Allosteric drugs: thinking outside the active-site box

Brian S DeDecker

Recently, a class of small molecules that thermally stabilize the tumor suppressor p53 was selected from a small-molecule library. This, and other recent work, demonstrates the feasibility of taking a lead from nature and selecting new classes of drugs that function by allosteric mechanisms.

Address: Harvard Institute of Chemistry and Cell Biology, 250 Longwood Avenue, Seeley G. Mudd Building, Room 604, Boston, MA 02115-5731, USA.

E-mail: brian_dedecker@hms.harvard.edu

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Conventional drug development has traditionally focused on the active sites of proteins, and on identifying molecules such as the HIV protease inhibitors that bind to the active sites of proteins and block directly interactions with the natural substrate. Besides this direct mode of enzymatic regulation, nature makes extensive use of allosteric (Greek: *allos*, other and *stereos*, solid or space) interactions to regulate the activities of proteins, especially in metabolic pathways and transcriptional regulation. These types of interactions allow much to be accomplished by a small molecule. For example, the late Paul Sigler's classic structural work on trp repressor showed how the free energy of binding a small molecule could pull the equilibrium from a state in which the transcription factor was unable to bind DNA to one in which it could [1] (Figure 1).

Allosteric mechanisms have several benefits. They allow a particular reaction step to be regulated by the products of other steps in the pathway or by other reaction pathways altogether. They also allow highly specific regulation of an enzyme. It is difficult, for example, to block directly a specific ATP-binding site by requiring the regulator to compete with high concentrations of ATP for the targeted enzyme and not bind the multiple related ATP-binding sites of other proteins. Allosteric molecules bind to the protein outside the highly conserved active site and can therefore have a wider range of possible binding interactions.

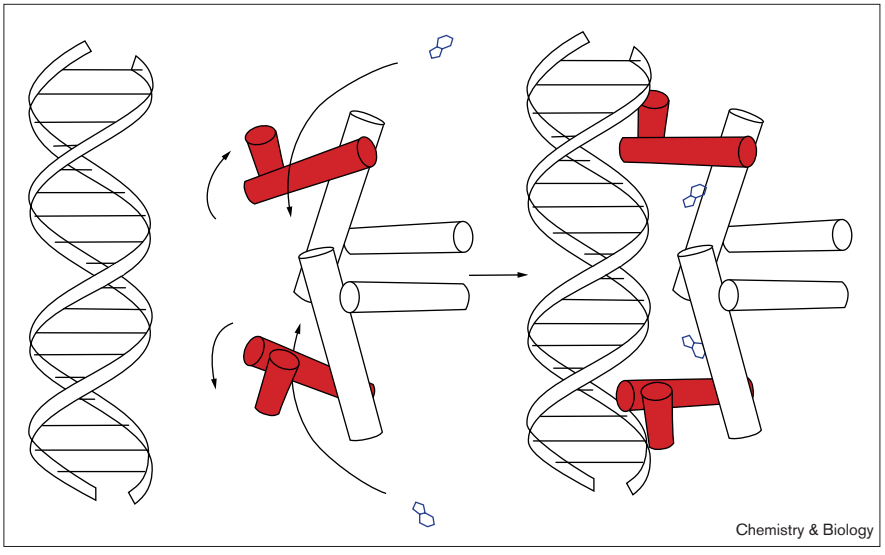
Allosteric regulation is frequently used by biological systems, as was predicted by Monod almost 40 years ago. 'While possession of this almost universal key (allostery) raises serious latent dangers for the experimenter, it is of such value to living beings that natural selection must have used it to the limit' [2]. But it is rare that an attempt is made to use the same principles in drug development. Two recent studies on mutant p53 and nitric oxide synthase are changing this trend. These studies demonstrate the feasibility of taking a lead from nature and 'designing' new classes of allosteric drugs.

A small-molecule 'Band-Aid' for mutant p53

The tumor suppressor p53 responds to DNA damage either by activating the transcription of regulatory genes

Figure 1

Stereochemical basis of trp repressor activation. Trp repressor in an inactive DNA binding state on the left and an active conformation bound to DNA and tryptophan to the right. Tryptophan (blue) binds to and stabilizes the DNA-binding conformation of the trp repressor; with both helix–turn–helix motifs (red) spread apart and aligned properly for interaction with the DNA major groove. Adapted from Zhang *et al.* [1].



that arrest cell growth until the damage is repaired, or by inducing apoptosis using both transcription dependent and independent pathways. A single missense mutation to the p53 allele can inactivate the protein and even cause a dominant negative inactivation of wild-type protein encoded by the unaffected allele. This inactivation results in unregulated cell division leading to tumor growth; indeed, over half of all human cancers can be attributed to mutations in p53 [3].

The p53 protein consists of 393 amino acids that can be divided into four major regions: a core DNA-binding domain of 200 residues that folds independently into the common immunoglobulin motif [4], an amino-terminal transactivation domain, a tetramerization domain, and a carboxy-terminal regulatory region. The core domain is the most evolutionarily conserved of the domains, highlighting the essential role of DNA binding in p53 function. Substitution mutations in the core domain make up 98% of the transforming mutations in p53, 40% of which are located in just six 'hot spots' [3].

Reverse protein engineering

To determine whether p53 mutants could, in principle, be 'repaired' by a small molecule, one could look at the results of protein-engineering studies. Such studies can give hints as to what is possible for a small-molecule interaction to accomplish. For example, the allosteric mechanism of trp repressor was confirmed by showing that the repressor could also be activated by a point mutation [5]. When an alanine that faces the tryptophan-binding pocket was mutated to a bulkier valine, the larger sidechain was shown to fill the binding-pocket cavity and activate the repressor as tryptophan would when bound. The mutation therefore mimicked the effect of a small molecule, and consequently the reverse will at least sometimes be possible.

Protein-engineering studies on model systems have shown that the energetic consequences of point mutations on thermodynamic stability are generally cumulative [6,7]. For instance, the stability gain resulting from one point mutation will be added to the stability gain of another in the double mutant (assuming they are distant in the structure). Or, if the first mutation is destabilizing and the second stabilizing, the overall free energy of unfolding may be little changed. Stabilizing mutations can therefore compensate for destabilizing mutations, such as those frequently seen in p53 core domain. Two such general suppressor mutations (Asn239→Tyr and Asn268→Asp) were identified using a random mutagenesis screen for mutations that restore transcription activity to a set of mutant p53s [8]. Thermodynamic studies demonstrated that these two particular suppressor mutations act as 'global stability' enhancers that indiscriminately stabilize the fold seen in the wild-type protein and counteract the destabilizing effects of tumorigenic mutations [8,9]. The small amount of stabilizing energy expected from mutations of this kind is similar to the levels that could reasonably be expected from a small-molecule–protein interaction. These studies suggested that allosteric stabilization of p53 mutants seemed possible in principle.

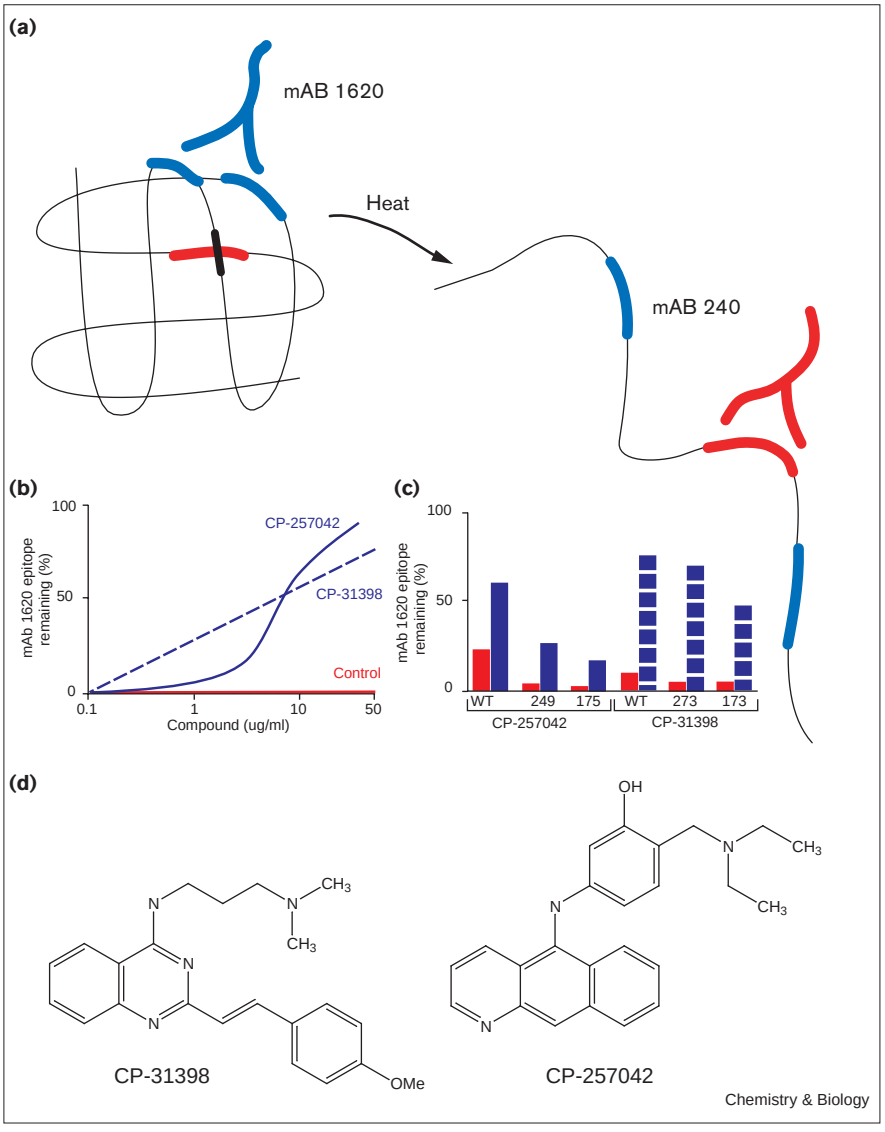
The needle in the compound library haystack

To identify candidate stabilizers, Foster *et al.* [10] selected small molecules from a random library using an assay that took advantage of the mAb 1620 antibody, which recognizes an epitope that is specific for the native conformation (Figure 2a). The core domain was immobilized in microtiter wells and heated to 45°C, 3°C above the apparent melting temperature of the isolated core domain [11], to cause the protein to unfold irreversibly and lose the conformationally sensitive mAb 1620 epitope after 30 minutes. A >100,000 member library of small molecules was then screened for candidates that would prevent this temperature-dependent loss of the mAb 1620 epitope and effectively hyperstabilize the domain. Indeed a class of relatively simple molecules was identified from the library that, as a consequence of binding, stabilize the native conformation (Figure 2b). Just as certain suppressor mutations both stabilize wild-type p53 and generally counteract destabilizing point mutations, the small molecules that were selected to stabilize wild-type p53 also stabilized a random subset of mutant p53 core domains (Figure 2c).

The selected compounds had some structural features in common: they all contain a polycyclic hydrophobic moiety attached by a linker of a specific length to a positively charged amine group (Figure 2d). How the form of these small molecules dictates their function remains to be determined. As the compounds specifically stabilize the native conformation they must bind to a surface of the protein with a stereochemistry unique to the native state. But the identification of this binding site and the chemical nature of this stabilizing interaction awaits structural studies.

Next, Foster *et al.* [10] tested the effectiveness of their compounds in cells. As expected, the level of folded mutant p53 increased; 4–6 hours after addition of compound, a fivefold increase in mAb 1620 reactivity was observed. The kinetics of this effect suggest that the hyperstabilized mutant p53 was not rescued from the pool of mutant p53 that had already become aggregated: instead, newly synthesized p53 was stabilized and therefore prevented from entering the quagmire of aggregated and chaperone-associated mutant p53. The stabilized mutant p53 was also able to restore some level of transcription of a luciferase reporter driven by a p53 promoter. Finally, a correlation was shown between administering the compounds and reducing tumor growth in mouse models. Although these results are promising, the doses required in mice are too high for the compound to be a

Figure 2



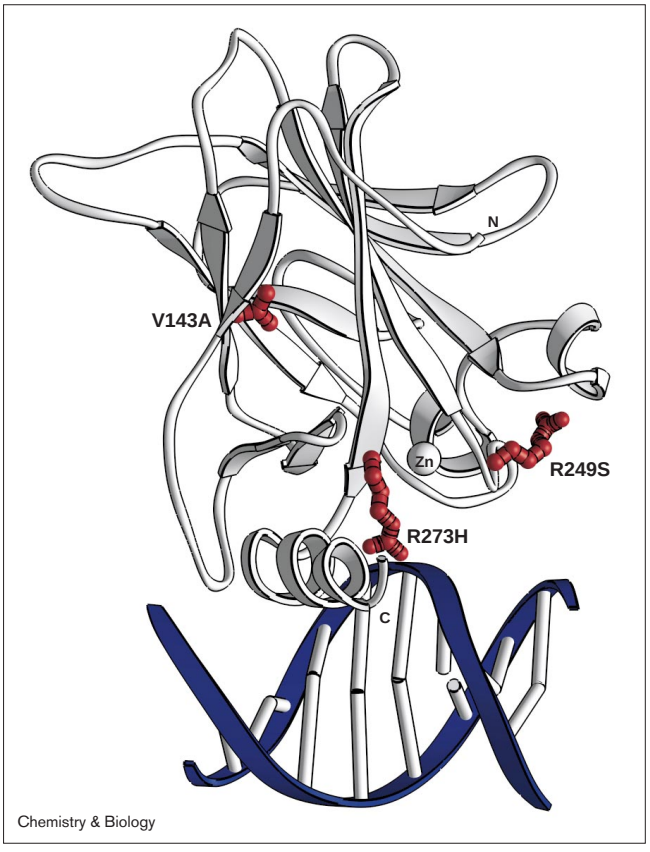
Selection strategy for p53 stabilizers used by Foster *et al.* [10]. **(a)** The wild-type p53 core domain loses the mAb 1620 epitope and gains mAb 240 reactivity upon heat denaturation. **(b)** Immobilized p53 core domain has no remaining mAb 1620 reactivity after 30 min at 45°C. Addition of compounds CP-31398 or CP-257042 stabilize the p53 fold, preserving mAb 1620 reactivity under the heat-denaturing conditions in a concentration-dependent fashion. **(c)** The two compounds that were selected to stabilize wild-type core domain also stabilize various p53 point mutants. The stability of various p53 mutants ia compared between the proteins alone (red) and in the presence of compound (blue) incubated at 37°C for 30 min. **(d)** Structures of two active compounds (CP-31398 and CP-257042): both show a polycyclic moiety attached to an amine.

practical therapy [12]. Could allostery-based approaches go further along the same lines?

A double-barrelled gun for a magic bullet

The small molecules found by Foster *et al.* [10] were selected to correct defects involved in thermal stability, and would be expected to be most effective against mutations that primarily affect protein folding, such as the temperature-sensitive mutant Val143→Ala [13] (Figure 3). This mutation is located in the core of the protein and does not appreciably affect the DNA contact residues: in this case, increasing the stability of the fold restores sufficient DNA binding. Other p53 mutants, however, such as Arg273→His (Figure 3) lack a crucial DNA contact. Improving the stability of these mutant proteins may not provide much functional benefit. Recent quantitative thermodynamic studies show that most p53 mutations affect both equilibria (protein folding and DNA binding) to varying extents [11,14]. For example, the aflatoxin-associated mutation Arg249→Ser shows highly decreased affinity for DNA and its folding equilibrium is significantly destabilized. Is it possible to use an extension of the allosteric approach to stabilize the DNA-bound state as well? Nature has accomplished this feat in stabilizing the active state of trp repressor with tryptophan. One way for a small molecule to stabilize the protein–DNA complex is to make bridging interactions across the protein–DNA interface; another possiblity is that the small molecule could stabilize local unfolding in the mutant protein, such as that caused by Arg249→Ser in the DNA-binding loop L3 (B.S.D. and A.R. Fersht, unpublished observations).

Figure 3



Ribbon diagram of the p53 core domain bound to a DNA half site [4,21]. Sites of representative mutations are shown: Arg273→His primarily reduces affinity for DNA, Val143→Ala mainly decreases the stability of protein folding, and Arg249→Ser affects both equilibria.

It may also be possible to find small molecules that relieve the inactivation of specific DNA binding by the carboxy-terminal regulatory region of p53 [15–17]. This region is used by the cell to regulate p53 activity by phosphorylation and acetylation events targeted to this domain. Antibodies that bind to this region, and peptides that mimic and compete with it, effectively counter its negative regulatory effects. These antibodies and peptides are the most effective against DNA-binding mutations such as Arg273→His. Perhaps a small molecule equivalent to these antibodies or peptides can be found.

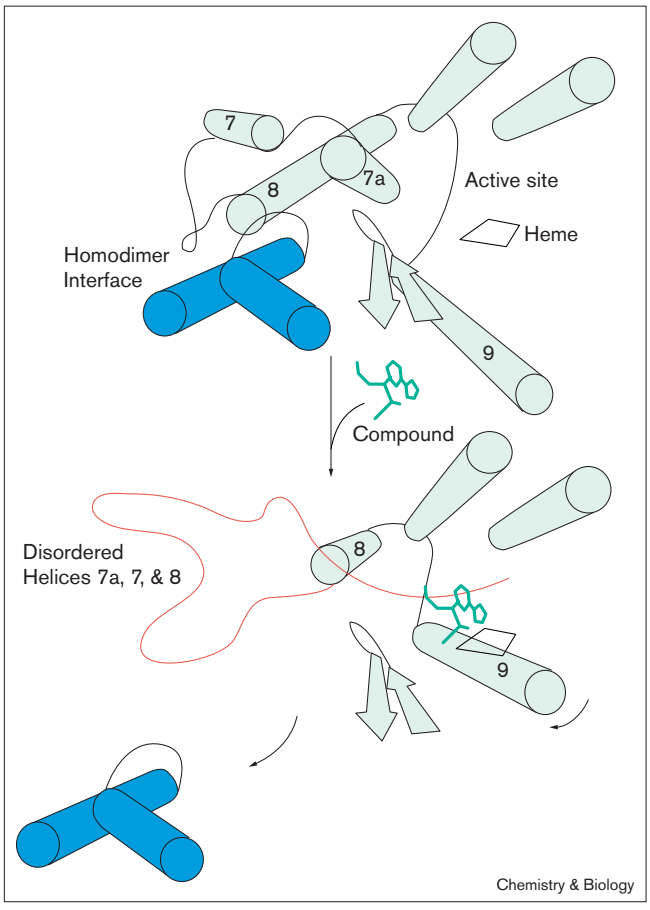
Of course, there is one possible caveat in this approach, altering the finely regulated DNA-binding equilibrium of p53 in a general and unspecific way may have disastrous consequences for healthy cells. As wild-type p53 core domain is ~99% folded at body temperature, changing the folding equilibrium would not be expected to affect normal cell function significantly, and indeed the compounds identified by Foster *et al.* [10] were not toxic. But the situation for DNA binding is quite different, because the DNA binding of p53 is normally downregulated in the cell. One solution may be to tailor DNA stabilizers to the specific mutation of interest. Once again, protein-engineering studies suggest that this may be possible; suppressor mutations that stabilize only the Arg249→Ser mutation have been identified [8,9]. The disadvantage of such mutation-specific molecules would be their limited applicability. Mutations can, however, correlate with the type of cancer or region of origin; for example, in certain areas of Africa and Asia over 50% of hepatomas carry the Arg249→Ser p53 mutation. In such regions a drug that specifically targets the Arg249→Ser mutation would be expected to have general success.

Allosteric modulation of protein–protein interactions in NO synthase

A second recent example of the allosteric approach arose serendipitously from a study that set out to identify inhibitors that compete directly with the substrate in the active site of an inducible nitric oxide synthase (iNOS) [18]. Instead, this study identified a compound that allosterically regulates the enzyme. The enzyme iNOS catalyzes the NADPH-dependent oxidation of L-arginine to citrulline and the important signaling molecule NO•. This inducible enzyme's role in signaling and its misfunction has been implicated in inflammatory and autoimmune diseases such as septic shock, hemorrhagic shock, rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and multiple sclerosis. Needless to say, these observations have made the specific inhibition of iNOS (but not the constitutive NOS isoforms) a therapeutic goal.

McMillan *et al.* [18] selected inhibitors of iNOS from a combinatorial library. The library was designed based on a pyrimidineimidazole core, a structural class of compounds known to bind the prosthetic heme group in the NOS active site and competitively inhibit the binding of the NOS substrate, L-arginine. The library was therefore biased to search for compounds that blocked directly the active site of the protein. The highly specific compounds selected did in fact bind the active site, as shown by a co-crystal structure, but they did not work in a straightforward competitive manner. Instead, they were found to block the initial step of protein homodimerization that is required to form active enzyme (Figure 4). In the iNOS–compound complex, helix 7a was found to be disordered, possibly because the compound displaced a residue of the helix (Glu371) from the active site. In the iNOS dimer structure, helix 7a buttresses helix 8 and residues 460–462 against the dimer interface, and the disruption of helix 7a by compound binding may dismantle this binding surface. Thus the fact that the compound-binding site happens to also be the active site is in some sense coincidental: the mechanism of action is the unexpected allosteric disruption of protein–protein interactions outside the active site.

Figure 4



(a) The iNOS active homodimer complex with the active site and part of the dimer interface of one NOS molecule (green) shown bound to the dimer interface of the other (blue). **(b)** The compound binds the arginine-binding site (near the heme) and displaces helix 7a, causing the region from the beginning of helix 7a to the middle half of helix 8 to be disordered (inferred by lack of electron density) and possibly disrupts the dimer interface by an allosteric mechanism. Helix 9 also shifts in position between the two structures. Adapted from McMillan *et al.* [18].

Prospects

Is selecting for allosteric molecules a generally plausible strategy in drug-design efforts? Flexibility in protein–protein interactions and protein–DNA interactions have been well demonstrated [19,20]. If fluctuating states are intrinsic to macromolecular interactions, then there is hope for an approach aimed at using small-molecule interactions to stabilize the state of interest, whether they be hyperactive or inactive. Thinking outside the active-site box requires a number of changes in experimental design, such as using more varied small-molecule libraries not biased for the active site and developing assays that specifically search for allosteric interactions. For instance, using enzyme assays that take reaction kinetics into account would allow one to distinguish between molecules that bind the active site (competitive inhibitors) and ones that do not (noncompetitive and uncompetitive inhibitors). Or, as in the iNOS sythase example, it may just require keeping an eye open for such unexpected activities. Nature has a way of devising elegant solutions to design problems, and allosteric control of protein function may be one solution worth trying to emulate.

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