Published in final edited form as:

Science. 2009 April 10; 324(5924): 213–215. doi:10.1126/science.1169378.

Trapping Moving Targets with Small Molecules

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Summary

Structure-based drug design traditionally uses static protein models as inspirations for focusing on “active” site targets. Allosteric regulation of biological macromolecules, however, is affected by both conformational and dynamic properties of the protein or protein complex, and can potentially lead to more avenues for therapeutic development. We discuss the advantages of searching for molecules that conformationally trap a macromolecule in its inactive state. Although multiple methodologies exist to probe protein dynamics and ligand binding, our current discussion highlights the use of nuclear magnetic resonance (NMR) spectroscopy in the drug discovery and design process.

Normal biological processes generally rely on a finely-tuned network of *dynamic* macromolecular interactions which allow signals to transfer throughout the cell, while misregulation of these signaling complexes often ends in disease states. For example, misregulated protein-protein interactions lead to unchecked cellular signaling in the case of multiple forms of cancer. In fact, many proteins that are intrinsically unstructured or have domains that fold into regular structural motifs upon binding to their partner proteins can be linked to human diseases (1,2).

To control the activity of these complexes in a disease state, efforts generally focus on small molecule ligands that competitively bind to a particular catalytic or active site, using a static model of the complex as a starting point. This method has identified and validated a multitude of viable active site therapeutics in use today. However, as reflected by the high failure rate of new drug compounds – only an estimated 8% of phase I clinical therapeutics eventually reach FDA approval at a conservative cost of \$800M per drug – many efforts are unsuccessful and often targets are abandoned once they are deemed undruggable (3). Focusing on active-site inhibitors limits the number of potential sites that can be targeted by therapeutics. Expanding the search for inhibitors that bind beyond the active site, or even at protein complex interfaces, offers “more shots on goal” for a particular target. These allosteric effectors can initiate a cascade of changes that alter macromolecular activity (4–6).

The energetics of macromolecular complexes involve enthalpic and entropic properties that govern activity by influencing the conformational rigidity and flexibility of macromolecules within the complex. The main theories linking allosteric regulation to alterations in protein conformation and dynamics, and ultimately protein function, usually focus upon the protein folding or ligand binding free energy landscape (7). The Monod-Wyman-Changeux (MWC) symmetrical model of allostery states that an unbound, or “apo”, target protein exists in an equilibrium that thermodynamically favors one of two distinct conformational states and that ligand binding initiates an interconversion between these two states in a *concerted* manner (8). Conversely, the Koshland-Nemethy-Filmer (KNF) induced-fit model of allostery suggests that conformational changes within the target protein propagate *sequentially* through the

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system after ligand binding (9). In other words, the unbound target protein is a static structure, and protein dynamics are induced.

More current theories extend the MWC two-state model and posit that in its apo form, proteins sample multiple pre-existing conformations, the majority of which are minor populations (10,11). A dynamic free energy landscape with shallow energy wells and transition barriers would allow the target protein to sample the conformation appropriate for efficient ligand-binding interactions (Fig.1). Upon ligand binding to a minor populated state, the dynamic free energy landscape would alter and shift the equilibrium to favor that bound conformational state (7,11). The concept of allostery can be extended to include such conformational trapping which might shift the equilibrium away from the active state. In some cases, allosteric effects may only involve alterations in protein dynamics in the absence of a conformational change, suggesting that ligand binding can be solely due to alterations in entropic factors (12).

Allosteric sites might also display more selectivity than active sites. More importantly, in contrast to competitive inhibitors, which generally focus on a single conformational binding state, different allosteric inhibitors could target multiple conformational states. This in turn provides more opportunities to inhibit the same macromolecular target. By targeting multiple sites on a particular macromolecule, rather than focusing on a single active site, allosteric therapeutics would encounter less drug resistance due to mutations within the receptor. Resistance to allosteric inhibitors can also eventually occur, but combination therapy against the same target helps alleviate this situation. An example of this type of combination therapy is found in the highly active antiretroviral treatment (HAART) drug cocktail for AIDS (13).

Several allosterically active drugs are either on the market or in late clinical testing stages. One example is imatinib (Gleevec®), discussed below. Other examples include non-nucleoside inhibitors that target HIV reverse transcriptase (14) and herpesvirus C polymerase (15): both inhibitor classes act by restricting conformational mobility in regions of the enzymes required for catalytic activity. Though preclinical, allosteric inhibitors of caspases, dimeric complexes implicated in inflammation and apoptosis, were shown to trap the enzyme in an inactive zymogen conformational state (16). X-ray crystallography, NMR, and computational studies of HIV protease have shown “closed” and “open” flap conformations which help regulate substrate access to the catalytic site (17). Inhibitors that restrict the motion of these dynamic flaps have potential for regulating the enzymatic activity of a well-validated target at a nonactive site.

Small molecule inhibitors may also regulate protein-protein interactions by restricting conformational mobility. Due to the larger and flatter surfaces associated with protein-protein interaction sites, discovering small molecule compounds that disrupt protein complexes are more difficult than those that bind to a monomeric target with well-defined binding pockets (18). Most protein-protein interactions exhibit nM to mM binding affinities, and finding the “high hanging fruit” that capture dynamic macromolecules in their inactive state remains a pathway of high potential (18,19). Herpesvirus proteases represent a homodimeric enzyme family, members of which are attractive targets for controlling several human viral pathogens with small molecule compounds (20). Each monomer contains two flexible helices at the dimer interface that undergo a conformational disorder-to-order transition which is critical for regulating enzymatic activity (21). Small molecules that capture an inactive conformation of the monomer and disrupt dimerization may provide new opportunities to make this previously intractable target druggable.

While there are several methods to discover inhibitors that target dynamic proteins, NMR is uniquely suited to sample multiple timeframes in order to characterize protein motion and is a powerful technique for detecting conformational changes in proteins and protein complexes

on a per-residue basis (see supplementary online material) (22). Traditionally, NMR studies were relegated to protein sizes less than 20–30 kDa. However, recent advances in NMR methodologies can now probe conformational and dynamic properties of protein complexes approaching 1 MDa (23). Collectively, NMR data can derive information reflecting the ligand binding energetics (ΔG_{bind}) of a particular complex. For example, differences in protein conformation upon the addition of a small drug-like molecule reflect enthalpic changes, while differences in dynamic properties indicate entropic changes (24).

Conformational changes in proteins labeled with ^{15}N and/or ^{13}C stable isotopes can be detected by two-dimensional heteronuclear single quantum coherence (HSQC) spectroscopy, with extensions that allow applications to larger proteins (23). Upon ligand binding, crosspeaks representing residues in or near binding sites will be perturbed, either by shifting locations or broadening out. This data is mapped onto a protein structure, providing a “fingerprint” and allowing easy differentiation between competitive and allosteric-site binders (Fig.1). By the same principle, protein dynamics in the absence and presence of small molecule ligands can be compared. This experiment is the basis for the well-established and widely-used structure-activity relationship (SAR) by NMR technique (25), which combines NMR and fragment-based approaches for drug design and development. Importantly, NMR can be used to quantitatively measure the binding affinities of ligands with sub- μM to mM K_d values. An extension of the SAR by NMR technique which holds potential for semi-high throughput ligand screening involves the simultaneous acquisition of multiple target protein spectra by differential labeling. NMR spectral editing techniques deconvolute the spectra to separate the data of the individual proteins, allowing determination of which, if any, targets interact with the ligand (26,27).

A combination of NMR spectroscopy and X-ray crystallography showed how imatinib (Gleevec[®]) allosterically inhibits the ABL tyrosine kinase associated with chronic myelogenous leukemia. Second generation ABL inhibitors currently on the market include dasatinib (Sprycel[®]) and nilotinib (Tasigna[®]). Proliferation assays involving Bcr-ABL expressing cells indicated imatinib IC_{50} values of 260 nM, with higher potencies reported for nilotinib (13 nM) and dasatinib (0.8 nM) (28). Enzyme activity is regulated by a structural motif that places the catalytic residues in an orientation required for efficient substrate phosphorylation. This motif contains an α -helix and two loops – the phosphate-binding (P) and activation (A) loops – with the A-loop adopting two major conformers corresponding to the active and inactive states (29). Although classified as competitive ATP inhibitors, X-ray crystallography studies of ABL-imatinib and ABL-nilotinib complexes indicate that ligand binding induces an allosteric re-orientation of the A-loop to its inactive state. Conversely, and counter to activity studies, complexes of ABL-dasatinib and two investigational compounds display the active conformation in their crystal structures (29). Recent NMR data confirms the X-ray crystallography data and suggest that the A-loop of the protein is still conformationally dynamic. Corresponding residues within all the ABL-inhibitor complexes display peak broadening and indicate that dasatinib imparts enhanced backbone dynamics relative to imatinib/nilotinib, and that it is able to sample both the active and inactive ABL loop conformations (29). This suggests that the major effect on enzyme activity is conformationally-based. Future drug discovery efforts can benefit from the HSQC fingerprints of the active and inactive loop conformations, by identifying promising new leads that impart diagnostic spectral signatures.

In addition to quickly mapping ligand binding-induced conformational changes of a macromolecule, NMR also detects disruptions in protein complexes by taking advantage of the overall dynamics of large oligomeric complexes. A small protein that displays sharp resonances in its monomeric state would exhibit extensive peak broadening once it is part of a larger macromolecular complex. If a small molecule is able to disrupt these complexes, the

protein would exhibit an HSQC spectrum closely associated with its monomeric state (30). A major advantage of this technique is that it does not require protein resonance assignments. Additionally, these assays are able to identify which protein partner binds to the small molecule. If the small molecule strongly associates with one of the protein partners, the final HSQC spectrum also acts as a starting point for further SAR by NMR studies.

This dynamic property of protein complexes for detecting small molecule ligands that disrupt protein-protein interactions was demonstrated with the well-studied p53-MDM2 complex (31). Activity of p53 is intimately involved with suppressing tumor growth and proliferation in a number of cancer types; unchecked p53-MDM2 binding results in the misregulation of p53 activity. Binding competition experiments were reported with nutlin-3 (see supplementary online materials) (30), a member of the nutlin family which inhibits p53-MDM2 binding with an IC₅₀ value of 90 nM (32). This affinity is comparable to that observed for p53-MDM2 binding as determined using peptide analogs and surface plasmon resonance (33). Interestingly, the N-terminal domain of p53 displays a dramatic disorder-to-order transition: in the apo state, it is intrinsically unstructured, while in complex with MDM2, it folds into an α -helix. Meanwhile, the apo form of MDM2 contains a short N-terminal “lid” region, which exists in slow exchange between a conformationally dynamic “open” (minor) state and a more structured helical “closed” (major) state closely associated with the p53 binding cleft of MDM2 (34). An examination of the backbone dynamics of the lid indicates higher conformational mobility in the p53-MDM2 dimer relative to the apo MDM2 and MDM2-nutlin-3 complex (34). Nutlin-3 does not conformationally trap an inactive state of either MDM2 or p53. However, these initial studies lay the groundwork for future drug discovery efforts, which may lead to compounds that trap either protein in an inactive state.

Protein-protein interactions involving cAMP response binding element (CREB) and CREB binding protein (CBP) also illustrate the use of NMR for screening small molecule conformational traps in a more highly conformationally dynamic complex. CREB is involved with cellular signaling, and misregulation of activity is associated with various neurological disorders and cancers, while CBP is classified as a co-activator protein. Interactions between the two proteins are governed by the KID domain of CREB and the KIX domain of CBP. The KID domain is unfolded in its apo state, but forms two helices when bound to KIX (35). Similarly, the KIX domain exhibits lowly populated conformers in its apo state, becoming less dynamic upon binding to KID (36).

Previous inhibition studies primarily focused on short peptides that competitively associated with the shallow hydrophobic KID binding cleft of KIX (37). However, NMR screening of 762 preselected small molecule ligands demonstrated that protein-protein interactions of this complex can be regulated by trapping an inactive conformer (38). Mapping the HSQC spectral differences onto the structures indicated that two of these compounds bound to sites in the KIX domain that were distal to the KID binding pocket. Residues of the KID binding cleft display smaller perturbations indicating that these small molecules trap the KIX domain in a conformation which is unable to effectively bind KID (38). Though still preclinical, these compounds, which exhibited fluorescence-based K_i values in the μ M range (38), highlight the possibility of making a challenging target amenable to drug intervention.

Along with the realization that biological macromolecules exist in binding free energy landscapes (39) comes a myriad of opportunities for drug discovery. Target receptors that were biologically validated but then deemed undruggable are now more attractive. In some cases, the less tractable targets, such as complexes involving protein-protein interactions, can be reconsidered or, as in the case of human herpesvirus proteases, even resurrected. The greater selectivity of these allosteric inhibitors we refer to as “conformational traps” could bode well for compounds facing the gauntlet of factors, such as efficacy, pharmacokinetics, and toxicity,

which result in the staggering attrition rate of current clinical lead compounds in the drug pipeline. Similarly, issues surrounding any drug lead such as sufficient affinity, ligand efficiency, binding free energy per atom, absorption and permeability must be addressed. Integrating multiple methodologies such as NMR spectroscopy and computational modeling (40) to complement the current approaches of rational drug design is needed. This will significantly increase the chances of success for effectively hitting a given target to antagonize or agonize its activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by grants from the NIH (grant #1R01A1067423) and the HIV Accessory and Regulatory Complex (HARC) Center (grant #P50 GM082250). GML is a recipient of the 2007–2008 UCSF-Gladstone Institute for Virology & Immunology/Center for AIDS Research Clinical Science Pilot Award (grant #P30-AI027763). We also thank K. Dill, J. Gross, D. Hostetter, and T. Shahian for discussion and proofreading of this manuscript. Due to space limitations, we could not include a larger number of relevant references.

References

1. Dyson HJ, Wright PE. *Nat Rev Mol Cell Biol* 2005;6:197. [PubMed: 15738986]
2. Uversky VN, Oldfield CJ, Dunker AK. *Annu Rev Biophys* 2008;37:215. [PubMed: 18573080]
3. Caskey CT. *Annu Rev Med* 2007;58:1. [PubMed: 17059362]
4. Kern D, Zuiderweg ER. *Curr Opin Struct Biol* 2003;13:748. [PubMed: 14675554]
5. Henzler-Wildman K, Kern D. *Nature* 2007;450:964. [PubMed: 18075575]
6. Goodey NM, Benkovic SJ. *Nat Chem Biol* 2008;4:474. [PubMed: 18641628]
7. Tsai CJ, Ma B, Nussinov R. *Proc Natl Acad Sci U S A* 1999;96:9970. [PubMed: 10468538]
8. Monod J, Wyman J, Changeux JP. *J Mol Biol* 1965;12:88. [PubMed: 14343300]
9. Koshland DE, Nemethy G, Filmer D. *Biochemistry* 1966;5:365. [PubMed: 5938952]
10. Weber G. *Biochemistry* 1972;11:864. [PubMed: 5059892]
11. Tsai CJ, Kumar S, Ma B, Nussinov R. *Protein Sci* 1999;8:1181. [PubMed: 10386868]
12. Tsai CJ, del Sol A, Nussinov R. *J Mol Biol* 2008;378:1. [PubMed: 18353365]
13. Martinez-Cajas JL, Wainberg MA. *Drugs* 2008;68:43. [PubMed: 18081372]
14. Martins S, Ramos MJ, Fernandes PA. *Curr Med Chem* 2008;15:1083. [PubMed: 18473804]
15. De Francesco R, Carfi A. *Adv Drug Deliv Rev* 2007;59:1242. [PubMed: 17869377]
16. Scheer JM, Romanowski MJ, Wells JA. *Proc Natl Acad Sci U S A* 2006;103:7595. [PubMed: 16682620]
17. Hornak V, Simmerling C. *Drug Discov Today* 2007;12:132. [PubMed: 17275733]
18. Wells JA, McClendon CL. *Nature* 2007;450:1001. [PubMed: 18075579]
19. Arkin MR, Wells JA. *Nat Rev Drug Discov* 2004;3:301. [PubMed: 15060526]
20. Babe LM, Craik CS. *Cell* 1997;91:427. [PubMed: 9390549]
21. Nomura AM, Marnett AB, Shimba N, Dotsch V, et al. *Nat Struct Mol Biol* 2005;12:1019. [PubMed: 16244665]
22. Boehr DD, Dyson HJ, Wright PE. *Chem Rev* 2006;106:3055. [PubMed: 16895318]
23. Sprangers R, Velyvis A, Kay LE. *Nat Meth* 2007;4:697.
24. Frederick KK, Marlow MS, Valentine KG, Wand AJ. *Nature* 2007;448:325. [PubMed: 17637663]
25. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW. *Science* 1996;274:1531. [PubMed: 8929414]
26. Golovanov AP, Blankley RT, Avis JM, Bermel W. *J Am Chem Soc* 2007;129:6528. [PubMed: 17458964]

27. Masterson LR, Tonelli M, Markley JL, Veglia G. *J Am Chem Soc* 2008;130:7818. [PubMed: 18512910]
28. O'Hare T, Walters DK, Stoffregen EP, Jia T, et al. *Cancer Res* 2005;65:4500. [PubMed: 15930265]
29. Vajpai N, Strauss A, Fendrich G, Cowan-Jacob SW, et al. *J Biol Chem* 2008;283:18292. [PubMed: 18434310]
30. D'Silva L, Ozdowy P, Krajewski M, Rothweiler U, et al. *J Am Chem Soc* 2005;127:13220. [PubMed: 16173750]
31. Hu CQ, Hu YZ. *Curr Med Chem* 2008;15:1720. [PubMed: 18673221]
32. Vassilev LT, Vu BT, Graves B, Carvajal D, et al. *Science* 2004;303:844. [PubMed: 14704432]
33. Pazgier M, Liu M, Zou G, Yuan W, et al. *Proc Natl Acad Sci U S A*. in press (Epub: Mar. 2, 2009).
34. Showalter SA, Bruschweiler-Li L, Johnson E, Zhang F, et al. *J Am Chem Soc* 2008;130:6472. [PubMed: 18435534]
35. Sugase K, Dyson HJ, Wright PE. *Nature* 2007;447:1021. [PubMed: 17522630]
36. Schanda P, Brutscher B, Konrat R, Tollinger M. *J Mol Biol* 2008;380:726. [PubMed: 18565542]
37. Volkman HM, Rutledge SE, Schepartz A. *J Am Chem Soc* 2005;127:4649. [PubMed: 15796530]
38. Best JL, Amezcua CA, Mayr B, Flechner L, et al. *Proc Natl Acad Sci U S A* 2004;101:17622. [PubMed: 15585582]
39. Verkhivker GM, Bouzida D, Gehlhaar DK, Rejto PA, et al. *Curr Opin Struct Biol* 2002;12:197. [PubMed: 11959497]
40. Mobley DL, Dill KA. *Structure*. 2009 in press.

Fig. 1.

(A) Shallow wells and transitional barriers of a dynamic free energy landscape allow a macromolecule to sample multiple pre-existing conformational states, corresponding to the catalytically active (green) and inactive (blue, red, yellow, and gray) forms. The global energy minimum shifts to favor the inactive conformation (red) when an inhibitor (cyan star) binds to an allosteric site. (B) The active (green) and inactive (red) conformations schematically represent a small enzyme, consisting of a flexible loop connecting two helices. Both conformations are in equilibrium while the enzyme is in the apo state. A native substrate (orange triangle) binds to the active site, located between the two helices. A small molecule (cyan star), binds to a region within the flexible loop, allosterically inhibiting the enzyme. (C) Overlaid theoretical ^{15}N -HSQC spectra display the backbone amides of residues in selected regions of the enzyme, numbered 1–4. As depicted, peak #1 of the active and inactive conformations are located in the same helix and are superimposable (purple box), while peaks #2–4 correspond to regions within the flexible loop region. Light green and pink peaks represent the apo active and inactive conformers, respectively (left); orange and cyan peaks represent the bound active and inactive conformers, respectively (right). Upon native substrate binding at the active site, only peak #1 of the active conformation shifts to a new location (orange box). Similarly, perturbation of peak #2 of the inactive conformation (cyan box) is observed when a small molecule inhibitor binds at an allosteric site.