) more tightly than to thrombin (pK_i = 5.68). Conversely, in the context of a similar scaffold, an inhibitor placing a methylsulfonyl group into this area shows an inverted selectivity profile for binding to thrombin (pK_i= 8.38) over trypsin (pK_i = 6.77).

In the case of thrombin and factor Xa, differences in electrostatics within the S1 pocket have been exploited to provide selectivity.[52] Position 192 is highly variable across the coagulation serine proteases and is a glutamate in thrombin but a glutamine in factor Xa. An inhibitor developed by Boehringer[58] provides a good example of position-192 dependent selectivity, where a high degree of selectivity for factor Xa (K_i = 41 nM) over thrombin (K_i > 2000 μM) was achieved by using negative design through electrostatic repulsion by introducing a carboxylate group near the Glu192 side chain. Crystal structure examination shows that the carboxylate is tolerated in factor Xa partially by hydrogen bonding with Gln192, which goes some way toward compensating the carboxylate desolvation. The corresponding methyl ester derivative of the inhibitor was nonselective. Quantum mechanical methods have also been exploited to elucidate the relative electrostatic potentials of the S4 subsite, locating a large negative potential that is present in factor Xa but absent in thrombin.[59] Combining these findings suggests that tuning the electrostatic properties of an inhibitor in these three regions of thrombin, factor Xa, and trypsin can be sufficient to gain selectivity for one of the targets.

Electrostatics has also proven key in selectivity for protein tyrosine phosphatases (PTPs). In the case of the drug target PTP1B, the negatively charged Asp48 presents an opportunity for narrow selectivity in ligand binding because many PTPs contain an uncharged asparagine at this position. This has been exploited by introducing a positive charge into an existing inhibitor at an appropriate position to form a salt bridge with the Asp48 in PTP1B. This was expected to decrease the affinity for other PTPs due to the lack of strongly compensating interactions with the Asn residue to balance the ligand desolvation penalty.[60] In agreement with this prediction, a new compound containing a basic nitrogen was found to have an increased affinity for PTP1B of about 20-fold and showed high selectivity for PTP1B versus all other PTPs tested. This can be explained by analyzing the interactions seen in Figure 3, showing the favorable charge complementarity between PTP1B and the basic nitrogen, which is absent in the other receptor−ligand pairs.[60]

Electrostatic Charge Optimization Applications for Selectivity. Developing a high-affinity inhibitor involves finding a balance between the favorable intermolecular interactions and the unfavorable desolvation penalty suffered when a ligand binds to a receptor. To achieve this, continuum electrostatic models have been developed to optimize the charge distribution of the ligand and yield the most beneficial balance of these opposing contributions.[45] This method of charge optimization can be used to minimize the electrostatic binding free energy[61] and has been applied in drug design to analyze and improve potency.[62−64] The concept of charge optimization is illustrated in Figure 4A. More recently, the charge optimization methodology has been applied to selectivity design using a formalism that simultaneously considers panels of desired targets and undesired decoy receptors. Within this framework it is possible to tailor a ligand for narrow selectivity, broad selectivity, or a combination of the two. The framework illustrates clearly the requirement that selectivity gains generally come at a cost in optimal target affinity, with greater gains requiring greater cost.[65] Specificity charge optimization is
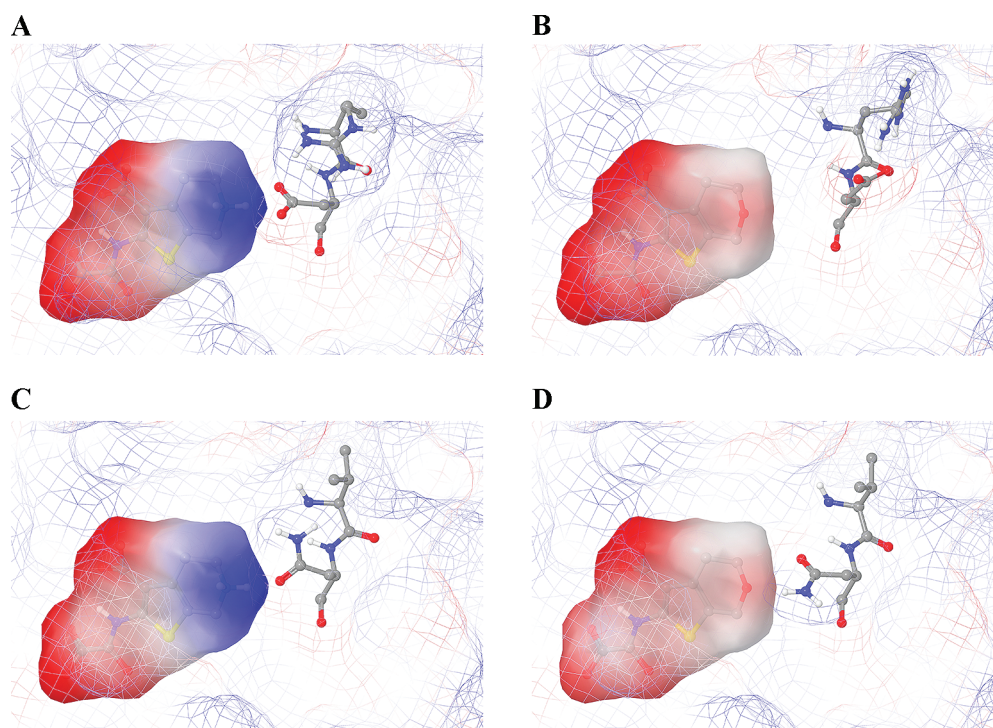
Figure 3. Electrostatic complementarity in specific PTP1B inhibition: (A) structure of PTP1B in complex with a PTP1B specific cyclic amine from PDB entry 1C88;[60] (B) structure of PTP1B in complex with a cyclic ether from PDB entry 1C87;[60] (C) structure of the PTP1B R47V/D48N double mutant in complex with a PTP1B specific cyclic amine from PDB entry 1C86;[60] (D) modeled structure of the PTP1B R47V/D48N double mutant in complex with a cyclic ether. The ligands are displayed as atom colored balls and sticks with green carbons and a transparent surface colored by electrostatic potential. The protein surfaces are displayed in wireframe and colored by electrostatic potential. Residues R47/V47 and D48/N48 are displayed in atom-colored ball and stick representation with gray carbons.

illustrated in Figure 4B. This approach has been applied to inhibitors of HIV-1 protease, where both broad and narrow
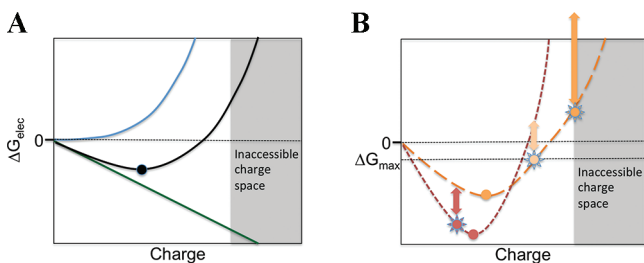

Figure 4. Charge optimization. (A) Affinity optimization, with a single well-defined minimum. The green line is the favorable Coulombic interaction between two opposite charges. The blue curve is the quadratic desolvation penalty, and the black line is the sum of the two (i.e., total electrostatic energy). Optimal charge is denoted with a black dot. (B) Specificity optimization with two proteins (red and orange curves). Only the total electrostatic energy is shown. The affinity optimal charge for each curve is denoted with a dot. The specificity optimal charge, which maximizes the energy difference between the curves, is denoted with a starburst. Note that the specificity optimum to the orange curve is theoretically unbounded but limits in chemical/biological reasonable charge space restrict the maximum charges. Furthermore, in most cases, high specificity is desirable but a baseline level of affinity (ΔG_{max}) to the primary target is needed to achieve efficacy, as shown by the light orange starburst.

selectivity were investigated.[28] Narrow selectivity was explored with the promiscuous aspartyl protease inhibitor pepstatin to predict modifications that would increase the relatively weak affinity of pepstatin for HIV-1 protease and decrease the affinity

for the related proteases pepsin and cathepsin D. The N-terminal portion of pepstatin was identified as the key specificity-determining region, in line with experimental work showing that N-acetyl pepstatin increases potency to HIV-1 protease (K_i =20 pM) but is not a known binder to pepsin or cathepsin D. In the same work, broad selectivity was explored with a set of clinically approved HIV-1 protease inhibitors to probe interactions that could broaden their affinity toward both wild-type HIV-1 protease and drug-resistant mutants. Saquinavir in particular was found to have a narrow selectivity profile toward the wild-type protease, in agreement with experimental data showing that saquinavir suffers markedly from resistance mutations.[66] Modifications to saquinavir and other approved HIV therapeutics were proposed to improve the broad selectivity binding profiles, although experimental validation of these compounds was not pursued.

Charge optimization has also been applied in a theoretical probe-based approach that simulates binding of a model ligand to a target receptor in order to understand general principles associated with selectivity.[67] The outcome of this analysis is a representation of the protein surface that gives the sign and magnitude of the complementary charge at a given location and also the strictness of selection for this optimal charge.[68] Highly selective sites have a steep curvature in the charge dependence of the binding free energy around the optimal charge, whereas sites with low selectivity have a shallow curvature. This analysis has been used to examine the change in binding affinity within a series of trypsin inhibitors. The trypsin profile shows one region with relatively low charge selectivity for a small and positive optimal charge, which is consistent with the

experimental data that show that p-carboxybenzamidine binds with an affinity of only 1.8 kcal/mol worse than p-amino-benzamidine. This indicates that trypsin prefers the neutral amino H-bond donor but will accept a negatively charged carboxylate group in this region with a relatively small loss in binding affinity. In contrast, there is a region of high selectivity for a positive charge predicted in the S1 subsite of trypsin. Experimentally, the binding affinity of P1-Met BPTI is 7.4 kcal/mol worse than P1-Lys BPTI, indicating the strong selectivity for a positively charged group in this site, in agreement with the charge optimization predictions. This concept was recently extended to predict a coupled charge selectivity (CSq), which is defined as the energetic cost of changing an atomic charge by one electron charge from its optimal value while allowing all other charges in the molecule to reoptimize.[69] The CSq method was applied to inhibitors of COX-2 such as celecoxib, which have nanomolar affinity for carbonic anhydrase II (CAII). The CSq analysis identified that the ionized sulfon-amide group of celecoxib was well optimized to bind CAII and was highly charge selective whereas there was little charge selec-tivity of this group binding to COX2. Studies have demonstrated that the sulfonamide group can be replaced with the isosteric sulfomethyl group without impacting the COX2 inhibition, in agreement with the computational predictions.[70]

The examples detailed above illustrate that charge comp-lementarity is an important design principle and can be used effectively in the lead-optimization process. In many cases, elec-trostatic complementarity design can be harnessed to achieve high affinity for the target(s) of interest as well as a desirable selectivity profile. However, it is often impossible to design a molecule with optimal charges, as the limits of chemical space restrict the range of charge distributions that can be attained within a molecule. Furthermore, even when a desirable charge distribution can be attained to design narrow selectivity toward a target receptor and against a panel of decoy receptors, it is possible for the decoys to relax to alleviate some of the un-favorable electrostatic interactions. This relaxation includes both conformational changes (i.e., induced fit) and tautomeric and ionization state changes (i.e., His, Asp, and Glu adopting difference protonation states). The range of relaxation effects has not been fully explored in previous applications of charge optimization and could add significant challenges to the application of the method. However, these relaxation effects can be accounted for within the charge optimization framework through the addition of multiple conformational states of each decoy receptor. It is also important to note that certain charge distributions may be chemically accessible but physiologically undesirable. For example, charged molecules and zwitterions are often undesirable for intracellular protein targets because of limited cell permeability. In addition, the optimal charges for selectivity may be undesirable for other reasons such as solubility, kinetics, or clearance.

In summary, differences in electrostatics between otherwise similar targets can be effectively exploited by utilizing tech-niques such as molecular field analysis and specificity charge optimization. The magnitude of selectivity gained through electro-static complementarity may be modest relative to introducing a shape change that creates a steric clash, but the effects of changes in electrostatics tend to be more predictable than the effects of changes in shape due to the smoother form of the energy surface and the long-range character of electrostatics relative to van der Waals interactions. Furthermore, the long-range nature of electrostatic forces allows for modulation of

binding affinity from interactions with residues distal from the binding site,[71−73] suggesting that binding selectivity can be derived from long-range electrostatic interactions as well. In short, relatively small receptor induced-fit effects can more easily eliminate unfavorable steric clashes than electrostatic incompatibility. This makes optimization of electrostatic interactions a general mechanism for improving selectivity whenever the target of interest and the decoys have differing charge profiles.

Conformational Selection and Flexibility. The above discussion focuses on the molecular properties of shape and electrostatics and describes examples in which similarities among targets and differences from decoys could be identified in these properties. It is interesting and perhaps under-appreciated that the molecular property of flexibility can differ sufficiently between proteins with similar binding sites to be a handle for attaining selectivity goals. One simple paradigm involves a target and a decoy that both have similar binding sites in terms of shape and electrostatic patterning, but the target is more deformable than the decoy. An inhibitor that binds to the deformed active site could then be designed to obtain selectivity for the target over the decoy. It is essential that the deformation has a relatively small energetic penalty in order to avoid too great a sacrifice in affinity. Predicting the energy associated with these structural rearrangements has been successful in a small number of very long time scale simulations run on specialty hardware,[74] but this remains a challenging area of research.

Perhaps the most renowned cases of selectivity deriving from protein flexibility come from kinases,[27,75] and a great deal of experimental data exist for kinase selectivity profiles.[21] A number of strategies have been used to achieve kinase selectivity by considering shape and protein flexibility.[9] One key notion has been to target an inactive conformation of a particular kinase,[76] which may be inaccessible or very energetically unfavorable for undesired targets. The primary structural change is a movement of the activation loop (also called the DFG loop), which opens up a deeper, more hydrophobic binding site that is adjacent to the traditional ATP binding site. While all kinases have the activation loop (which typically contains the DFG amino acid motif), the transition to the inactive DFG-out state has not been observed in all kinases, thereby offering a potential mechanism to gain selectivity. In the development of imatinib, it was found that selectivity was achieved by binding to the DFG-out conformation of the Abl kinase,[77] which also produced a desirable pharmacological profile.[9] Another com-pound that binds to P38 MAP kinase, doramapimod (BIRB796),[78] also targets an inactive kinase conformation and had great promise for its affinity and selectivity profile. Unfortunately, clinical success has not been on par with imatinib. Doramapimod was subsequently discontinued from clinical trials because of lack of efficacy for the primary indications and the development of liver function abnormalities.[79] However, a number of compounds that target kinases with known DFG-out conformations are actively being pursued. These targets include Aurora A,[80,81] cFMS,[82] EGFR,[83,84] KIT,[85] and PYK2.[86] A relatively recent computational method has been published to convert kinase structures to the DFG-out form,[87] which can then be used for virtual screening and structure-based lead optimization. In theory, this is an excellent idea, but it is difficult to know whether the converted kinase structure is energetically accessible, and therefore, the utility of such a method still needs to be proven in prospective studies.

Selectivity originating from protein flexibility has been observed in many other protein classes as well. For example,
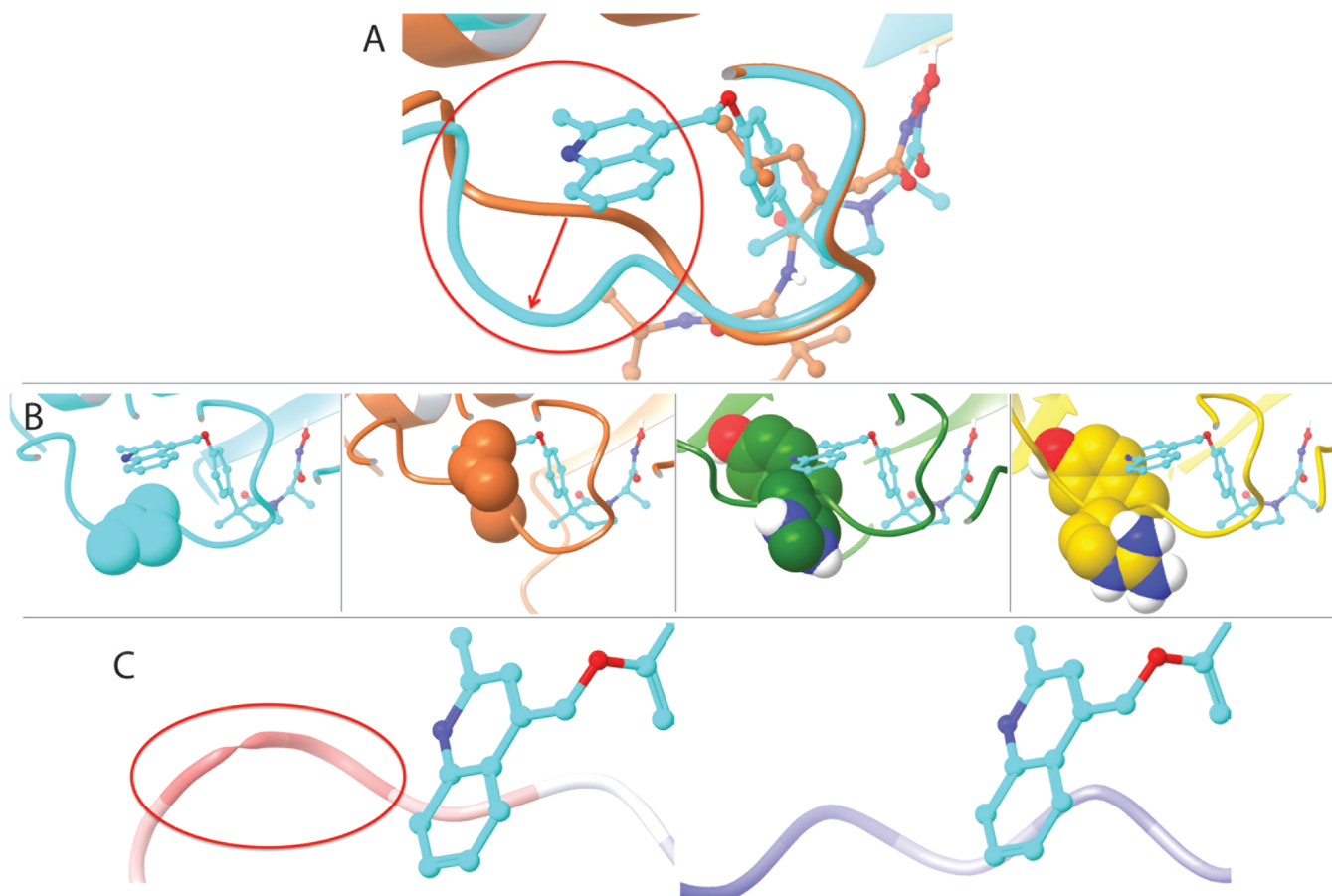
Figure 5. Protein Flexibility of TACE and MMPs. S1′ loop in TACE and related MMPs showing conformational flexibility that leads to selectivity. (A) TACE structure 2FV5 (cyan) shows significant movement in the S1′ loop (red oval) to accommodate the larger quinolone ring of the 2FV5[91] inhibitor relative to the 3KMC[92] (orange) structure. (B) Overlays of TACE and MMP structures with the ligand from 2FV5 for reference showing side chains proximate to the quinolone ring in space filling representation. TACE crystal structure before induced fit (orange) shows clashes with the ligand. The small side chains in TACE allow loop movement that can accommodate the quinolone ring (cyan). The MMP-3 structure 2JT5[93] (green) and MMP-9 structure 2OW0[94] (yellow) with larger residues show that the ligand could not fit without substantial rearrangement of the S1′ loop, which might not be possible because the larger side chains make interactions with other protein residues that stabilize the loop (adjacent residues not shown for clarity). (C) TACE (3KMC, left) and MMP-9 (2OW0, right) with S1′ loop colored by B-factor (blue = low; red = high). Gly442 in TACE (circled in red) allows for increased flexibility of the S1′ loop.

crystal structure analysis and docking studies have shown that selectivity between different species of thymidylate synthase (TS) can be attributed to protein flexibility.[88] In this case, the objective was to target bacterial TS proteins and not the corresponding human protein. The most selective inhibitors in this study were found to bind 35-fold tighter to *L. casei* and 24-fold tighter to *E. coli* compared with human TS. Studies of rigid receptor docking to previously known crystal structures were not able to accurately predict the pose for the most selective compounds. However, a crystal structure of *E. coli* TS solved by the authors of this work revealed substantial rearrangements of the protein, both in the binding site and distal to the ligand. The greatest backbone movements were in excess of 6.0 Å, highlighting the challenge that protein flexibility presents. Variations in protein flexibility have also been proposed as the origin of selectivity of carboxamide analogues of zanamivir binding to influenza virus sialidase type A preferentially over type B.[89] In this case, the increased potency of some analogues was attributed to the formation of an intramolecular salt bridge in the ligand. Interestingly, molecular dynamics (MD) simulations predict that there is substantially more rearrangement of sialidase type B than type A in order to accommodate the intramolecular

salt bridge. The authors propose that this additional rearrangement in type B in order to accommodate the intramolecular salt bridge comes at a significant energetic cost, thereby reducing the potency of the zanamivir analogues to sialidase type B even though they can still match the shape of the binding site.

Finally, researchers at Bristol-Myers Squibb were able to develop TNF-α converting enzyme (TACE) inhibitors with high selectivity versus other similar matrix metalloproteinases (MMPs) by taking advantage of differences in protein flexibility.[90] For example, the inhibitor in PDB structure 2FV5[91] uses flexibility in the loop, forming the S1β pocket (Pro437-His444) of TACE to gain selectivity over other MMPs. The movement in the 2FV5 structure is substantial and unique compared with other TACE structures, such as 3KMC[92] (Figure 5A). Interestingly, this inhibitor has a slow k_{off}, a factor that is important in controlling pharmacodynamics. The observed kinetics may be related to the induced fit required for binding. In order to understand this selectivity, the authors built a homology model of TACE on the crystal structure of atrolysin, a related member of the reprolysin family. They identified that the S1′ pocket shows substantial differences when compared with MMP-3, such as an alanine residue in

TACE replaced by a tyrosine in MMP-3. After several TACE crystal structures were solved, it became apparent that the selectivity toward TACE was due not only to the shape difference but also to the additional flexibility of the TACE loop in the S1′ pocket that was allowed by the smaller residues in TACE. Larger residues in other MMPs, such as MMP-3 (PDB code 2JT5[93]) and MMP-9 (PDB code 2OW0[94]), retard this flexibility, disfavoring ligand binding. This can be seen in Figure 5B. Interestingly, these differences in flexibility are suggested by analysis of the B-factors in the loop residues of the crystal structures, as shown in Figure 5C. With careful analysis of crystallographic data, consideration of such difference in B-factors may prove useful for gaining selectivity in other systems.

These examples, in addition to other published work,[25] highlight the importance of considering multiple protein conformations when modeling selectivity in order to sample different binding site shapes effectively. Numerous methods have been developed to account for protein flexibility, generally through a combination of protein sampling and ligand docking, although they have not been applied directly toward selectivity design.[95−99] Furthermore, the success of these methods depends heavily on the complexity of the motion in the receptor required to accommodate the ligand, where side chain rotamer changes are generally more successful to predict than large-scale backbone movements. Once a reasonable receptor structure (or ensemble of receptor structures) is generated, techniques for estimating binding free energy can be applied to predict differences in potency.

Explicit Water Molecules Bound at Target Site. Just as similarities and differences in shape, electrostatics, and flexibility among targets and decoys can form the basis of selectivity enhancing design efforts, so can differences in the location and thermodynamics of binding-site water molecules.[100] Even in cases where the binding sites are highly similar, there can still be key differences in the location and thermodynamic profile of water molecules.[101] A simple paradigm illustrating this idea is a decoy active site with a tightly bound (favorable) water molecule at a position in which the target has a loosely bound (unfavorable) one; an inhibitor that displaces each of the water molecules to make identical interactions with the target and decoy active sites gains a selectivity advantage in binding target over decoy due to the relative water-displacement costs. This newly appreciated role for water molecules in selectivity is in addition to their involvement in playing key roles in molecular recognition,[102,103] computational drug design,[104] and metabolism prediction.[105] A review by Cozzini et al. presents a number of examples of rational methods that have been used to understand the role of water in binding affinity.[106] In most cases, visualization of crystal structure water molecules cannot explain their thermodynamic properties and it is difficult to use simple empirical rules for determining whether to displace a water molecule or form a bridging interaction.[107] Furthermore, bridging interactions with water molecules can be either favorable[108,109] or unfavorable,[110] depending on the system. Therefore, more sophisticated methods for characterizing water molecules have been developed, as described below.

Free Energy Simulations. The most direct approach to compute the thermodynamic stability of a water molecule in a given environment is to use rigorous free energy methods,[111,112] such as free energy perturbation (FEP)[113−115] or thermodynamic integration (TI).[115,116] These methods are general and can be applied to any molecule of interest or any part of a molecule. It is thus possible to grow or annihilate a water

molecule to determine its thermodynamic contribution to binding. An FEP approach has been applied in the Jorgensen group by Michel et al. to assess the contribution of water molecules to binding affinity.[117,118] While their aim was not solely to determine the free energies of binding site water molecules, they demonstrated that incorporation of the water energetics could lead to improved reproduction of experimental binding energies when combined with their FEP implementation in the program MCPRO. However, it is important to note that both FEP and TI are very sensitive to the implementation details. Without the proper constraints on the system it is possible for the annihilation of one water molecule to leave a hole that is filled by another water molecule. This yields an uninformative or even misleading result regarding the energetic contribution of the presence or absence of the water molecule.

While the application of explicit solvent free energy methods to selectivity has been limited, there are cases where calculations have been helpful in providing qualitative and quantitative insights. Of particular interest is the case of differential binding of a single compound to a wild-type and mutant protein. For example, Pearlman and Connelly were able to accurately compute the energetic difference of tacrolimus (FK506) binding to wild-type and Y82F mutant FKBP-12.[119] The authors attributed the higher affinity of tacrolimus for the wild-type protein to a more favorable entropy change associated with the release of water molecules when the ligand binds.

Inhomogeneous Solvation Theory. Another computational approach to assess the thermodynamic properties of binding site water molecules, inhomogeneous solvation theory, was proposed by Lazaridis[120] and has been applied to ordered water molecule in HIV-1 protease[121] and concanavalin A.[107] In the case of HIV-1 protease, the water molecule bound between the flaps of the dimer subunits was computed to be stable relative to bulk water, suggesting that the contribution for displacing this water molecule should be unfavorable to binding, although contributions due to the displacing group or other differences between inhibitors can counterbalance this effect, which complicated comparison to available experiments. In the case of concanavalin A, the authors performed a more complete thermodynamic analysis of binding and the computational results were consistent with experimental binding affinities. In both cases, the authors highlighted the complexities associated with water molecules and the fine balance between enthalpy and entropy, which necessitates a careful analysis of water energetics that is not readily predicted by simple empirical rules. Inhomogeneous solvation theory has recently been used to identify binding hot spots at a protein surface.[122]

Qualitative Assessment of Water Molecule Locations. The application of free energy and inhomogeneous solvation methods validates the idea that differences in water thermodynamics can be used to improve affinity and selectivity, but they can be expensive and complex to implement and run and they require pre-existing knowledge of water placement, which may not be available experimentally. Although MD simulations can be used to predict the positions of observed water molecules[123] and hypothesize their importance,[124] this does not improve the issues of computational complexity and expense. Thus, considerable benefit can result from faster and less computationally demanding methods of identifying the same effects.

An alternative approach to study the role of water molecules is to look exclusively at properties of water molecules around a conformation of a protein, thereby reducing the variability

associated with the other components of the binding free energy. This approach has been taken by Fernández and colleagues with the development of a concept of a "dehydron", which is a region of a protein that is not adequately hydrated.[125] The suggestion is that backbone amide hydrogen bonds are in a globally stable state when ideally packed by hydrophobic groups. Backbone amide hydrogen bonds that are incompletely or suboptimally packed are termed dehydrons, and potency can be gained by interacting in these dehydron sites to improve the hydrophobic packing. Furthermore, selectivity can be gained by taking advantage of differences in dehydrons between similar proteins. Indeed, this approach was used to engineer selectivity into a c-Kit kinase inhibitor by finding a dehydron that was present in c-Kit but not the related Abl kinase, making it more potent and less toxic.[126]

Hydration Site Prediction and Thermodynamic Characterization. An approach that combines the prediction of water molecule locations (called hydration sites) and thermodynamic characteristics (entropy and enthalpy) has been described in recent years and has been applied to affinity and selectivity predictions.[127−129] The method, called WaterMap, determines water molecule positions by clustering water molecules from an MD simulation. Once the hydration site locations are identified, the enthalpy and entropy of each hydration site is determined using inhomogeneous solvation theory as developed by Lazaridis.[120] The advantage of this approach, in comparison with other free energy methods, is that a single simulation can provide information about all binding site water molecules for a given protein conformation. In a study on peptides that bind to PDZ domains, it was shown that the displacement energies of water molecules were able to explain why the tightest binding peptides had very broad selectivity to wild-type Erbin and variants.[130] Alanine mutants of Erbin did not affect the potency of Trp at the P-1 position of the peptide, which is consistent with the finding that the high-energy water molecule pattern in this region was preserved across the Erbin alanine mutations. In the same paper, the authors presented an example in which water energetics were able to explain the narrow selectivity of a peptide, where a tryptophan-to-alanine mutation in the peptide had a substantial effect on binding to the PDZ domains HTRA2 and HTRA3 but little effect in HTRA1. In the case of both HTRA2 and HTRA3, there was a substantial cluster of high-energy hydration sites that was displaced by the Trp, whereas in HTRA1 the energetics of the related hydration sites were not as highly unfavorable (Figure 6).

The same method has more recently been applied to kinase selectivity, where the authors studied general Src-family selectivity as well as three cases comprising pairs of kinases (Abl/c-Kit, CDK2/4, and Syk/ZAP-70).[26] It was found that in all cases, the differences in the water molecule locations, energetics, or both were able to explain the experimentally observed selectivity trends. For example, in the case of the Src-family kinases, it was shown that the water molecules at the hinge are conserved, suggesting that selectivity cannot be gained here. However, the back pocket (now known as the selectivity pocket) shows a difference in the position and energetics of Src water molecules compared with GSK3-β. An interesting prediction is that it is not necessary for an inhibitor to extend deeply into the selectivity pocket to gain differential binding affinity toward Src because the high-energy water molecule in Src resides at the opening of the selectivity pocket. A further consequence of this is that an inhibitor that enters the selectivity pocket to any degree risks hitting Src-family kinases.
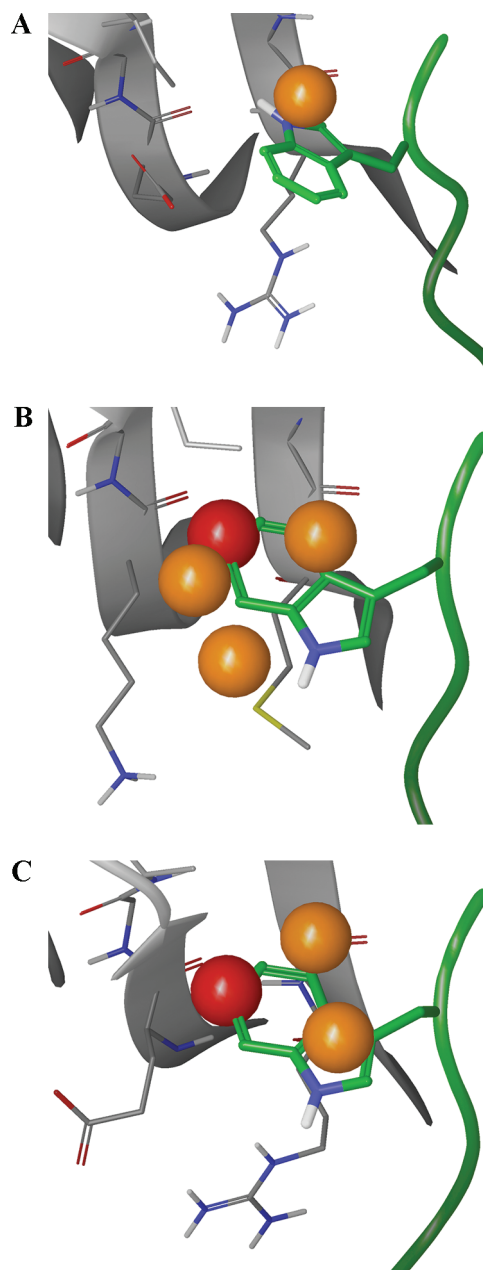
Figure 6. Water molecules in PDZ domains HTRA1, HTRA2, and HTRA3. Selectivity in the HTRA family of PDZ domains is predicted to arise from differences in binding site waters. HTRA1 (A, PDB entry 2JOA)[182] does not have a strong preference for Trp at the P-1 position, losing only 6-fold in potency when mutated to Ala. However, HTRA2 (B, PDB entry 2PZD)[183] and HTRA3 (C, PDB entry 2P3W)[182] lose considerable binding potency when Trp is mutated to other residues, such as Ala (over 300-fold for HTRA2 and 450-fold for HTRA3). Hydration site free energies are computed with the WaterMap program, and only high-energy hydration sites in the P-1 pocket are shown. Red sites are greater than 4.0 kcal/mol and orange sites are greater than 2.0 kcal/mol unfavorable relative to bulk water. HTRA2 and HTRA3 are computed to gain a substantial amount of free energy from the displacement of high-energy hydration sites, whereas HTRA1 gains significantly less. Importantly, Trp is the only side chain that is able to displace all of the high-energy hydration sites in the P-1 pocket of HTRA2 and HTRA3. The peptide backbone is shown in green with only the P-1 Trp side chain displayed.

The periplasmic oligopeptide-binding protein (OppA) has been studied for many years as a test case for selectivity; highly

selective ligands have been found in recent years,[131] and water molecules have been implicated in the broad selectivity of this and related proteins.[132,133] It has been proposed that the large number of interfacial water molecules allows the binding site to accommodate a wide variety of ligand shapes, sizes, and polarity.[134] It was noted that crystal structures with peptides having small amino acids have a higher number of crystallographically resolved water molecules at the interface, and it is thought that the water molecules fill the volume between the smaller peptides and the protein. Furthermore, selectivity between OppA and dipeptide binding protein (DppA) was proposed to stem from a difference in direct ion pairing in DppA (more favorable) versus water-mediated ion pairing (less favorable) in OppA. Finally, differential potency between di- versus tripeptides and tri- versus tetrapeptides was proposed to arise from the gain in entropy associated with the displacement of three structured water molecules by the larger peptide. A detailed series of calculations using quantum mechanics and molecular mechanics with Poisson−Boltzmann implicit solvent (MM-PBSA) suggested that the broad selectivity resulted from a fine balance between many energetic contributors to binding, including indirect desolvation effects.[135]

Another interesting system in which water molecules are proposed to play a crucial role in binding selectivity is that of growth factor-bound protein 2 (Grb2), which is involved in the Ras-MAPK signaling cascade. Researchers have used MD to explore the binding of two selective ligands to the SH2 domain of Grb2 and found that water molecules play a key stabilizing role in binding.[136] They also proposed that destabilizing interactions with bulk solvent played a role. Although the authors did not explicitly explore selective binding of these two ligands to other targets, it was also hypothesized that the key water molecule interactions would contribute an important part to the ligand selectivity. In another study on the indirect role of water molecules in binding to SH2 domains, the authors used the change in solvent accessible surface area upon binding to predict binding thermodynamics.[137] Although explicit water molecules were not used in binding energy predictions, the authors did explore the possibility that explicit water molecules could impact the calculations, and they included combinations of the interfacial water molecules in the solvent accessibility calculations. The authors then related changes in polar and nonpolar surface area to changes in heat capacity, which can be directly related to the entropy of binding. The approximations and parameters used in this study built on previous work to generate empirical models for binding energy predictions based on solvent accessibility described by Baker and Murphy.[138]

Importance of Water in "Hard" Cases of Selectivity Design. The reason that water alone can explain selectivity in the difficult cases presented above can be understood by an analysis of the thermodynamic process of binding. One can rigorously decompose the binding process into a number of steps, where a series of events takes the ligand and receptor from their relaxed unbound state in solution to the bound complex state. The total free energy of binding is the sum of the energies for each step. For cases of selectivity that are typically considered to be difficult (i.e., the binding sites of the two receptors exhibit high similarity), many of these energetic terms approximately cancel.

For example, a ligand that binds to two similar receptors will lose approximately the same amount of conformational freedom (ligand entropy) and will pay approximately the same desolvation cost when binding to each receptor. In fact, all

of the ligand-only thermodynamic properties should approximately cancel. Furthermore, the interactions between the ligand and receptor should be similar in difficult cases, where the binding site has roughly the same shape, electrostatic properties, and hydrogen bonds between the ligand and receptor. The receptor terms are thus the key determinants of selectivity. The terms with the largest magnitude include the receptor desolvation (discussed in this section) and the receptor reorganization and strain energy (discussed in the earlier section on flexibility).

Thus, the location of explicit water molecules and the conformation of the receptor play key roles in influencing binding affinity and selectivity. For the majority of methods used in structural modeling and virtual screening, it is necessary to predefine both of these features before beginning. This determines both the binding site shape and electrostatics and is thus an important choice that must be made. Some methods include the ability to switch known water molecules on or off[139] or to include limited receptor flexibility,[99,140] but in many cases this is not sufficient. Methodological advancements must be focused in these areas in order to model selectivity in a thorough fashion.

Allosteric Pockets and Noncompetitive Binding. The traditional view of inhibition is the blockage of a primary binding site that is involved in the recognition of natural binding partners. However, selectivity can also arise from noncompetitive allosteric inhibition involving differences in protein flexibility in sites distal to the primary inhibition site, and identifying and exploiting similarities and differences in allosteric pockets and interactions across targets and decoys can be another mechanism for attaining selectivity. For example, a highly selective PTP1B compound was found that binds to a site 20 Å from the catalytic site. It was proposed that binding to this distal site reduces mobility of the catalytic loop and thereby inhibits PTP1B enzyme function.[141] Interestingly, this allosteric site has not been detected in related tyrosine phosphatases, which provides a mechanism for designing highly selective compounds that target this site. Allosteric sites have also been identified for a number of other drug targets.[142−144]

Targeting allosteric sites is an attractive proposition. However, the prediction of such allosteric sites in the absence of experimental data remains a challenging problem for computational tools. There are a few examples where MD has been used to reveal cryptic sites,[145−149] but to our knowledge all of the previous studies have been retrospective and there are no examples of calculations predicting allosteric sites that were later confirmed experimentally. We see this as an area of great potential, as methods for enhanced sampling are developed in conjunction with increasing computational capacities.

■ HIGHER-LEVEL CONCEPTS

The previous section on structure-based approaches was applicable to cases in which there exists an explicit set of targets and decoys together with appropriate structural information, and the goal is to identify strategies for crafting families of ligands with the ability to cover the targets while largely avoiding the decoys. Here we consider two different classes, one in which all the targets are not explicitly known and the other for which the targets and decoys are the same molecules but the goal is to bind to them only in some tissues or environments and not in others.

Substrate Envelope Hypothesis. For therapies to be useful against rapidly mutating targets, they must avoid the
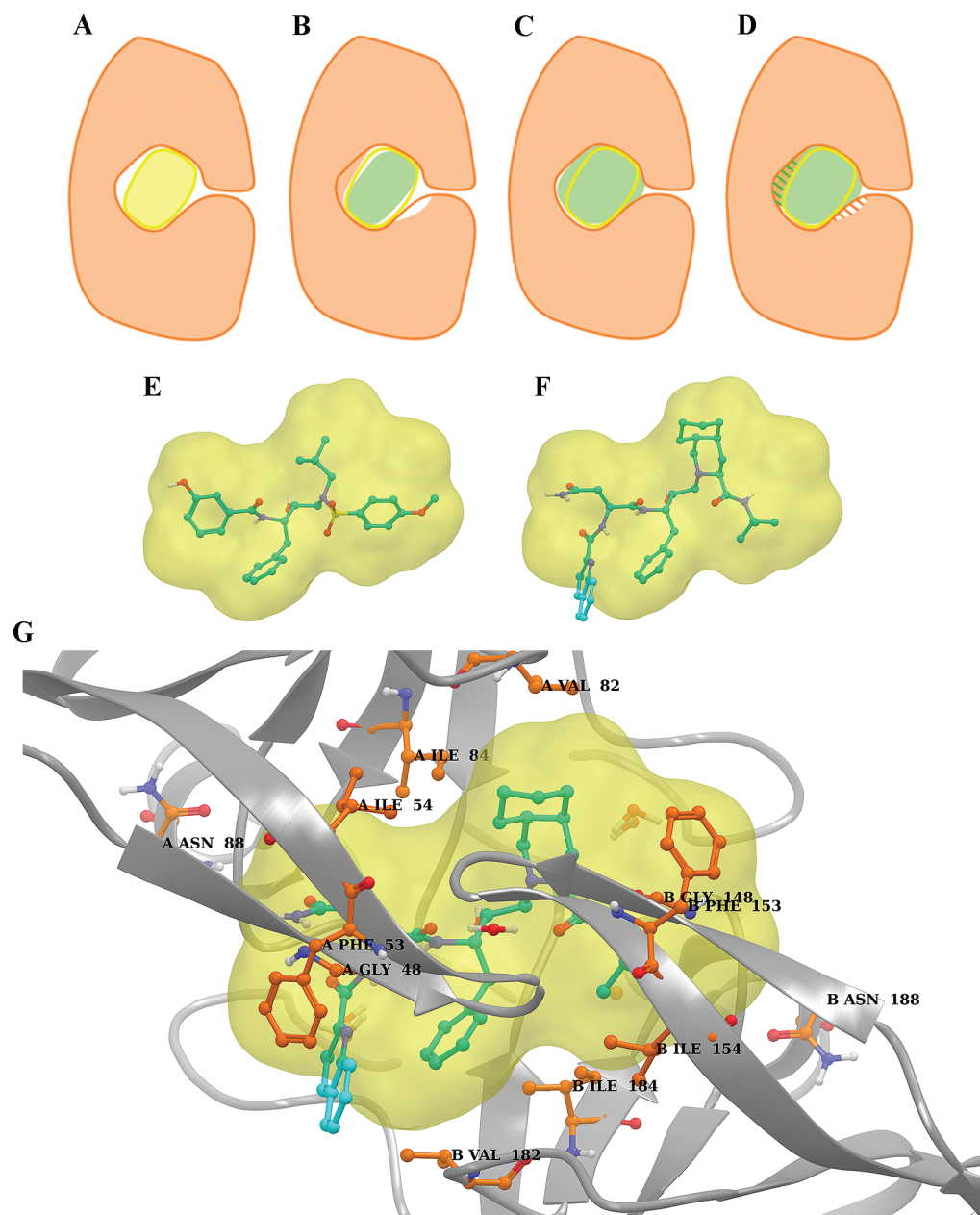
Figure 7. Substrate envelope hypothesis. To achieve broad binding selectivity against an enzyme target and the collection of its functional mutants, a useful approach has been to develop inhibitors that bind within and do not extend beyond the envelope created by the outer shape of the substrate (or a collection of substrates) bound to the active site. The idea is illustrated in panels A−D, and an example from HIV-1 protease is given in panels E−G. (A) The parent target protein is shown in orange outline and shading, and a bound substrate is shown in yellow with the substrate envelope indicated by the yellow outline. (B) An inhibitor (green shading) that binds within the substrate envelope (yellow outline) binds not only the parent target (orange outline) but also a mutant (orange shading) that includes positions that protrude further into the active site (left side) and that retreat away from the site (right side). (C) A different inhibitor (green shading) that extends beyond the substrate envelope (yellow outline) might make better interactions with the parent target (orange outline and shading) and even bind with higher affinity than other inhibitors. (D) However, such an envelope-violating inhibitor may bind poorly to protein mutants (orange shading) that differ from the parent (orange outline) by protruding further into the active site and introduce a potential clash with the inhibitor (left side, green hatching) or by retreating away from the active site and remove a stabilizing interaction (right side, orange hatching). Interestingly, there is a preponderance of the "retreating" mutations over the "protruding" ones for HIV-1 protease, perhaps because of molecular plasticity issues. (E) An HIV-1 protease inhibitor[66] that binds with high affinity to wild-type HIV-1 proteases as well as to mutants is shown to reside within the substrate envelope (yellow surface) in its crystal structure in the protein complex (the protein has been removed for clarity). (F, G) HIV-1 protease inhibitor saquinavir from PDB entry 3OXC,[184] which binds well to wild-type HIV-1 proteases but is susceptible to resistance mutants, is shown to extend outside the substrate envelope (yellow surface) in its crystal structure (the protein has been removed for clarity in panel F but is present in panel G, in which some side chains associated with resistance mutations have been highlighted and labeled).

development of resistance mutants that no longer bind the therapeutic molecule. Such cases are especially important in infectious disease and cancer, and such considerations are paramount in HIV. Application of the previously discussed structure-based concepts first requires knowledge of *all* the potential targets, which can be daunting in these situations.

The substrate envelope hypothesis elegantly avoids this difficulty for cases in which the target is an enzyme, by acknowledging that all targets must still bind and process substrate; mutants that fail to process substrate are lethal, if the target is truly valid. The substrate envelope hypothesis is one implementation of the notion that inhibitors sufficiently similar to substrate will bind to all enzyme variants capable of binding and processing substrates. The specific similarity criterion applied is that candidate inhibitors, when bound to the active site, must reside within and not extend beyond the molecular envelope of substrates when productively bound at the active site (Figure 7).

The substrate envelope hypothesis has been applied to the protease from HIV-1, a rapidly mutating target presenting significant drug resistance.[66] Early clinically approved inhibitors lopinavir and saquinavir are highly susceptible to resistance mutations. Once these mutants were identified, it became clear that lopinavir and saquinavir had overly narrow selectivity across the true but initially unknown set of targets. Lopinavir and saquinavir bind wild-type enzyme relatively strongly (K_i of 0.005 and 0.65 nM, respectively) but lose over 1000-fold affinity to resistance mutants (L10I/G48V/V82A for lopinavir and L10I/G48V/I54V/L63P/V82A for saquinavir).[150] Consistent with the substrate envelope hypothesis, both lopinavir and saquinavir extend outside the substrate envelope when bound at the active site (Figure 7F and Figure 7G illustrate this for saquinavir).[151,152]

To test the substrate envelope hypothesis as a design methodology rather than as an analysis tool, computational molecular design was undertaken with the constraint that all designed inhibitors be required to respect the substrate envelope. Some but not all of the resulting high-affinity inhibitors had broad binding profiles to a panel of drug-resistant mutants, which provides strong support for the substrate envelope hypothesis as a useful design approach.[66] X-ray crystal structures on a selection of compounds showed that all ligands successfully bound within the substrate envelope, as seen in Figure 7E for one example ligand. Interestingly, the result that some envelope-respecting high-affinity inhibitors were susceptible to resistance mutations suggests that the substrate envelope hypothesis represents one dimension of substrate similarity and that other dimensions may also be necessary to ensure that a designed molecule is sufficiently substrate-like to avoid resistance.

The substrate envelope hypothesis has more recently been applied to HCV protease[153] and appears to be effective in that system as well. Further application and validation of the substrate envelope hypothesis could lead to a new way of developing inhibitors with a broad selectivity profile with respect to potential drug-resistance mutations. Designing inhibitors to fit within the substrate envelope is a key design strategy in avoiding the problems of drug resistance and highlights the importance of shape in controlling selectivity and promiscuity. While the exact mechanism of achieving broad selectivity depends on the system of interest, the idea of trying to replicate the shape and flexibility of the natural substrates is helpful when dealing with enzymes that are prone to resistance mutations.

Local Cellular Environments. In all of the above examples, the primary determinant of selectivity has been the thermodynamics of binding. However, drug targets exist in a complex environment and there are approaches to design for selectivity that rely on the nonequilibrium nature of cells, organs, and organisms. For example, the ability to control the rate at which a compound enters or exits the cell can provide a mechanism to achieve increases in local concentrations and thereby offers an opportunity to tune selectivity. Membrane transporters that span cell membranes and control the influx and efflux of endogenous substrates are also known to be crucial in controlling the transport of xenobiotics such as drugs. Indeed, it has been suggested that carrier-mediated and active mechanisms represent the major mode of drug uptake.[154] Extensive genome analysis has recently provided a comprehensive list of drug transporters, and experimental screening systems have been suggested to pick appropriate transporters that can be used for drug delivery.[155]

There are a large number of transporters that act on existing drugs, a subset of which are shown in Table 1. Drug transporters

Table 1. Known Drug Transporters along with Their Natural Substrates and a Subset of the Identified Drug Substrates[a]

transporter	natural substrates	drug substrates
PEPT1	dipeptides, tripeptides	ampicillin, temocapril, enalapril, midodrine, valacyclovir,
PEPT2	dipeptides, tripeptides	amoxicillin, cefadroxil, cefaclor, bestatin, valganciclovir
OCT1	organic cations	zidovudine, acyclovir, ganciclovir, metformin, cimetidine
OCT2	organic cations	memantine, metformin, propranolol, cimetidine, quinine
OAT1	organic anions	quinidine, pyrilamine, verapamil, valproate, cephaloridine
OAT2	organic anions	zidovudine, tetracycline, salicylate, methotrexate, erythromycin
OATP-A	organic anions	fexofenadine, rocuronium, enalapril, temocaprilat, rosuvastatin
OATP-B	organic anions	pravastatin, glibenclamide, atorvastatin, fluvastatin, rosuvastatin
OATP-C	organic anions	benzylpenicillin, rifampicin, cerivastatin, pitavastatin, methotrexate

[a]The table is based on data from Sai and Tsuji[155] and Dobson & Kell.[154]

play key roles in drug absorption, distribution, and excretion and are differentially expressed in many tissues such as the intestine, liver, kidney, and brain. This is neatly illustrated by the quinolone antibacterial olamufloxacin (HSR-903). Many derivatives of quinolone are known to cause severe central nervous system side effects, such as convulsion. However, olamufloxacin is actively effluxed by P-glycoprotein (P-gp) at the blood–brain barrier (BBB), circumventing these potential side effects.[156] Furthermore, it is well absorbed from the intestine and actively taken up by the lung, where it performs its function.[157] Harnessing such knowledge of drug transporters should allow us to target transporter proteins in specific organs and thus develop improved methods of selective drug delivery.[158] In fact, recent computational work has shown that a structure-based method based on induced-fit docking[99] is capable of predicting P-gp binding selectivity.[159] The approach was able to consistently differentiate between P-gp binders and nonbinders, both in retrospective and prospective studies. Accounting for receptor flexibility, as discussed in the above section, was critical in obtaining accurate structural models and predictions, which are likely to have a significant impact on future drug development efforts.

Another method to gain selectivity for specific tissues is by regulating cellular trafficking.[160] This is exemplified in the
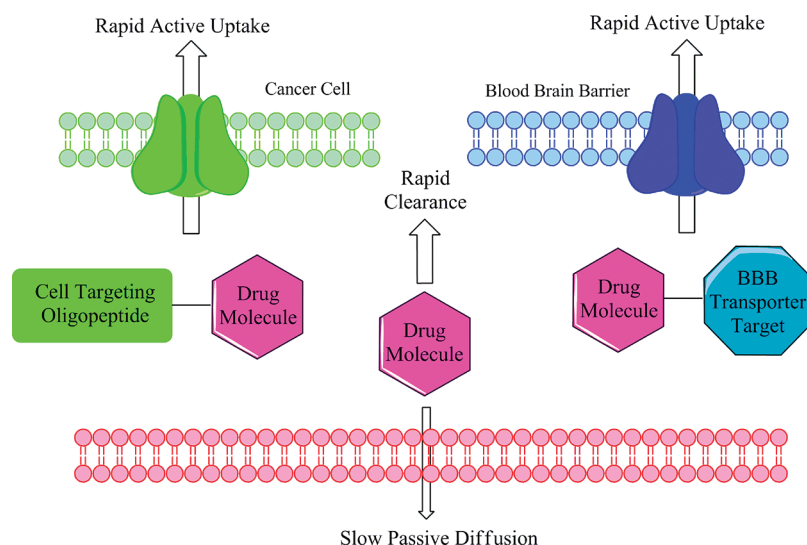
Figure 8. Targeting drugs to cellular transporters. A cartoon illustrating the mechanism by which selectivity is achieved from linking drug molecules to targets of membrane transporters. Passive transport of molecules across membranes is a slow process and is in competition with rapid clearance (bottom). Active uptake by membrane bound transporters such as GRP78 (top left) or LAT1/GLUT1 (top right) allows drug molecules to be targeted toward particular cells or organs.

design of novel cytokines that target cancer cells using the iron-binding protein transferrin (Tf). When bound with iron, Tf binds to the Tf receptor (TfR) on cell surfaces, where the complex is endocytosed. The acidic environment of the endosome then stimulates iron release. This process can be exploited, as cancerous cells express higher levels of TfR than normal cells. Thus, cancer cells can be specifically targeted by conjugating drugs to Tf.[161] The same phenomenon of local cellular pH is also an important regulator of protein structure and function in other systems.[162]

The peculiarity of the tumor environment has also been exploited by other methods. Solid tumors commonly contain regions with very low concentrations of oxygen, and cancerous cells in these hypoxic regions are often resistant to both radiotherapy and chemotherapy. However, hypoxic conditions provide an opportunity for tumor-selective therapy,[163] including prodrugs activated by hypoxia such as tirapazamine[164] and banoxantrone (AQ4N).[165] Banoxantrone is a prodrug with two dimethylamino *N*-oxide groups that is converted to a topoisomerase II inhibitor by reduction of the *N*-oxides to dimethylamino substituents. It appears that banoxantrone is reduced by the cytochrome P450 enzymes CYP2S1 and CYP2W1 under hypoxic conditions in vivo.[166] These two extrahepatic P450 enzymes are expressed in hypoxic tumor cells at much higher levels than in normal tissue. Evidence from phase I trials shows that banoxantrone penetrates hypoxic tumors and accumulates selectively in cancer cells, providing a potentially useful therapeutic window.[167]

It is clear that targeting drugs to specific cells offers a direct route to achieving selectivity. In addition to capitalizing upon the effect of cell trafficking on drug molecules, it is also possible to direct drugs to specific cells by coupling them with cell-targeting oligopeptides. The glucose-regulated protein 78 (GRP78) is overexpressed on the surface of human cancer cells, and the recently identified peptide Pep42 binds to GRP78 and is selectively internalized.[168] Thus, Pep42 can potentially act as a carrier for cytotoxic drugs to specifically target human cancer cells in a GRP78-dependent manner. Linkage with Pep42 was shown to enrich the presence of quantum dots in

tumor tissue in a xenograft mouse model.[169] Pep42 has also been used to transport paclitaxel and doxorubicin through a connection with a cathepsin B-cleavable linker to facilitate intracellular release.[170] Such an application has the potential to minimize the adverse side effects associated with conventional cancer therapeutics, as the drugs are effective at a lower concentration. The effectiveness of such cell-targeting peptide has also been improved by coupling to liposomes. PIVO-8 (sequence SNPFSKPYGLTV) is one of a series of peptides that binds to non-small-cell lung cancer cell lines but not to normal cells.[171] PIVO-8 was coupled to the polyethylene glycol terminus of a stabilized liposome containing doxorubicin. This targeted delivery of liposomal doxorubicin was shown to increase cancer cell apoptosis and decrease tumor angiogenesis in mice.[172]

Rational design to control pharmacokinetics also shows promise in the development of drugs targeting the central nervous system (CNS). The market for CNS drugs is one of the fastest growing in the pharmaceutical sector, but CNS drugs show the poorest success rates in clinical development.[173] One of the key problems is that drugs have to penetrate the BBB to exert their action in the brain.[174] This can be achieved if the compound is highly lipophilic and able to penetrate the BBB by passive diffusion or if it is the substrate of an influx transporter.[156] One caveat is that the compound cannot also be a substrate of efflux transporters such as the ABC or amino acid transporters.

Two of the most important families of influx transporters are the large neutral amino acid transporters such as LAT1 and the glucose transporters such as GLUT1. LAT1 is responsible for transporting amino acids such as valine and tyrosine, but it has also been found to transport drugs such as baclofen, levodopa, gabapentin, melphalan, and thyroxin. This has recently been exploited for the purpose of drug delivery by coupling ketoprofen to L-tyrosine. This prodrug has been shown to cross the rat BBB by a LAT1-mediated mechanism.[175] A similar approach has been used to target drugs for BBB uptake via the GLUT1 glucose transporter.[176] The idea of targeting drugs by coupling with transporter substrates is illustrated in Figure 8.

There have been many recent advances in controlling drug pharmacodynamics and pharmacokinetics, and the number of examples discussed here indicates their significance to drug development and importance for selective drug delivery. Such selectivity can be gained either from the inherent properties of a molecule or by coupling with a specific targeting species. Both techniques have proven to be useful, and exploiting them successfully should lead to improved drug delivery and higher success rates in drug development.

CONCLUSIONS

We have described the key principles of rational selectivity design and presented real-world examples of how these principles have been successfully applied in achieving selectivity. While selectivity is always desired in a drug discovery campaign, often it is not explicitly considered during the discovery process. Furthermore, it is important to think of selectivity along the continuum of narrow (hitting only a single target) to broad (hitting a panel of desired targets). The majority of drug discovery projects includes aspects of both broad and narrow selectivity, to varying degrees. By continuation of expansion of the knowledge base of experimental information related to interaction networks and cellular processes within biological systems, the definition of desirable targets and undesirable decoys will become increasingly clear.

The key aspects of this paper are illustrated in Figure 1. We discuss five structure-related design principles that can be leveraged to achieve selectivity. Shape complementarity provides one way of gaining selectivity, particularly when the binding site of the target is larger than that of the decoy. In this case, generating a clash with the decoy that is not present in the target can be worth many log units in selectivity. Selectivity can also be gained when the binding site of the target is smaller than that of the decoy, but in this case the gains may be only modest. Electrostatic complementarity also provides a direct means of gaining selectivity. This can be particularly effective when the target or the decoy binding site is charged or highly polar. Modulation of the ligand electrostatic potential field provides an attractive means of attaining selectivity because of the long-range nature of electrostatic interactions.

Protein flexibility is another crucial aspect to consider in selectivity design. With respect to predicting selectivity, it is particularly important to understand the plasticity of the undesirable decoy structures, since induced fit effects may confound simple predictions based on static crystal structures. However, such plasticity can also provide a mechanism for gaining selectivity in cases where the target is flexible and the decoy is rigid. Modeling of explicit water molecules is another area that requires careful consideration in selectivity design. Interfacial water molecules have been implicated in cases of both selective and promiscuous binding, and recently developed computational methods allow the effect of water molecules on binding to be probed. Finally, allosteric modulation of the target can be used to gain selectivity in cases where the decoy lacks an allosteric site. There are a number of proteins for which allosteric sites have been identified, thereby offering an opportunity to gain selectivity.

In addition to these five structural properties, two higher-order concepts are presented. The substrate envelope hypothesis postulates that a drug molecule designed to fit within the consensus volume of natural substrates will evade problems due to resistance mutations, as mutations that adversely affect binding of the ligand will also adversely affect substrate processing. The hypothesis has been utilized to design inhibitors that show broad selectivity and are effective against both the wild-type protein and resistance mutants. The second higher-order concept is to alter the drug molecule to control pharmacokinetics and target specific organs or cell types. Carrier-mediated uptake of drug molecules is an area that is now being explored and has recently been used to target hypoxic tumors, cancer cells, the lungs, and the brain. These developments have the potential to yield higher success rates in drug development by rational design of selective drug delivery.

While we have focused this work primarily on structure-based determinants of selectivity, recent work has highlighted the relationship between the nature of molecular scaffolds and the promiscuity of molecules containing those scaffolds.[177] It was also found that molecules with increased log P tend to be more promiscuous binders, in agreement with previous work.[178] Smaller molecules with a large number of terminal ring systems were also found to be more promiscuous. This agrees with other work, suggesting that larger and more complex molecules have a lower probability of exhibiting perfect shape and electrostatic complementarity with any given target and are thus expected to show narrower selectivity.[179,180] Indeed, using ligand information can be valuable in improving selectivity and can be used in conjunction with the structure-based techniques described in this work.

One important consideration not explored in this work is the process of target selection itself. In some cases it is possible to choose targets that are less likely to raise challenging selectivity problems. For example, when multiple biologically viable targets are available, one can use protein sequence analysis to choose the target that is least similar to other targets, especially in the binding site. Correspondingly, if other proteins that are highly similar to the target of interest have been previously shown to have selectivity problems, this can raise an early red flag in a discovery program.

We believe that the current structure-based drug design methods have great power when the right approach is taken for the appropriate problem. Conversely, it is easy to overextend the applicable domain of a method and deem the computation to have failed when indeed the method may not be suitable to address the problem of interest. As methods are improved and computational power is increased, we will see the applicability of the methods expand. With the aforementioned advances and the growing number of successful applications of rational selectivity design appearing in the literature, the decisions about which method to apply and when they are appropriate will become more straightforward. At present, selectivity design remains an immensely important and challenging problem in the drug discovery process. We hope that the principles laid out in this work and the associated examples will help make the practice of selectivity design more transparent and lead to more explicit consideration of how selectivity can be improved in the process of rational drug design.

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Bruce Tidor received his Ph.D. (1990) in Biophysics from Harvard University, MA, under the supervision of Professor Martin Karplus, where he studied protein folding and binding with free energy simulations and normal mode calculations. In 1990 he started his independent research as a Whitehead Fellow at the Whitehead Institute for Biomedical Research, and in 1994 he was appointed to the faculty at Massachusetts Institute of Technology, MA, where he is currently Professor of Biological Engineering and Computer Science. His research focuses on the analysis of complex biological systems at the molecular and cellular levels. He is studying the structure, function, and interactions of proteins. Using cell-level models, his group is exploring the relationship between network structure and biological function. He is actively applying knowledge from modeling studies to rational design.

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ABBREVIATIONS USED

PDB, Protein Data Bank; hERG, human ether-a-go-go-related gene; HIV, human immunodeficiency virus; COX, cyclooxygenase; PTP1B, protein tyrosine phosphatase 1B; TS, thymidylate synthase; TACE, TNF-α converting enzyme; MMP, matrix metalloproteinase; MD, molecular dynamics; FEP, free energy perturbation; vdW, van der Waals; OppA, oligopeptide-binding protein; HCV, hepatitis C virus; BBB, blood–brain barrier

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