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Review

Review

# Computational allosteric ligand binding site identification on Ras proteins

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## Abstract

A number of computational techniques have been proposed to expedite the process of allosteric ligand binding site identification in inherently flexible and hence challenging drug targets. Some of these techniques have been instrumental in the discovery of allosteric ligand binding sites on Ras proteins, a group of elusive anticancer drug targets. This review provides an overview of these techniques and their application to Ras proteins. A summary of molecular docking and binding site identification is provided first, followed by a more detailed discussion of two specific techniques for binding site identification in ensembles of Ras conformations generated by molecular simulations.

**Key words:** molecular dynamics, binding site identification, probe-based simulation, Ras proteins

## Introduction

Ras proteins are guanosine triphosphate (GTP) binding enzymes that regulate a number of key signaling pathways involved in the control of cell division, proliferation, and development [1,2]. Activating somatic mutations in Ras proteins occur in 15%–25% of all human tumors [3]. There are three major Ras proteins in humans: N-, H-, and K-Ras. These proteins share essentially the same catalytic machinery yet differ in their ability to drive cancer formation (e.g. [4]). K-Ras mutations represent 85% of all oncogenic Ras mutations and are frequently found in lung, colorectal, and pancreatic carcinomas [5–8]. N-Ras mutations are common in melanomas, hepatocellular carcinomas, and hematologic malignancies [9–11]. Although less frequent, H-Ras mutations are found in bladder, kidney, and thyroid carcinomas [3,12,13]. Therefore, Ras proteins remain one of the most crucial anticancer drug targets [14].

The nucleotide-binding site would have been a logical target for structure-based ligand design for Ras. However, it is conserved in other families of GTPases and therefore targeting this site would likely lead to problems of selectivity and toxicity. Although there are ongoing efforts toward developing guanosine diphosphate (GDP) analogs for covalent binding to the G12C mutant of K-Ras [15,16], such an inhibitor would not be applicable to the vast majority of oncogenic Ras mutations. Even if selective inhibitors of the active site were identified, the high intracellular concentrations of GTP and GDP and their high (picomolar) affinity for Ras would make competitive inhibition impractical. Therefore, alternative strategies are needed to abrogate abnormal Ras function.

Over the years, a number of different approaches have been used to try to find ligands that inhibit Ras function. These include indirect ways of perturbing membrane binding of Ras, and blocking the activity of Ras activators and downstream effectors. Examples of the former include development of farnesyltransferase inhibitors (FTIs) [17–20] and farnesyl analogs [21–24], as well as compounds that modulate plasma membrane lipid distribution [25–27], induce cytoplasmic accumulation of inactive Ras [28,29] and inhibit trafficking proteins responsible for localizing Ras on membranes such as PDEδ [30,31]. The goal is to somehow disrupt Ras membrane binding that is required for its biological activity. While the impact of the latter four classes of compounds is yet to be determined, FTIs turn out to be a major disappointment [32]. This is because K- and N-Ras can be alternately prenylated by geranyl-geranyl transferase in cells treated with an FTI [33], and combination therapy with multiple prenylation inhibitors leads to severe toxicity [19]. Similarly, inhibition of Raf, a downstream effector of Ras, leads to a paradoxical activation of Ras [34,35]; nonetheless

efforts are still ongoing to inhibit other Mek- and Erk-related pathways [36,37]. It remains unclear as to which of these approaches, if any, will turn out to be most successful in preventing abnormal Ras function. We favor approaches that aim at directly inhibiting oncogenic mutant Ras.

Recent work from our group [38–41] and that of others [42–53] demonstrated that Ras is an allosteric enzyme. In very simple terms, allostery is defined as an observed effect on a distant region of a protein, such as its active site, as a result of perturbation of another region upon ligand binding. Several earlier studies suggested an allosteric communication between the Ras active site and its membrane-facing surface and existence of multiple conformational substates [38–56]. Together, these observations opened up new possibilities of inhibiting Ras activity (see [57,58] for recent reviews) and, upon further computational studies [38,39], provided the initial clues into the potential druggability of Ras [59]. This was followed by the prediction of four previously uncharacterized allosteric ligand binding sites [38], some which subsequently confirmed by the discovery of a number of allosteric Ras binders [42,44–47] (discussed in detail in the 'Discovery and Validation of Four Allosteric Binding Sites on Ras' section). These ligands either interfere with GDP–GTP exchange [42,46,47], stabilize the inactive GTP-bound conformation of Ras (state 1) [44,45], or prevent Ras–effector interaction [46] (**Fig. 1**). It is important to note here that most of these ligands were discovered through *in silico* screening of ligand libraries [46] or via nuclear magnetic resonance spectroscopy (NMR)-based screening of molecular fragments [42,44,45].

The primary focus of this review is on the role of computational approaches to aid in efforts toward direct inhibition of Ras. We begin with a general overview of structure-based computer-aided drug discovery. We then discuss the four allosteric ligand binding sites obtained from computational and experimental studies, followed by a comparatively more detailed discussion of two recently developed computational methods that are likely to play a key role in ongoing searches for novel allosteric ligand binding sites on the soluble and membrane-bound Ras and related drug targets.

## Overview of Structure-based Computer-aided Drug Discovery

Given the involvement of mutant Ras in almost every major cancer type, the need for discovering drugs that abrogate abnormal Ras signaling cannot be overstated. Drug discovery, however, is a very complex and expensive process that takes years and costs billions of dollars [60]. Structure-based computer-aided drug design (CADD) approaches can expedite the process and reduce cost [61]. As a result, high-throughput virtual screening (HTVS) of ligand libraries has become an integral part of drug design programs in both industry and academic laboratories [62,63].

A typical modern CADD workflow is shown in **Fig. 2**. One of the key starting points for CADD is a well-characterized target whose atomic structure has been determined to a sufficiently high resolution [64]. Ras meets this condition with more than 150 high resolution crystal structures available in the protein data bank. Also of key importance is knowledge about the potential drug binding site or pocket(s) on the surface of the target [65,66]. In most cases, the target site for docking is the functionally most responsive orthosteric site, which, for example, can be the active site of an enzyme [67] or the agonist/antagonist-binding site of a G-protein-coupled receptor (GPCR). In some cases, active site inhibition is either ineffective or leads to toxicity if the site is highly conserved among related proteins. For such targets, allosteric inhibition is the preferred (or only) option to achieve enhanced selectivity or reduced toxicity. Moreover, some of the most effective drugs on the market are allosteric inhibitors (e.g. MK-2206 [68]). The first crucial step for a successful structure-based discovery of an allosteric inhibitor is the identification of an allosteric ligand binding site [69]. We will return to this issue in subsequent sections.

After target selection and binding site identification, one can conduct HTVS of ligand libraries against the target site. There are a number of knowledge-based and/or physics-based algorithms to perform HTVS using various energy functions for docking and scoring. Many excellent recent reviews have discussed current HTVS techniques as well as their advantages, limitations and potential for improvements [61,63,70,71]. In short, HTVS requires a careful selection of a small-molecule ligand library [72], which involves among other things setting up criteria for molecular size, solubility, and cell permeability [73–75]. There is a wealth of freely available data on small-molecule ligands in public databases such as ZINC [76] and PubChem [77]. These depositories of large numbers of drug-like small molecules

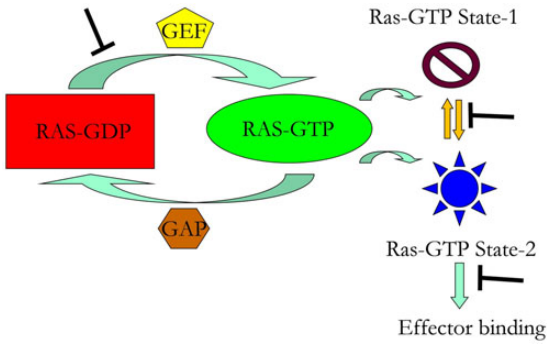


**Figure 1. Modulation of the GTPase cycle and effector interaction of Ras by weak allosteric inhibitors that have been discovered in the past several years** The catalytic cycle of Ras (left) and the two substates of GTP-bound Ras (right) are highlighted in cartoon diagram. The inverted T sign indicates the site of action of different ligands. GEF, guanine nucleotide exchange factor; GAP, G-protein activating protein.

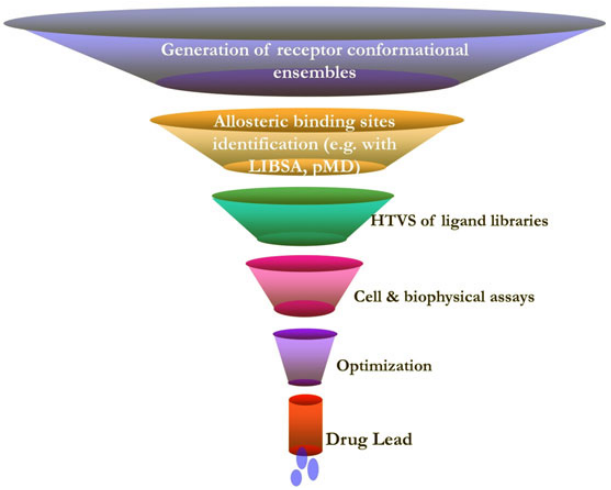


**Figure 2. Overview of a typical ensemble-based computer-aided drug design strategy** Shown here is a simplified workflow of a structure-based computer-aided drug design process involving target selection, binding site identification, high-throughput virtual screening of ligand libraries, experimental validation, and optimization for potency and selectivity.

provide ready-to-use, downloadable files of ligand libraries [63,77]. A focused library biased toward a given set of compounds can also be generated based on known high affinity binders via similarity searches and knowledge-based culling [63]. Once the desired ligand library is chosen, HTVS can be conducted using a number of programs such as GLIDE, DOCK, and Autodock [78–88]. The output of these algorithms includes docking scores (typically an estimate of the binding free energy) and the structure of the predicted target–ligand complex. The final result is a list of predicted hits ranked by binding free energy score, ligand pose, or both. It is often useful to obtain a consensus score from multiple docking runs and different programs to reduce false positives that usually arise from limitations in the scoring functions [89,90]. The predicted hits should be validated by experimental methods before being subjected to a series of optimization steps that are required to generate a lead compound with the desired potency and selectivity (**Fig. 2**).

## Discovery and Validation of Four Allosteric Binding Sites on Ras

As noted in the first section, a number of computational [38,39,54,55] and experimental [42–53] studies suggested that Ras is an allosteric enzyme. This conceptual advance led to the prediction of four allosteric ligand binding sites based on different computational approaches, primarily ensemble-based blind docking and FTMap [38,48]. The ensemble-based approach accounts for Ras flexibility and conformational transitions using molecular dynamics (MD) sampling of configurational space, followed by blind docking of drug-like molecules on the entire surface of each receptor conformation to search for allosteric binding sites. FTMap uses a continuum approach for docking of molecular fragments on the surface of a rigid receptor [91]. The four allosteric ligand binding sites predicted by these methods were subsequently confirmed by NMR or X-ray crystal structures of Ras in complex with small-molecule ligands [42–47]. **Table 1** and **Fig. 3** summarize the location of these pockets and the ligands that have been determined or predicted to bind to them. Because all of these sites have been discussed in detail in previous reports [39,42–47], we provide only a brief summary of their key features that will facilitate future drug design.

Three of the four pockets (p1, p2, and p4) are located near the functionally critical switch regions. Since the switch regions interact with Ras activators and effectors, ligands that target any of these pockets can be expected to directly or allosterically modulate binding to proteins either upstream or downstream of Ras. This is indeed the case [39,42,44–47]. As indicated in **Table 1**, residues on switch 2 and the β1–3 strands were shown to be involved in stabilizing ligands bound to p1 [42,46,47], and p1 is the target site for the majority of NMR and X-ray structures of Ras-ligand complexes solved to date. These include 4,6-dichloro-2-methyl-3-aminoethyl-indole (DCAI) [42], the Kobe-family ligands [46], and other ligands with specific chemotypes including indoles, phenols, sulfonamides, and their analogs [47]. Crystal structures of ligands bound to p2 (broadly defined) that form a covalent bond to the Cys of a G12C mutant Ras have been solved recently [43]. These ligands span either of two subpockets lying toward switch 2 or helix 3. Evidence from NMR and MD studies suggest that p4 is a viable drug target in Ras structures with open switch 1 conformation [39,45]. Finally, pocket p3, which has been shown to be targeted by metal-cyclens, is unique in terms of its distant location from the active site as it lies near the C-terminal end of the protein [44,45]. Therefore, it is likely to be more significantly affected by membrane binding.

## Recent Methodological Advances for Allosteric Binding Site Identification

Over the years, a number of useful computational techniques have been developed to expedite ligand binding site identification in the structure of target proteins [91–94], including SiteMap [95], MDpocket [96], FTMap [91], and others [97–99]. Here, we focus on two techniques that take advantage of MD simulation for efficient incorporation of protein motion into the site identification process:

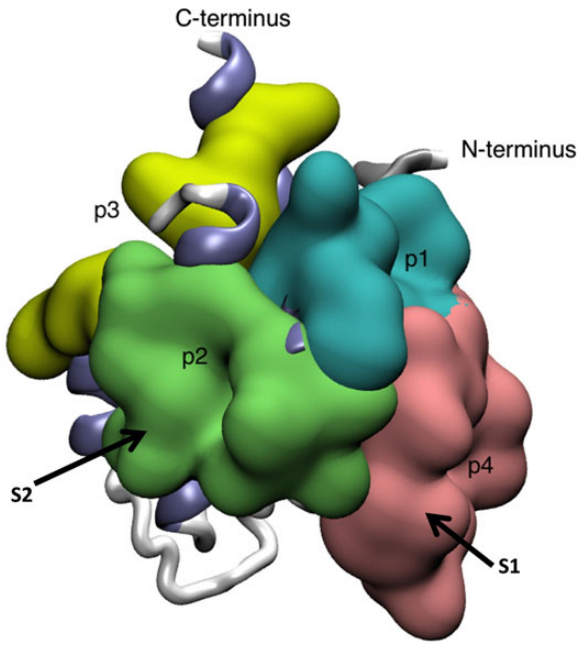


**Figure 3. The location of allosteric ligand binding sites on Ras** Four ligand binding sites are shown in different colors and labeled as p1 (light blue), p2 (green), p3 (yellow), and p4 (pink). The residues that define these pockets are listed in [92]. Note that in this illustration we used a single structure of G12D K-Ras in which some of the pockets are not fully open. As discussed in the main text, opening of some pockets in Ras requires relaxing the protein through MD simulation.

**Table 1. Binding sites/pockets of Ras characterized by experimental and computational studies**

| Binding site | Region | Ligand |
|---|---|---|
| p1 | β1–3, switch 2 | DCAI [42], Kobe ligands [46], indole, phenol, sulfonamide-containing ligands [47] |
| p2 | Loop2, switch 2, and helix 3 | Compounds specific for G12C Ras [43] |
| p3 | Loop7 and helix 5 | $M^{2+}$-BPA [45], $M^{2+}$-cyclen [44] |
| p4 | switch 1 | $M^{2+}$-BPA [45], andrographolide derivatives [39] |

ligand binding specificity analysis (LIBSA) [94] and probe-based MD (pMD) [92].

## Conformational ensembles and MD simulations

A number of excellent recent reviews have discussed the importance of incorporating structural flexibility to increase the success rate of CADD [100–102]. One of the most widely used methods to achieve this is MD simulation. When the ligand binding site is already known, MD can be used to generate ensembles of receptor conformations against which site-directed docking can be performed via the 'relaxed complex' scheme [103]. This paradigm of coupling protein dynamics to HTVS through MD is being widely used [104,105]. A relatively new application of MD in drug discovery is for allosteric binding site identification [106]. Because MD is highly effective in sampling conformations that are not readily captured by experimental methods, it has the potential to reveal pockets hidden in an averaged X-ray structure [92].

## LIBSA

The LIBSA protocol (see **Fig. 4**) has been developed in our laboratory with the goal of quantifying the relative binding preference of a ligand to a given target site versus all other sites [94]. It was validated on a number of different systems with known ligand binding sites, including kinases, G-proteins, and a GPCR. LIBSA relies on MD to generate conformational ensembles for the target protein, followed by common techniques such as RMSD-based clustering [107] to generate representative structures for docking (**Fig. 4**). A drug-like molecular probe is then docked onto the entire surface of the protein using available docking programs such as Autodock [83]. Three simple tools were developed to remove docking noise and compute binding preference based on the idea that binding consistency (rather than just affinity) can be used as a metric for identifying hits. The first tool, termed affinity filtering, uses the distribution of docking scores to reduce noise by assigning more weight to the high frequency high affinity poses. In this procedure, the inherently large error in scoring functions can be accounted for by including ligand poses whose predicted affinity lies within a certain range of the high frequency poses. The second filter, termed high pass filter, reduces noise by applying a digital filter on the distribution of contacts involving the probe molecule and residues of the receptor. Following the application of one or both of these filters, a signal-to-noise ratio (SNR) is used to quantify binding preference. The current implementation of the SNR essentially refers to the sum of contacts that the probe molecule makes with a set of residues within a predetermined binding patch divided by the sum of all other probe–residue contacts. Each of these three tools can be applied on data generated by any method that can scan the surface of a target with a drug-like molecular probe.

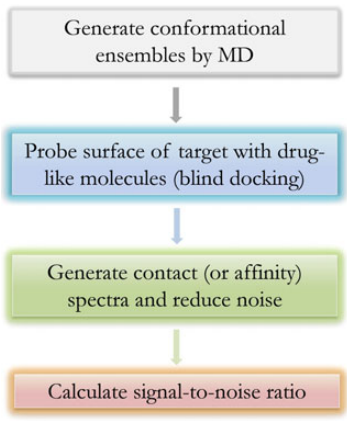


**Figure 4. Workflow of LIBSA** LIBSA enables determination of ligand binding preferences to allosteric sites on an ensemble of receptor conformations. The protocol involves optional preprocessing of MD-generated protein conformers based on clustering or other methods, followed by docking of a drug-like molecule on the entire surface of the target, quantification of contact (and/or affinity) frequency distributions, and determination of SNR for each site of interest.

LIBSA was applied on Ras in a couple of different ways. First, as an additional test of robustness, the method was used to re-identify binding sites of ligands that were solved in complex with Ras. Then, it was applied on MD-derived ensembles of Ras structures in solution to rank the binding preference of andrographolide derivatives for different pockets. These ligands were found to exhibit distinct pocket preferences despite their chemical/structural similarity [94].

## pMD

First proposed by Seco *et al.* [108], pMD is a mixed solvent MD approach for the identification of novel binding pockets (e.g. [108,109]). This method incorporates protein motion directly, thus allowing for effects of conformational selection or induced fit to be captured during the pocket identification process [109,110]. In pMD, MD simulation is conducted in the presence of probe molecules as part of the solvent environment (typically a 20:1 water-to-probe ratio) [92,108,109,111–114]. The most common probes include ethanol, isopropanol, isobutanol, acetone, acetaldehyde, and benzene [91]. These molecules represent some of the most common fragments found in marketed drugs [109]. The average distribution of the probe occupancy is used to compute the druggability index of various sites based on grid binding free energies [92]. The method can also be used to estimate the maximal binding affinity ($K_d$) per site, although this must be used with caution as the results can be sensitive to the cutoff used to group interaction spots (see [92] for more details). Probe-based MD has been tested on a number of systems [108,109,111–114], including the isolated catalytic domain of K-Ras in solution [92] where it was able to identify all four allosteric pockets described in the 'Discovery and Validation of Four Allosteric Binding Sites on Ras' section (**Fig. 5**). In addition, binding sites that may be involved in protein–protein or protein–membrane interaction were predicted. These additional sites may be useful for engineering lead compounds to augment binding to the primary sites p1–p4.

As mentioned earlier, membrane binding is essential for the biological function of Ras [115,116], and several studies suggested that there exist differences in dynamics between Ras in solution and when bound to membrane [54–56,59,117,118]. Therefore, it is important to examine if the allosteric ligand binding sites characterized in solution might still be accessible to ligands when Ras is membrane bound. In addition, it is possible that new druggable sites might open during fluctuations of Ras in membrane. However, until recently, we lacked the necessary tools to computationally probe these issues. While pMD can be used in principle, the typically apolar probe molecules tend to partition into the membrane and affect its structure and dynamics. We have developed an approach referred to as pMD-membrane that overcomes this limitation [118]. The method entails modifying the van der Waals interaction potentials between selected atoms of the probes and the lipid molecules without affecting the structural properties of the bilayer and the target protein. Application of pMD-membrane to G12D K-Ras in two different modes of membrane binding revealed that, in one of these, only three of the four

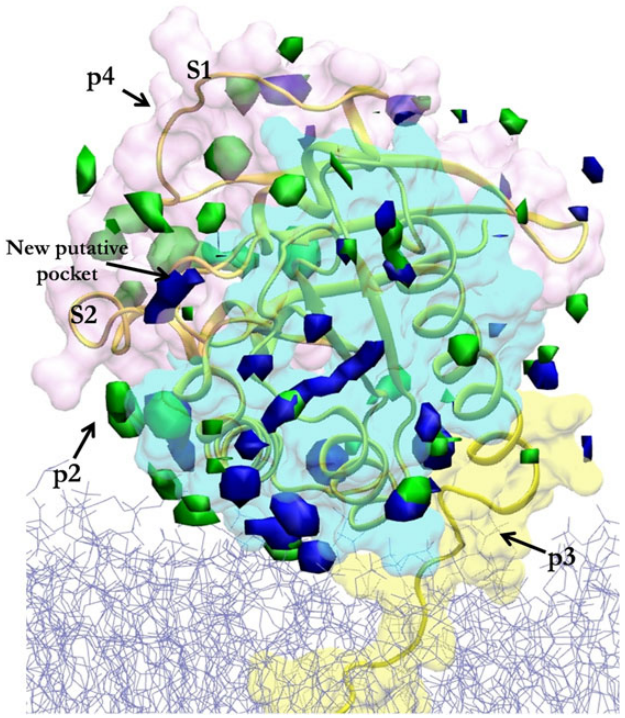


**Figure 5. Allosteric ligand binding sites on Ras identified by pMD** Four ligand binding sites are shown by black arrows and labeled as p1, p2, p3, and p4. All four allosteric pockets are observed by pMD-solution (green solid spheres) while a new site emerges and p1 disappears in pMD-membrane (blue solid spheres). Lobe 1 is in pink and lobe 2 in cyan. A portion of the bilayer is shown in ice blue lines. Hypervariable region is shown in yellow.

pockets identified in solution are still visible; instead a new putative pocket emerged (**Fig. 5**). This suggests that membrane binding indeed affects pocket accessibility (**Fig. 5**). Similarly, we found differences in the accessibility of sites to probe molecules when we compared G12D and G13D mutations [118]. These examples demonstrate that pMD-membrane is a promising new technique that has the potential to quantify differences in the ligand binding potential of Ras in solution and membrane environments, and between different mutant forms.

### Similarities, advantages, and limitations of LIBSA and pMD

These two MD-based ligand binding site identification techniques share a number of common features. To name a few, both use molecular probes to search for binding sites on the surface of the target protein, and both place major emphasis on probe-target contact frequency rather than binding score to estimate binding preference. This means that the probability of interaction of a probe molecule with a given site determines the druggability of the target site (e.g. [92,109]). The notion that the ligand binding potential of a site is proportional to the frequency of probe binding to 'hotspot' residues also forms the basis for fragment screening by NMR [119,120], where for example, difference spectra of an $N^{15}$-labeled protein before and after the addition of molecular probes can be used to determine the ligand binding potential of a site [119]. Similarly, fragment-based computational ligand screening methods such as SEED [84] rely on docking of comparatively few chemical building blocks derived from drug-like molecules to serve as scaffolds for the construction of novel ligands [120]. The same basic principle applies to FTMap [91].

Despite the shared features noted above, LIBSA and pMD also differ in some important ways. First, the tools in LIBSA allow for an efficient search for allosteric pockets utilizing data from conventional MD simulations, whereas pMD directly couples the pocket identification process with the simulation. Second, the probe in LIBSA is a drug-like molecule, preferably a known ligand whose binding site on the protein of interest is unknown. In contrast, pMD primarily uses as probe small organic molecular fragments rather than the drugs themselves. Therefore, the two methods can be used in slightly different ways depending on need. For instance, if an allosteric ligand has been already identified but not its binding site, LIBSA can use the known ligand as a probe to search for its binding site. In the absence of a known ligand, pMD is a better option. Note that although in principle a known drug can also be used as a pMD probe, issues of solubility and aggregation could be a concern. Thus, LIBSA is potentially more appropriate for retrospective design and pMD for prospective design. pMD would be a better alternative if nothing is known about a potential ligand or its binding site also because, in some cases, the probe molecules themselves may facilitate pocket opening [118]. Third, whereas pMD is exclusively used in conjunction with MD, LIBSA can be applied on an existing (experimental) receptor structure or on an ensemble of conformers derived from MD or other simulation methods. Finally, pMD can be extended to the native environment of the target protein of interest, such as membrane, more easily than does LIBSA. We note, however, that the two techniques are not mutually exclusive and can be regarded as complementary tools for identifying consensus allosteric ligand binding sites on ensembles of receptor conformations.

Although the two techniques do not differ much in terms of computational cost, LIBSA requires molecular docking after MD simulation and therefore can be marginally more expressive than pMD. As expected, in both cases computational cost increases with the size of the target protein but the size of molecular probes has little impact on computational cost.

## Conclusion and Perspective

Recent advances in computational methods have been instrumental in the identification of allosteric ligand binding sites on Ras, an elusive anticancer drug target. While the search for ligand binding sites on Ras in solution might be regarded as over with the identification and validation of four allosteric sites, it remains unclear if these binding sites and their accessibility to ligands are modulated by interaction of Ras with membranes. Moreover, the potential of the reported hits to become a successful lead compound is limited by their poor selectivity and weak affinity. Therefore, the search for hits that can eventually become drug leads must continue. We believe future efforts in structure-based drug discovery for Ras must pay special attention to structural fluctuations of Ras in its native environment of lipid membranes; the discovery of isoform- and mutation-specific inhibitors would likely depend on our ability to account for small differences in dynamics and membrane-organization. Our new method, pMD-membrane, would facilitate these efforts. Furthermore, selectively stabilizing one of the two substates of GTP–Ras, particularly the substate with weak effector binding potential, may lead to selective inhibition. Finally, the approaches discussed in this review are also applicable to other challenging targets, including the many members of the large Ras superfamily.

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