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Discovery of hidden allosteric sites as novel targets for allosteric drug design

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Hidden allosteric sites, as a novel type of allosteric site, are invisible in ligand-unbound (apo) crystal structures, but can emerge in ligand-bound (holo) crystal structures when a specific ligand binds to, and stabilizes, a unique conformation favored by the ligand. However, the identification of these sites is a significant challenge. Several computational and experimental approaches have been developed to identify such sites in proteins. Here, we outline these approaches, with a focus on examples of the successful use of such techniques. The discovery of hidden allosteric sites offers a new avenue for facilitating drug design by greatly expanding the repertoire of available drug targets, contributing to the search for allosteric drugs for the treatment of human diseases.

Introduction

Biomolecules, such as proteins, in solution are not static. In fact, they are dynamic and exist as ensembles of multiple conformations with varying energies. Each conformation in the ensemble has a specific distribution that can be portrayed by the free energy landscape; lower energy conformations are sampled (or populated) more frequently than are those of the higher energy [1,2]. However, the distribution of the different conformations is tunable, and can alter in response to environmental changes, such as ligand binding, point mutations, covalent modifications, pressure, and temperature [3,4]. The redistribution or 'population shift' of the ensemble is regarded as the 'nature' of the allosteric effect [5,6].

Allostery, or allosteric regulation, known as 'the second secret of life' [7], tweaks innumerable biological processes, including signal transduction, enzymatic catalysis, metabolism, and transcription [8]. Dysregulation of protein allostery is intimately associated with the etiology of a broad spectrum of human diseases, such as cancer, mental disorder, diabetes, and inflammation [9,10]. Therefore, the recovery of malfunctional proteins by allosteric modulators provides an enriched landscape for innovative therapies [11–13]. By targeting the structural diversity of allosteric sites that are spatially and topologically distinct from the highly conversed orthosteric active sites, allosteric modulators have distinctive advantages of higher selectivity and fewer adverse effects compared with orthosteric ligands [14–18]. These advantages render the design of allosteric modulators as an appealing strategy in pharmaceutical research and development (R&D). Therefore, targeting allosteric sites has been the focus of recent interest in drug design [19–23], exemplified by the discovery of an increasing number of allosteric modulators [24,25].

X-ray crystallography is the main way of uncovering the 3D structures of biomacromolecules. Although it only captures a static snapshot, which represents the most populated average state in an ensemble, most protein structures in the Protein Data Bank (PDB) are determined by X-ray crystallography [26], leaving low-populated, high-energy conformations undisclosed. However, these minor conformations are desirable in structure-based drug design because they have potential hidden allosteric sites that can be targeted by allosteric modulators for therapeutic purposes. Hidden allosteric sites that are not visible in the ligand-unbound (apo) crystal structures can emerge in the conformational transition of proteins. Such sites are of functional importance because they can act as bona fide targets for drug design, particularly for currently

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considered undruggable proteins, which lack pharmacologically tractable sites for drug design. For instance, in the undruggable oncogenic K-Ras4B protein, andrographolide derivatives were discovered when they attached to the hidden allosteric site, resulting in the inhibition of nucleotide exchange and oncogenic K-Ras4B-mediated signaling [27]. Nonetheless, detection of hidden allosteric sites is difficult, because they are invisible in a single apo crystal structure and have often only been identified serendipitously when a specific small molecule binds to, and subsequently stabilizes, a unique conformation favored by the molecule.

Owing to the importance of hidden allosteric sites in drug design, many research groups have recently invested immense efforts to develop methods to detect these sites [28–35]. Fortunately, in tandem with computational methods, such as large-scale molecular dynamics (MD) simulations, accelerated MD simulations, MD-based Markov state analysis, and ensemble-based docking screening, in addition to experimental methods, such as cysteine trapping, high-throughput screening, and room-temperature X-ray crystallography, a considerable number of hidden allosteric sites in proteins have been identified, leading to the discovery of new allosteric modulators. Consequently, there is a need to highlight the methods used to discover hidden allosteric sites to increase the awareness of the benefit of these sites in drug design.

In the review, we discuss the recent incorporation of computational and experimental approaches to discover hidden allosteric sites in proteins, including clinically relevant proteins, such as oncogenic K-Ras4B and H-Ras GTPase, β2-adrenergic receptor, TEM-1 β-lactamase, Src kinase, neurotensin receptor NTR1, and interleukin-1 receptor 1. We then highlight the challenges to detecting hidden allosteric sites and their promise as a new source of allosteric binding sites. Discovery of as-yet-unknown hidden allosteric sites provides a promising opportunity for facilitating drug design by greatly expanding the repertoire of available drug targets, which is expected to guide the rational design of allosteric drugs for the treatment of human diseases.

Computational approaches for identification of hidden allosteric sites

Large-scale unbiased MD simulations

In theory, large-scale unbiased MD simulations of proteins can sample a diversity of protein conformations. Several low-populated conformations sampled by MD simulations are incapable of capturing by experimental approaches. These minor conformations might have hidden allosteric sites that can be exploited in the design of allosteric modulators.

G-protein-coupled receptors (GPCRs) constitute the largest class of drug targets, with one-third of all marked drugs bound to GPCRs. Using the β_2-adrenergic receptor (β_2AR), Dror *et al.* [36] performed numerous long-timescale unbiased MD simulations in which several beta blockers (i.e., propranolol, alprenolol, and dihydroalprenolol; Fig. 1) were placed away from the orthosteric binding site and the receptor surface. The simulations revealed that the drug molecules bound spontaneously to the orthosteric binding site of β_2AR in a manner similar to crystallographically solved poses. However, in the beta blocker–β_2AR binding pathway, several metastable, hidden binding sites in the extracellular vestibule were observed, which could function as targets for allosteric modulators. The presence of this corresponding hidden allosteric site was revealed by the crystal structure of the human M2 muscarinic acetylcholine receptor, in which a positive allosteric modulator LY2119620 (Fig. 1) binds to the extracellular vestibule [28].

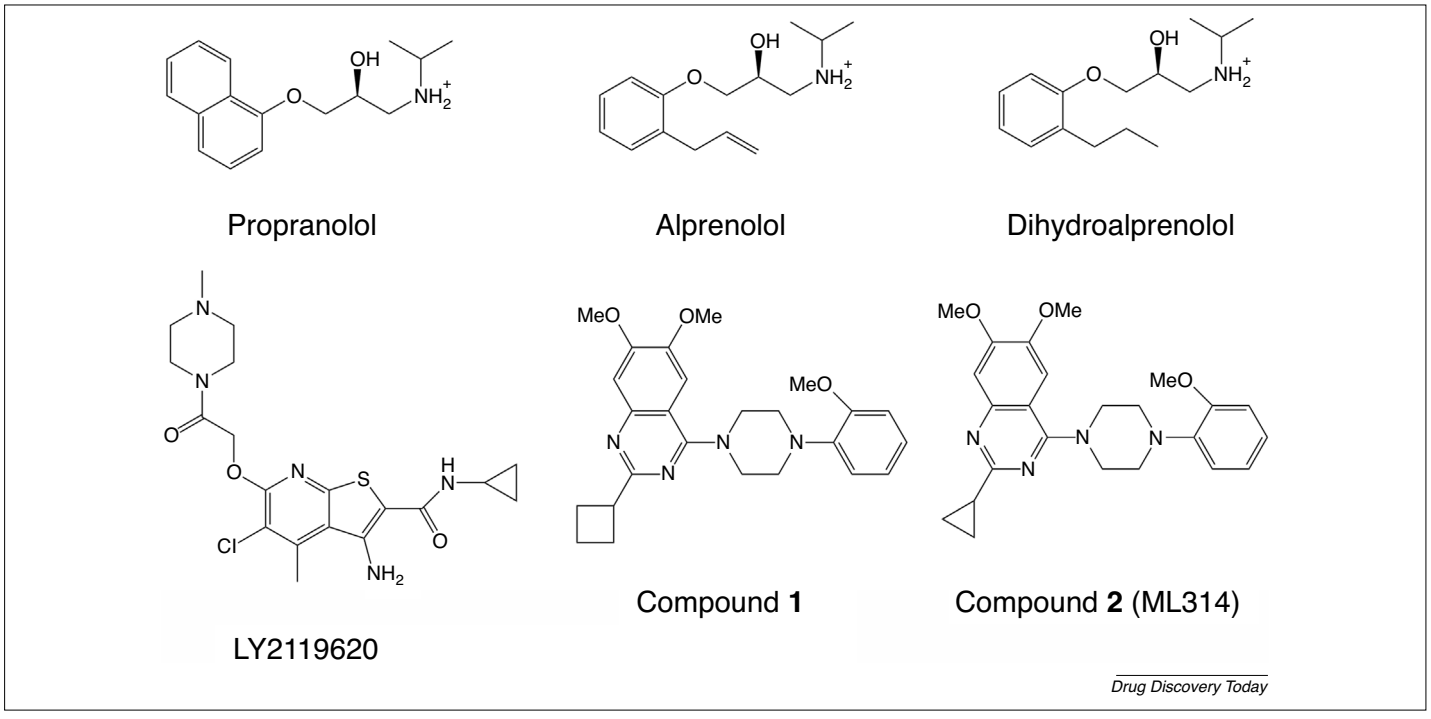

FIGURE 1

Chemical structures of propranolol, alprenolol, dihydroalprenolol, LY2119620, and compounds **1** and **2** (ML314).

Combined ensemble-based docking and MD simulations

As a small GTPase, K-Ras4B acts as a molecular switch that toggles between inactive GDP-bound and active GTP-bound states [37,38]. It regulates multiple signaling pathways that are critical for cellular growth, proliferation, and differentiation, encompassing the prototypical PI3K and MARK signaling cascades. Overactivation of K-Ras4B induced by oncogenic mutations occurs in various human cancers, such as lung and pancreatic cancers [39]. However, it is refractory to inhibition despite three decades of research and currently no drugs targeted to K-Ras4B are available, rendering it an undruggable protein [40].

Recently, based on an ensemble of 75 unique K-Ras4B conformers that were selected using root-mean-square deviation (RMSD) clustering of the MD trajectory of GTP-bound K-Ras4B^{Q61H}, Hocker *et al*. [27] performed a blind docking of andrographolide (AGP) and its benzylidene derivatives(SRJ09, SRJ10, and SRJ23) to the K-Ras4B ensemble (Fig. 2a). These K-Ras4B conformers are not frequently observed in the known crystal structures. To rule out any bias, blind docking was executed on the entire K-Ras4B surface, without specifying a pocket. The three pockets most frequently targeted by these ligands were identified: (i) pocket 1 includes the switch I domain, strand β2, and several residues in helix α1; (ii) pocket 2 contains β-stands 1 and 2, and the switch I and II domains; and (iii) pocket 3 is located at the C-terminal domain. These pockets, nonoverlapping with the active site, can be considered as hidden allosteric sites that are invisible in crystal structures. To further unravel the most probability of ligand binding, multiple MD simulations of K-Ras4B in complex with SRJ23 at distinct pockets were performed with different initial velocities. The results demonstrated that SRJ23 preferentially binds to pocket 1 (Fig. 2b). Principal component analysis of the K-Ras4B–SRJ23 trajectory suggested that binding of SRJ23 to the hidden pocket 1 allosterically promoted the K-Ras4B in a unique conformation (state 1-like state) that is resistant to GEF binding. *In vitro* cell-based assays demonstrated that SRJ ligands significantly reduced GTP binding to K-Ras4B and subsequently inhibited cancer cell growth. However, further X-ray crystallographic or nuclear magnetic resonance (NMR) studies are required to validate the pocket 1 as a high-fidelity target for SRJ ligands.

Accelerated MD simulations

Compared with conventional MD simulations, accelerated MD (aMD) simulations [41], which introduce a non-negative boost potential to the potential energy surface, enable the facilitation of conformational transitions between the low-energy states and the effective enhancement of the conformational sampling space of a protein, in particular for proteins with slow timescale dynamics.

Interleukin-1 receptor 1 (IL-1R1) is an important member of the cytokine family and is a target for the treatment of immune-dependent diseases and inflammation. It binds to cytokines through protein–protein interactions and might dysregulate cytokine production, which results in the aberrant activation of immune cells [42]. However, it is challenging to design small-molecule inhibitors targeted at the IL-1R/cytokine protein–protein interface. Based on the crystal structure of the IL-1R1 ectodomain, Yang [43] carried out aMD simulations to sample the conformational diversity of the protein, with the aim to determine potential hidden allosteric sites in unique conformations. Represented conformations from the aMD trajectory were chosen using cluster analyses and the small-molecule binding sites in these conformations were assessed using the Sitemap algorithm. One hidden allosteric site, located between the D2 and D3 domains, was

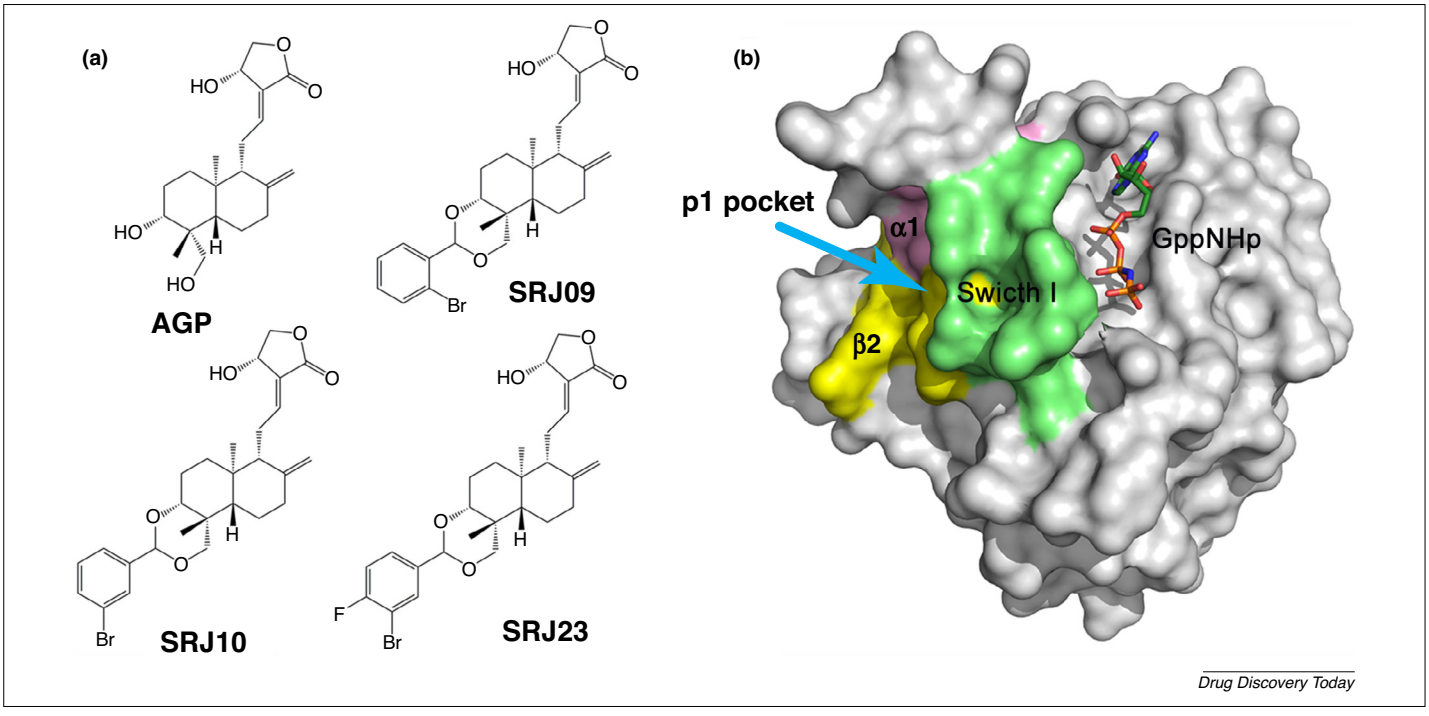

FIGURE 2

The hidden allosteric pocket of H-Ras^{T35S} and its binding compounds. (A) Chemical structures of andrographolide (AGP) and its benzylidene derivatives SRJ09, SRJ10, and SRJ23. (B) Surface representation of the pocket 1 hidden allosteric site in the crystal structure of H-Ras^{T35S}–GppNHp (Protein Data Bank ID 3KKN).

identified. Eight potential allosteric inhibitors were identified through virtual screening against this site. MD simulations of the IL-1R1 ectodomain in complex with each allosteric inhibitor revealed that, among them, four allosteric inhibitors were able to disturb the allosteric communication between the predicted hidden allosteric site and the active site, constraining the IL-1R1 ectodomain in its inactive conformation. However, *in vitro* biochemical experiments and X-ray or NMR spectroscopies are further needed to corroborate this computationally determined hidden allosteric site in the IL-1R1 ectodomain.

MD-based Markov state analysis

It is challenging to fully capture the ensemble of a biomolecule from a single MD simulation. Alternatively, Markov state models (MSMs), constructed based on numerous extensive MD simulations, provide a reduced view of an atomically detailed map of the ensemble of a biomolecule [44]. Such models have advantages in that they are able to identify hidden allosteric sites commonly located in the minor conformations that can be captured from multiple simulations rather than a single simulation.

TEM-1 β-lactamase catalyzes the hydrolysis of β-lactam antibiotics, such as penicillins, carbapenems, and cephamycins, to break the β-lactam ring, leading to deactivation of the antibacterial properties of the antibiotics and, subsequently, antibiotic resistance. X-ray crystallographic studies demonstrated that inhibitors bind to a hidden allosteric site of TEM-1 β-lactamase [45]; this site is distant (16 Å) from the active site and is invisible in the apo crystal structure (ligand-unbound TEM-1 β-lactamase) (Fig. 3a). Based on 1000 simulations of the apo TEM-1 β-lactamase (PDB ID 1JWP) with a total simulation time of 81 μs, Bowman *et al.* [29] performed a MSMs construction with MSMBuilder and obtained approximately 5000 states. The known hidden allosteric site observed in the crystal structure of inhibitor-bound TEM-1 β-lactamase was identified using a LIGSITE pocket-detection algorithm to each state in the MSMs for the apo TEM-1 β-lactamase. In addition to this known hidden allosteric site, three additional hidden sites were detected (Fig. 3b). Thiol labeling experiments were further deployed to confirm that the mutations in all three hidden sites affected the allosteric communication between these sites and the active site, strongly supporting these computationally determined sites as true hidden allosteric sites.

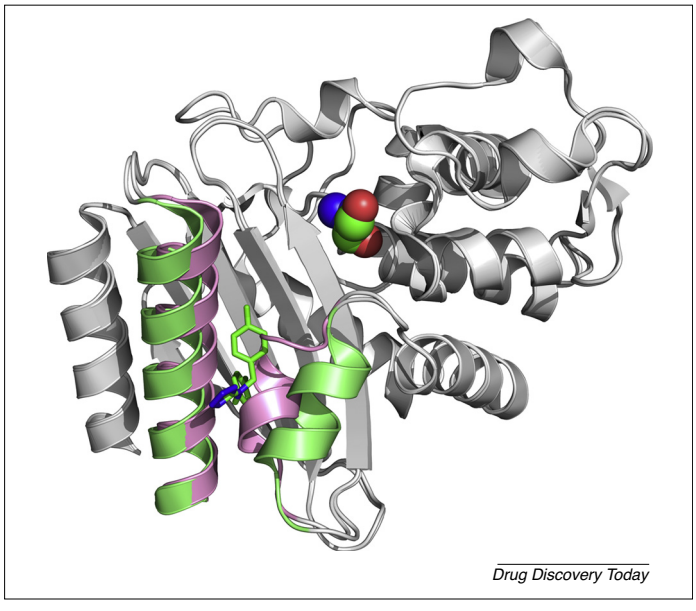

FIGURE 3

Superimposition of the crystal structure of TEM-1 β-lactamase in the absence of any ligand [Protein Data Bank (PDB) ID 1JWP, pink] to that of an inhibitor (sticks) bound to a hidden allosteric site (PDB ID 1PZO, green). The catalytic Ser70 in the active site is depicted by spheres.

As with TEM-1 β-lactamase, in the c-src tyrosine kinase, extensive MD simulations (550 μs) were performed with different initial structures generated using the string method. MSM analysis of c-src conformational dynamics uncovered the existence of a hidden allosteric site in the transition pathway connecting the active to the inactive states [30]; this site could be used to design novel kinase inhibitors.

Experimental approaches for the identification of hidden allosteric sites

Cysteine trapping

Cysteine trapping (or tethering) is a site-directed approach that designs compounds bearing a reactive, electrophilic warhead to react with an artificially introduced, or naturally occurring cysteine at the allosteric site [46]. This leads to the formation of a covalent allosteric modulator with the dual pharmacological advantages of covalent ligands (improved binding affinity, extended duration of drug action, and low drug resistance) and allosteric modulators (high specificity and few adverse effects) [47,48]. Overall, the tethering method has been established as an effective strategy to unearth hidden allosteric sites.

The G12C mutation of K-Ras4B is an oncogenic mutation that occurs in 20% of non-small cell lung cancers and is one of the most frequent *Ras* mutations [39]. Based on the introduced Cys12 at the P-loop domain of the K-Ras4B^{G12C} mutant, Ostrem *et al.* [32] constructed a library of tethering compounds (disulfide-based compounds, carbon-based electrophiles, vinyl sulfonamides, and acrylamides) to irreversibly bind to the Cys12. Crystallographic studies were further performed to characterize the detailed binding models between these tethering compounds and K-Ras4B^{G12C}. Via covalent modification with the Cys12, all these compounds bound to an adjacent pocket comprising mainly the switch-II domain, named the switch-II pocket (S-IIP). S-IIP does not overlap with the nucleotide GDP- or GTP-binding site, suggesting it is an allosteric site. As an example, comparison of the crystal structure of the acrylamide covalent modification of K-Ras4B^{G12C} with the previous structures of GDP- and GTP-bound K-Ras4B showed that the S-IIP is not apparent in the GDP- and GTP-bound K-Ras4B (Fig. 4). Therefore, S-IIP represents a hidden allosteric site that might appear in the conformational reaction path of K-Ras4B during GTP-to-GDP hydrolysis.

High-throughput screening

High-throughput screening is a method that allows a researcher to rapidly deal with millions of chemical tests, thereby leading to the identification of active compounds against a unique biomolecular target. Through medicinal chemistry optimization of lead hits and crystallographic determination of protein–ligand complexes or biochemical mutations, the binding sites of optimized ligands can be well characterized.

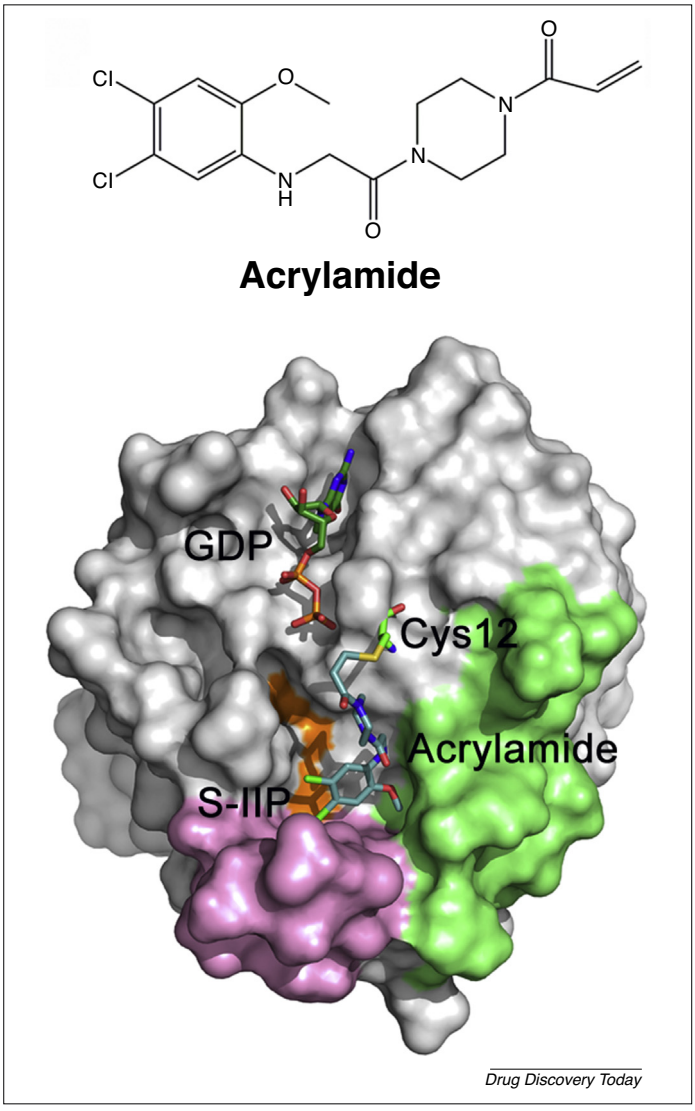

FIGURE 4

The hidden allosteric pocket of K-Ras^{G12C} and its binding compound Acrylamide. Acrylamide is covalently modified to Cys12 of K-Ras4B^{G12C} – GDP (PDB ID 4M21) and extends into a hidden allosteric site (S-IIP) beneath the switch II domain.

Neurotensin (NT) 1 receptor (NTR1), a GPCR, involved in the dopaminergic neurological pathway, is of prime importance for the treatment of methamphetamine abuse [49]. The peptide NT agonist [Lys(CH_2NH)lys-Pro,Trp-*tert*-Leu-Leu-OEt] has many adverse effects, which limit its clinical use. Recently, based on a high-throughput screening of the Molecule Libraries Small Molecule Repository (MLSMR) library (containing 331 861 compounds) using a NTR1 β-arrestin recruitment assay, Peddibhotla *et al.* [35] found a partial agonist, a quinazoline derivative (compound **1**, Fig. 1), with an EC_{50} value of 10 μM against NTR1. Following optimizations of this hit yielded the compound **2** (ML314, EC_{50} = 2.0 μM) (Fig. 1), the first nonpeptide small-molecule full agonist that selectively binds to NTR1 rather than to NTR2 and GPR35. Biochemical characterization of the involvement of ML314 in the potential pathways illustrated that it is a biased allosteric agonist of NTR1, which activates the β-arrestin rather than the G_q coupled pathway. Further investigation of the *in vivo* mechanism of action of ML304 suggested that it acts as a positive allosteric modulator that increases orthosteric ligand affinity by virtue of binding to the hidden NTR1 allosteric binding site [50]. However, the co-crystal complex of NTR1-ML314 is required to unmask the characteristics of this hidden allosteric binding site.

Room-temperature X-ray crystallography

Modern X-ray crystallographic experiments are based on the collection of diffraction data of protein crystals at cryogenic temperatures (generally 100 K) [26]. Cryocooling of crystals provides the benefit of introducing minor bias in the determination of 3D structures, because it alleviates perturbations of the overall protein backbone fold at cryogenic temperatures. However, cryogenic data only capture a representative set of protein conformations that are populated at room temperature. Collection of X-ray diffraction data at room temperature creates an opportunity to capture minor conformations [33], which might harbor hidden allosteric sites that are useful for discovering allosteric modulators.

As in the case of H-Ras, the determination of H-Ras in complex with a nonhydrolyzable GTP analog [guanosine-5′-(β,γ-imido) triphosphate, GppNHp] at cryogenic temperatures generated a catalytically incompetent conformation in which the catalytic residue Gln61 is not poised for hydrolysis [37,38]. However, at room temperature, in addition to a major conformation of the catalytically incompetent state of the H-Ras – GppNHp complex, a minor, alternative conformation was obtained [34]. This minor conformation represents a catalytically competent conformation in which the catalytic residue Gln61 interacts with a catalytic water molecule that is positioned to attack the γ-phosphate of GTP. The rotation of Gln61 triggers the perturbation of neighboring residues that extend to the C-terminal helix α3. The resulting movement of residues Arg97, Glu98, and Lys101 at the helix α3 at room temperature creates a hidden allosteric site that is invisible in the cryogenic structure. As with H-Ras, Fischer *et al.* [33] also observed that the hidden binding sites in cytochrome c peroxidase that are present only at room temperature disappeared at cryogenic temperatures. Therefore, shifting the diffraction environment from cryogenic temperatures to room temperature enables researchers to visualize the hidden allosteric sites in the minor, alternative conformations of proteins.

Concluding remarks: current challenges and future directions

Hidden allosteric sites offer a new source of allosteric sites that can be exploited as targets for sculpting novel therapeutics, particularly for orphan or undruggable proteins, which are not amenable to common regulation. Compared with allosteric sites, the discovery of hidden allosteric sites is more challenging, because of the invisibility of these sites in apo proteins. From a computational aspect, the prediction of hidden allosteric sites is largely based on the conformational sampling of proteins using large-scale MD simulations with different simulation techniques [51]. However, large-scale MD simulations are not only extremely time-consuming, but also incapable of fully sampling the protein space that represents the ensemble of the protein. The noncomprehensive capture of a protein ensemble by MD simulations yields false negatives in the prediction of hidden allosteric sites. From an experimental aspect, high-throughput screening enables us to

single out hidden allosteric sites, although the inability to identify allosteric modulators does not rule out the presence of allosteric sites. Cysteine trapping provides a site-directed way of searching for hidden allosteric sites by means of the formation of a stable covalent adduct between tethering compounds and native or introduced cysteines at the target site. This method has high specificity for the detection of hidden allosteric sites, because the position of the targeted cysteines is established. However, the cysteine trapping method can suffer from false negatives as a consequence of the lack of small-molecule ligands in the compound library that will occupy an allosteric site tightly enough. Room-temperature X-ray crystallography is useful for unmasking alternative structures harboring hidden allosteric sites, but further techniques should be developed that are feasible for any given protein.

Overall, the success of detecting hidden allosteric sites is rooted in the rapid, efficient conformational sampling of proteins. Indeed, the construction of an easy-to-use platform to detect hidden allosteric sites is already underway, one that integrates the normal mode analysis (NMA) to rapidly sample protein conformations and subsequently utilizes a previously developed Allosite server [52] to predict allosteric sites based on the conformations captured by NMA. *In silico* screening of large compound libraries can then be conducted against the predicted allosteric sites. Additional developments in this area include the construction and updating of the Allosteric Database (ASD) [53] and the benchmarking sets of allosteric sites (ASBench) [54], the development of a tool for predicting allosteric ligand–protein interactions (Alloscore) [55], and the prediction of somatic mutation-driven cancers at allosteric sites (AlloDriver) [56]. Moreover, the recently developed structure-based statistical-mechanical model of allostery by Berezovsky *et al.* [57] can predict an allosteric site via evaluation of the allosteric free energy change per residue, and can quantify the effects of allosteric signaling induced by mutations or ligand binding at allosteric sites [58,59]. Altogether, these computational advances will enable us to predict hidden allosteric sites with improved accuracy. In addition, recent breakthroughs in isotope-labeling and pulse sequence techniques in NMR spectroscopy can help researchers to investigate dynamics and allosteric mechanisms, and to determine hidden allosteric sites in large protein systems [60]. Cumulatively, the discovery of hidden allosteric sites will greatly expand the repertoire of available drug targets, providing an opportunity for allosteric drug discovery.

Acknowledgments

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References

1 Nussinov, R. and Wolynes, P.G. (2014) A second molecular biology revolution? The energy landscapes of biomolecular function. *Phys. Chem. Chem. Phys.* 16, 6321–6322
2 Boehr, D.D. *et al.* (2009) The role of dynamic conformatinal ensembles in biomolecular recognition. *Nat. Chem. Biol.* 5, 789–796
3 Nussinov, R. and Tsai, C.J. (2014) Free energy diagrams for protein function. *Chem. Biol.* 21, 311–318
4 Kornev, A.P. and Taylor, S.S. (2015) Dynamics-driven allostery in protein kinases. *Trends Biochem. Sci.* 40, 628–647
5 Hilser, V.J. (2010) An ensemble view of allostery. *Science* 327, 653–654
6 Motlagh, H.N. *et al.* (2014) The ensemble nature of allostery. *Nature* 508, 331–339
7 Fenton, A.W. (2008) Allostery: an illustrated definition for the 'second secret of life'. *Trends Biochem. Sci.* 33, 420–425
8 Goodey, N.M. and Benkovic, S.J. (2008) Allosteric regulation and catalysis emerge via a common route. *Nat. Chem. Biol.* 4, 474–482
9 Nussinov, R. and Tsai, C.-J. (2013) Allostery in disease and in drug discovery. *Cell* 153, 293–305
10 Jiang, H. *et al.* (2017) Peptidomimetic inhibitors of APC-Asef interaction block colorectal cancer migration. *Nat. Chem. Biol.* 13, 994–1001
11 Changeux, J.P. and Christopoulos, A. (2016) Allosteric modulation as a unifying mechanism for receptor function and regulation. *Cell* 166, 1084–1102
12 Wenthur, C.J. *et al.* (2014) Drugs for allosteric sites on receptors. *Annu. Rev. Pharmacol. Toxicol.* 54, 165–184
13 Pei, J. *et al.* (2014) Systems biology brings new dimensions for structure-based drug design. *J. Am. Chem. Soc.* 136, 11556–11565
14 De Smet, F. *et al.* (2014) Allosteric targeting of receptor tyrosine kinases. *Nat. Biotechnol.* 32, 1113–1120
15 Wootten, D. *et al.* (2013) Emerging paradigms in GPCR allostery: implications for drug discovery. *Nat. Rev. Drug Discov.* 12, 630–644
16 Guarnera, E. and Berezovsky, I.N. (2016) Allosteric sites: remote control in regulation of protein activity. *Curr. Opin. Struct. Biol.* 37, 1–8
17 Wu, P. *et al.* (2015) Allosteric small-molecule kinase inhibitors. *Pharmacol. Ther.* 156, 59–68
18 Lu, S. *et al.* (2014) The structural basis of ATP as an allosteric modulator. *PLoS Comput. Biol.* 10, e1003831
19 Qi, Y. *et al.* (2012) Identifying allosteric binding sites in proteins with a two-state Gō model for novel allosteric effector discovery. *J. Chem. Theory Comput.* 8, 2962–2971
20 Lu, S. *et al.* (2014) Recent computational advances in the identification of allosteric sites in proteins. *Drug Discov. Today* 19, 1595–1600
21 Schueler-Furman, O. and Wodak, S.J. (2016) Computational approaches to investigating allostery. *Curr. Opin. Struct. Biol.* 41, 159–171
22 Goncearenco, A. *et al.* (2013) SPACER: server for predicting allosteric communication and effects of regulation. *Nucleic Acids Res.* 41, 266–272
23 Panjkovich, A. and Daura, X. (2014) PARS: a web server for the prediction of protein allosteric and regulatory sites. *Bioinformatics* 30, 1314–1315
24 Lu, S. and Zhang, J. *et al.* (2017) Allosteric modulators. In *Comprehensive Medicinal Chemistry III* (3rd edn) (Chackalamannil, S., ed.), pp. 276–296, Elsevier
25 Lu, S. *et al.* (2014) Harnessing allostery: a novel approach to drug discovery. *Med. Res. Rev.* 34, 1242–1285
26 Garman, E. (2003) 'Cool' crystals: macromolecular cryocrystallography and radiation damage. *Curr. Opin. Struct. Biol.* 13, 545–551
27 Hocker, H.J. *et al.* (2013) Andrographolide derivatives inhibit guanine nucleotide exchange and abrogate oncogenic Ras function. *Proc. Natl. Acad. Sci. U. S. A.* 110, 10201–10206
28 Kruse, A.C. *et al.* (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* 504, 101–106
29 Bowman, G.R. *et al.* (2015) Discovery of multiple hidden allosteric sites by combining Markov state models and experiments. *Proc. Natl. Acad. Sci. U. S. A.* 112, 2734–2739
30 Shukla, D. *et al.* (2014) Activation pathway of Src kinase reveals intermediate states as targets for drug design. *Nat. Commun.* 5, 3397
31 Bowman, G.R. and Geissler, P.L. (2012) Equilibrium fluctuations of a single folded protein reveal a multitude of potential cryptic allosteric sites. *Proc. Natl. Acad. Sci. U. S. A.* 109, 11681–11686
32 Ostrem, J.M. *et al.* (2013) K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* 503, 548–551
33 Fischer, M. *et al.* (2015) One crystal, two temperatures: cryocooling penalties alter ligand binding to transient protein sites. *ChemBioChem* 16, 1560–1564
34 Fraser, J.S. *et al.* (2011) Accessing protein conformational ensembles using room-temperature X-ray crystallography. *Proc. Natl. Acad. Sci. U. S. A.* 108, 16247–16252
35 Peddibhotla, S. *et al.* (2013) Discovery of ML314, a brain penetrant nonpeptidic β-arrestin biased agonist of the neurotensin NTR1 receptor. *ACS Med. Chem. Lett.* 4, 846–851

36 Dror, R.O. *et al.* (2011) Pathway and mechanism of drug binding to G-protein-coupled receptors. *Proc. Natl. Acad. Sci. U. S. A.* 108, 13118–13123
37 Lu, S. *et al.* (2016) Ras conformational ensembles, allostery, and signaling. *Chem. Rev.* 116, 6607–6665
38 Lu, S. *et al.* (2016) Drugging Ras GTPase: a comprehensive mechanistic and signaling structural view. *Chem. Soc. Rev.* 45, 4929–4952
39 Prior, I.A. *et al.* (2012) A comprehensive survey of Ras mutations in cancer. *Cancer Res.* 72, 2457–2467
40 Cox, A.D. *et al.* (2014) Drugging the undruggable RAS: mission possible? *Nat. Rev. Drug Discov.* 13, 828–851
41 Bucher, D. *et al.* (2011) Accessing a hidden conformation of the maltose binding protein using accelerated molecular dynamics. *PLoS Comput. Biol.* 7, e1002034
42 Harris, F. and Pierpoint, L. (2012) Photodynamic therapy based on 5-aminolevulinic acid and its use as an antimicrobial agent. *Med. Res. Rev.* 29, 1292–1327
43 Yang, C.Y. (2015) Identification of potential small molecule allosteric modulator sites on IL-1R1 ectodomain using accelerated conformational sampling method. *PLoS One* 10, 1–25
44 Chodera, J.D. and Noé, F. (2014) Markov state models of biomolecular conformational dynamics. *Curr. Opin. Struct. Biol.* 25, 135–144
45 Horn, J.R. and Shoichet, B.K. (2004) Allosteric inhibition through core disruption. *J. Mol. Biol.* 336, 1283–1291
46 Hardy, J.A. and Wells, J.A. (2004) Searching for new allosteric sites in enzymes. *Curr. Opin. Struct. Biol.* 14, 706–715
47 Nussinov, R. and Tsai, C.-J. (2015) The design of covalent allosteric drugs. *Annu. Rev. Pharmacol. Toxicol.* 55, 249–267
48 Lu, S. and Zhang, J. (2017) Designed covalent allosteric modulators: an emerging paradigm in drug discovery. *Drug Discov. Today* 22, 447–453
49 Vincent, J. *et al.* (1999) Neurotensin and neurotensin receptors. *Trends Pharmacol. Sci.* 20, 302–309
50 Barak, L.S. *et al.* (2016) ML314: a biased neurotensin receptor ligand for methamphetamine abuse. *ACS Chem. Biol.* 11, 1880–1890
51 Perilla, J.R. *et al.* (2015) Molecular dynamics simulations of large macromolecular complexes. *Curr. Opin. Struct. Biol.* 31, 64–74
52 Huang, W. *et al.* (2013) Allosite: a method for predicting allosteric sites. *Bioinformatics* 29, 2357–2359
53 Shen, Q. *et al.* (2016) ASD v3.0: unraveling allosteric regulation with structural mechanisms and biological networks. *Nucleic Acids Res.* 44, D527–D535
54 Huang, W. *et al.* (2015) ASBench: benchmarking sets for allosteric discovery. *Bioinformatics* 31, 2598–2600
55 Li, S. *et al.* (2016) Alloscore: a method for predicting allosteric ligand–protein interactions. *Bioinformatics* 32, 1574–1576
56 Shen, Q. *et al.* (2017) Proteome-scale investigation of protein allosteric regulation perturbed by somatic mutations in 7,000 cancer genomes. *Am. J. Hum. Genet.* 100, 5–20
57 Guarnera, E. and Berezovsky, I.N. (2017) Structure-based statistical mechanical model accounts for the causality and engergetics of allosteric communication. *PLoS Comput. Biol.* 12, e1004678
58 Kurochkin, I.V. *et al.* (2017) Toward allosterically increased catalytic activity of insulin degarding enzyme against amyloid peptides. *Biochemistry* 56, 228–239
59 Guarnera, E. *et al.* (2017) AlloSigMA: allosteric signaling and mutation analysis server. *Bioinformatics* http://dx.doi.org/10.1093/bioinformatics/btx430 Published online July 6, 2017
60 Jiang, Y. and Kalodimos, C.G. (2017) NMR studies of large proteins. *J. Mol. Biol.* 429, 2667–2676