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J Mol Biol. Author manuscript; available in PMC 2023 September 15.

Published in final edited form as:
J Mol Biol. 2022 September 15; 434(17): 167569. doi:10.1016/j.jmb.2022.167569.

Allostery: Allosteric Cancer Drivers and Innovative Allosteric Drugs

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Abstract

Here, we discuss the principles of allosteric activating mutations, propagation downstream of the signals that they prompt, and allosteric drugs, with examples from the Ras signaling network. We focus on Abl kinase where mutations shift the landscape toward the active, imatinib binding-incompetent conformation, likely resulting in the high affinity ATP outcompeting drug binding. Recent pharmacological innovation extends to allosteric inhibitor (GNF-5)-linked PROTAC, targeting Bcr-Abl1 myristoylation site, and broadly, allosteric heterobifunctional degraders that destroy targets, rather than inhibiting them. Designed chemical linkers in bifunctional degraders can connect the allosteric ligand that binds the target protein and the E3 ubiquitin ligase warhead anchor. The physical properties and favored conformational state of the engineered linker can precisely coordinate the distance and orientation between the target and the recruited E3. Allosteric PROTACs, noncompetitive molecular glues, and bitopic ligands, with covalent links of allosteric ligands and orthosteric warheads, increase the effective local concentration of productively oriented and placed ligands. Through covalent chemical or peptide linkers, allosteric drugs can collaborate with competitive drugs, degrader anchors, or other molecules of choice, driving innovative drug discovery.

Graphical Abstract

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CRediT author statement
Ruth Nussinov: Conceptualization, Investigation, Writing - Original Draft, Supervision, Project administration. **Mingzhen Zhang**: Resources, Visualization. **Ryan Maloney**: Resources, Visualization. **Yonglan Liu**: Resources, Visualization. **Chung-Jung Tsai**: Resources, Visualization. **Hyunbum Jang**: Conceptualization, Investigation, Writing - Review & Editing, Supervision.

Declaration of interests
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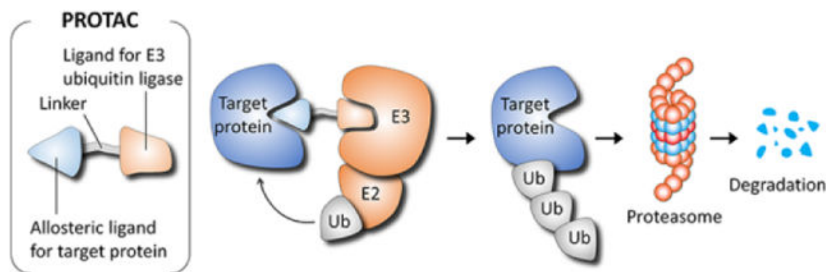

Keywords

ensembles; allosteric PROTAC; allosteric molecular glues; autoinhibition; kinase drug discovery; enzyme catalysis; cellular network

Introduction

Classically, throughout past decades, the life sciences aimed at morphological descriptions of organisms, their classification, and their habitats. The emergence of the structure – function paradigm was inspired by the recognition that even living things must conform to the laws of physics, including the laws of motion and structural chemistry [1]. The gradual conceptual change merged with technological advances that were able to capture the conformational heterogeneity [1–5]. Allostery, which involves dynamic shifts of the distributions of the conformational ensembles prompted by some triggering events, is at the center of regulated molecular behavior [6–15]. Changes in the conformational distributions provoked by allosteric PTMs (post-translational modifications), interactions with ions, cofactors, mutations, allosteric drugs, changes in concentrations, and more [16–30], result in conformational and dynamic changes [12,13,31–41] and can be captured by the dynamic free energy landscapes [37,42]. The resulting energetic frustration, or local energetic conflicts in the structure [43–46] reinforced by the allosteric conformational changes, propagate and are the key to protein function [47–52]. Allostery signals at short ranges. If the domains are separated by long disordered linkers, as in the case of Raf kinase [53], the relatively similar free energies of the conformational states (the depths of the basins in the landscape) characteristic of disordered ensembles, support multiple routes through which the allosteric signals can travel [54]. Funnels of folding, binding and function [55–58] showed that folding progresses via multiple routes, with some dominantly populated, which may not be the case for allosteric propagation or folding of disordered states.

Catalysis and regulation are the beating heart of biology [59]. In catalysis, ensembles of activated conformations can catalyze multiple reaction steps [60]. The transition-state ensemble [61] can also employ multiple paths from the rugged saddle region as shown for the yeast chorismate mutase [62,63]. Thus, conformational ensemble concepts [64] extend from folding to binding to enzymatic catalysis, and below we apply them to allosteric cancer drivers and innovative allosteric drug concepts. Considering that to have a functional impact allostery-prompted signals of activating mutations need to propagate downstream, we further take up the vital and formidable question of measurements of signal thresholds and outline some principles [65].

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Allosteric driver mutations in cancer

Here, we consider diseases that are the outcome of allosteric driver mutations [33,66–79]. Not all drivers are allosteric [80]; mutations at the functional (or active) site are not, as exemplified by oncogenic Ras drivers at G12, or Q61. More on this below. Allosteric drivers work by destabilizing the inactive protein state, stabilizing the active state, or both [33]. In repressors, allosteric mutations stabilize the inactive state [70]. The differences in energy between the inactive and active states are typically relatively small. To get stabilized, that is, to become the favorable state, the protein needs to undergo a conformational change. Mutations can do this by breaking the interactions that stabilize it, and/or forming alternative stabilizing interactions, such as salt bridges or hydrophobic cores. By definition, these are allosteric events [6] that can lead to significant increase in protein activity over the basal state. Driver mutations promote cancer since they bestow a growth edge [81]. Current therapeutic regimens seek knowledge of the patient's driver mutations to guide treatment [82–85]. Driver mutations are commonly identified by their high frequencies of occurrence, making detection of rare allosteric driver mutations that may be equally potent a daunting task [86]. Thus, in addition to the statistics of occurrence, strategies to identify driver mutations include structural location and organization, and functional consequences [81]. Clustering of mutations not only in protein sequences [87] but especially in structures [88–93] is one such strategy. Allosteric drivers often cluster since clustering intensifies their effect. A classic example of clustered residue hotspots is the "hot regions" [94,95]. The residues are structurally highly conserved residing within locally tightly packed regions, where they form a network of interactions making their contributions to the stability cooperative. Identification of drivers can also be helped by molecular dynamics (MD) simulations, which can observe conformational changes. Residue interaction networks [96] can further reveal whether they lie on the same propagation pathway of the intramolecular allosteric signal, which can strengthen the signal outcome. Other approaches include (i) machine learning, which can identify dynamic signatures (e.g., [40,97–100]), and whose vast contributions to the field will be reviewed elsewhere, (ii) dynamic residue networks and (iii) perturbation response scanning which were used to identify allosteric hot spots of human Hsp90 as cancer drug target, and it was shown that both approaches are in agreement [101]. A recent review summarized the approaches to identify allosteric regions/hotspots. It includes residue interaction networks and other approaches. It also extensively discusses allosteric drugs [102]. Additional approaches have also been devised [103–109]. From the conformational standpoint, the hallmark of allosteric activity is the behavior of the ensemble. If it is shifted toward an active (or inactive in repressors) state, then the mutations are activating. Ensemble shifts can be captured through, e.g., schematic, funnel-like "function diagrams" [110], nuclear magnetic resonance (NMR), and MD simulations [111–114]. Recently, we proposed that a key mechanism of allosteric driver mutations is relieving autoinhibition and provided a few mechanistic examples, such as protein kinase B (AKT) and mammalian target of rapamycin (mTOR) protein kinases, phosphoinositide 3-kinase α (PI3Kα) lipid kinase, SH2 domain-containing phosphatase 2 (SHP2) phosphatase, NIMA-related kinase 7 (NEK7) and 9 (NEK9) protein kinases, and engineered mutations in MAP/microtubule affinity-regulating kinase 1 (MARK1) kinase-associated-1 (KA1) domain [66]. Mutations that relieve autoinhibition are allosteric drivers, whether they are frequent

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or statistically rare [86]. Below, we go deeper into PI3Kα with recent data, which now also include double/multiple mutations, Raf, and phosphatase and tensin homolog (PTEN) phosphatase for comparison and discuss Ras non-allosteric mutations.

PI3Kα lipid kinase

PI3Kα is a lipid kinase. It is a key component in the PI3K/AKT/mTOR signaling pathway, phosphorylating signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) at the membrane [115,116]. Phosphorylation events play a key role in PI3Kα autoinhibition and activation [117]. PI3Kα has two domains, p85α and p110α. Single, double, and multiple cancer driver mutations have been identified in PI3Kα (Figure 1). They can be strong, functionally weak, or relatively rare. When co-occurring they can collaborate to more potently transform cells [118–120]. Strong drivers include E542K, E545K in the p110α helical domain and H1047R in the kinase domain. E726K and M1043V/I are weak drivers in the kinase domain. The other weak mutations are N345K, C420R, E453K/Q in the C2 domain and R38H/C, R88Q, R93Q, R108H, and G118D in the adaptor binding domain (ABD). Their actions can be additive or cooperative. The driver mutations activate PI3Kα by relieving the autoinhibition exerted by the nSH2 domain of the p85α subunit, which covers the active site and blocks access to membrane, and by positioning the kinase domain appropriately at the membrane. Under physiological conditions, the favorable interaction of the nSH2 with a phosphorylated tyrosine motif at the C-terminal of an insulin receptor, a receptor tyrosine kinase (RTK), promotes conformational changes that lead to exposure to the active site. The E542K and E545K driver mutations in p110α disrupt the interfacial salt bridges, relieving the autoinhibition and leading to a reorganization at the active site. In the absence of an incoming physiological signal from the RTK and Ras activation, the H1047R strong driver promotes the interaction with the membrane. The weak drivers (e.g., E453K/Q and E726K on the surface of the N-lobe, and M1043V/I in the interior of the C-lobe of the kinase domain) can couple with the driver hotspots [118,121]. They too help promote population shift toward the active state by enhancing the activation mechanisms, all of which mimic the physiological activation of PI3K, which involves two components: release of autoinhibition by the nSH2 domain and attaching favorably to the membrane. Some mutations (e.g., M1043V/I) enhance the population shift by stabilizing the hydrophobic core. For further details and the roles played by other driver mutations see [115].

Raf kinase

Raf is a key protein kinase in the mitogen-activated protein kinase (MAPK) signaling cascade [122–126]. It consists of three conserved regions (CRs). CR1 contains the Ras binding domain (RBD), which binds activated Ras at the membrane [53,127,128], and the cysteine rich domain (CRD), which anchors at the membrane [129,130]. Wild-type active Raf is a dimer [131]. In the inactive autoinhibited state, it is a monomer [132–134]. Both RBD and CRD are involved in Raf's autoinhibition by binding CR3, which results in occluding the dimerization surface of the kinase domain (Figure 2). Binding to Ras and the membrane exposes the catalytic kinase domain surface for dimerization and activation. CR2 has a flexible linker between CR1 and CR3 which contains a serine/threonine rich region. In the autoinhibited state, it is phosphorylated and binds the 14-3-3 protein. The binding

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stabilizes the ternary autoinhibited state [135–137]. CR3 is the kinase domain, which consists of two lobes connected by a hinge. When activated, it phosphorylates mitogen-activated protein kinase kinase 1/2 (MEK1/2), a key action in MAPK signal propagation. The activation loop (A-loop) runs from the conserved DFG motif to the APE motif in the C-terminal lobe. Phosphorylated Thr599 and Ser602 in the A-loop [138–144] of active, dimeric B-Raf destabilize the inactive and stabilize the active state. The switching from the inactive to the active state involves allosteric structural changes [145].

V600E, the most frequent cancer mutation in B-Raf, is a potent driver mutation [146,147]. MD simulations provided mechanistic details as to how it allosterically shifts B-Raf from the inactive to the active state by mimicking the mechanism of activation of the wild-type [120,123]. The A-loop is extended, and when the DFG motif orients such that Phe595 rotates away from the αC-helix, the helix moves inward (Figure 3). This allows formation of a salt bridge between Lys483 and Glu501. The mutation further acts by destabilizing the inactive state. It breaks the hydrophobic interactions and stabilizes the active state a salt bridge between Glu600 and Lys507.

PTEN tumor suppressor

PTEN is a tumor suppressor phosphatase [117]. It dephosphorylates signaling lipid PIP_3 at the membrane to PIP_2, opposing PI3K phosphorylation of PIP_2, thus suspending signaling. Its activation and catalytic reaction have been described [148]. PTEN consists of the PIP_2-binding motif (PBM, residues 1–15), the phosphatase domain (residues 16–185), the C2 domain which anchors at the membrane (residues 190–350), the C-terminal tail (CTT), and the PDZ binding motif (Figure 4). In the autoinhibited state, the phosphorylated residues in CTT interact with the arginine loop in the phosphatase domain, and the CBR3 and Cα2 loops in the C2 domain. Being a tumor suppressor, its driver mutations reduce the vital membrane interactions (S10N, K13E, G20E, L42R, and F90S) [149], obstruct the catalysis (R130Q/G), obstruct the essential phosphatase/C2 interface (S170N/G/I/R and R173C/H/L) [150], and relieve the autoinhibition. Its pathway is among the most highly mutated in cancer, particularly when involving mutations in PTEN, and to lesser extent, mutations in PI3Kα and AKT1 [151]. PTEN deletion mutations are also common in cancer [152] as are S170N/G/I/R and R173C/H/L [153]. Some of the mutations are allosteric and our on-going work aims to reveal their detailed mechanisms. Notably, some of PTEN's mutations have been observed to promote autism spectrum disorder (ASD) [154]. Neurodevelopmental disorders have also been associated with mutations in PI3K, Raf, Ras, and more [155,156]. Recently, we resolved the tantalizing question of how to understand same gene, and even same mutations, promoting both cancer and neurodevelopmental disorder [157].

Not all driver mutations are allosteric: The Ras example.

Wild-type Ras regulates cell growth and division [158–165]. It binds multiple effectors and signals through multiple pathways. Among these, MAPK and PI3K/AKT/mTOR stand out as the major, and most consequential in cell life, including development, and disease. Above we have already discussed its two main effectors, PI3K and B-Raf. These, along with Ras activators, regulators and its other effectors, and signaling cascades (e.g., PTEN), including events such as mutations, have been studied and reviewed in multiple publications (e.g.,

[54,128,130,136,137,166–189]). The MAPK signal enters the G1 phase of the cell cycle, where cyclin-dependent kinases promote passage to the S (synthesis) phase. Together with the Ras/PI3K/AKT/mTOR pathway signal [190,191], they promote cell growth and division, accelerating proliferation [192–194]. Ras inactivation is mediated by GTPase-activating proteins (GAPs) that hydrolyze the GTP to GDP [195]. Driver mutations such as those at G12 hinder GAP-assisted hydrolysis, keeping Ras in the active GTP-bound state [196–199]. Weaker drivers enhance the exchange of GDP by GTP, e.g., A146T in KRas4B [200] as well as influence the intrinsic hydrolysis. In the steady state of the cell, about seventy five percent of KRas^{G12C} is GTP bound, reflecting its high, millimolar-range concentration and picomolar affinity. G12C shows relatively higher rate of intrinsic hydrolysis as compared to other mutations such as G12D, G12V, G13D, and Q61H [201].

Different than kinases, in Ras activating mutations work by blocking deactivation, not by stabilizing the active and (or) destabilizing the inactive state; that is, not by shifting the equilibrium which results in increasing the number of the active molecules [158]. In contrast, kinases switch from the inactive αC-helix-out to the active αC-helix-in, movements that involve rotation and shift. In epidermal growth factor receptor (EGFR), the driver L858R mutation in the A-loop destabilizes the inactive αC-helix-out conformation with Arg breaking the hydrophobic interactions in the αC-helix-out, and the driver T790M mutation stabilizes the active αC-helix-in conformation by stabilizing the hydrophobic R-spine. T315I in Bcr-Abl, T334I in c-Abl, T341I in Src, T670I in Kit, and T674I in platelet-derived growth factor receptor α (PDGFRα), all also stabilize the hydrophobic R-spine [33].

Allosteric drugs: Bcr-Abl, allosteric molecular glues and allosteric PROTACs

Targeting Bcr-Abl

Allosteric drugs have been extensively reviewed by us and others (e.g., [12,31,33,34,202–210]). Below following a brief overview, we touch on some recent examples focusing on Bcr-Abl, a fusion of *BCR* and *ABL* genes present in most patients with different phenotypes of leukemia, including acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and neutrophilic-chronic myeloid leukemia (CML-N) (Figure 5). This fusion results from the reciprocal translocation of chromosomes 9 and 22. CML has been treated by tyrosine kinase inhibitors (TKIs), especially imatinib that binds to Abl *inactive state* [211]. Drug resistance eventually emerges, either by mutations at the active site or elsewhere in the kinase domain, allosterically altering the active site. Overcoming such mutational events requires a drug with higher affinity than the substrate or cofactor, which can be challenging to achieve. In the quintessential kinases example, this requires higher affinity than the low micromolar ATP [212]. It also requires sufficiently high dosage. High dosage leads to binding to related kinases with conserved active sites, thus toxic side-effects. Allosteric drugs bind at sites other than the active, or functional site, which are not conserved. Their resulting high specificity has low chance of side effects. Covalent allosteric drugs combine the pharmacological merits of covalent drugs and the high specificity of allosteric drugs [202,212]. Cooperativity between the orthosteric and allosteric ligand binding sites has been

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proposed [6] and recently observed for RAR-related orphan receptor γ isoform 2 (RORγt, a.k.a. RORγ2) [213] and discussed further in a number of works (e.g., [214] and references therein). How to quantify design parameters [18,215,216] was also overviewed.

A recent study reported that multiple patient-derived imatinib-resistant Abl kinase domain mutants still bound imatinib [217]. Kinetic analyses suggested that the allosteric drug resistance mutations resulted in considerably faster drug dissociation from the mutant as compared to the wild type. These observations can be explained by considering that the difference in energy between the active and inactive states of the kinase is small. The conformational changes promoted by the allosteric oncogenic mutations shift the free energy landscape from the inactive kinase to populate the now more stable active state, which is not imatinib binding competent. These changes destabilize imatinib binding [218–220], lowering its affinity which is outcompeted by the high affinity of ATP. Gene duplication, a frequent event in cancer, will increase Bcr-Abl expression, which will aggravate the plight. On a related note, resistance may also be augmented by upregulation of a redundant kinase [217,219,221–228].

In kinases, the A-loop, the DFG motif, the regulatory spine, and the gatekeeper residue are all key elements in identification of the active/inactive states. Recently two inactive states have been detected by NMR for the Abl kinase domain [218]. The studies revealed that the kinase domain interconverts between one active, populated 90% of the time, and two discrete inactive states, each transiently populated 5% of the time. One of these is imatinib binding competent. Resistance mutations shift the ensemble toward the active state facilitated by the small difference in energy. As to the two inactive states, in principle both can be used for drug design if new features are captured in the second. Additional Abl allosteric strategies have been discussed as well (e.g., see [223,229]).

Innovative allosteric drugs: from heterobifunctional PROTACs to molecular glues

Allosteric drugs have recently taken an innovative turn [230,231] through the powerful concept of molecular glues [232] and heterobifunctional PROteolysis TArgeting Chimeras (PROTACs) [233]. Molecular glues are small molecules that bind at the interface between two proteins and induce their interactions [234]. Induction of proximity is a groundbreaking concept in drug discovery, with molecular glues promising to broaden the therapeutic landscape. Their potential has been validated by natural products (e.g., rapamycin, tacrolimus (FK506), and sanglifehrin A), synthetic small molecules (e.g., IMiD, immunomodulatory imide drug) including thalidomide and its analogues, lenalidomide and pomalidomide), and anticancer sulfonamides (e.g., tasisulam, indisulam, and chloroquinoxaline sulfonamide (CQS)) (Figure 6). Uncompetitive molecular glues acting as active state stabilizers were shown to expand protein-protein modulation drugs for peptide hormone receptors [235]. Interfacial binding of the IMiD ligands induces a protein-protein interaction e.g., between cereblon (CRBN) and the target proteins [236]. The 'molecular glue' connotation was attributed not only to heteromeric protein-protein interactions but also to homomeric sigma-1 receptor (Sig1R) interactions to explain how allosteric modulators could increase the number of Sig1R in the agonist state conformation, thus activity [237]. Molecular glue compounds can stabilize weak protein interactions as

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shown in the case of the Cdc34A (a.k.a. UBE2R1, ubiquitin-conjugating enzyme E2 R1) where molecular glue compounds inhibit a noncovalent E2 enzyme–ubiquitin complex [238], and promote cyclin-dependent kinase 12 (CDK12)–DDB1 (CUL4 adaptor protein) interaction to trigger cyclin K degradation [239].

The PROTACs do not inhibit proteins [230,240–242]. They destroy them. While conceptually resembling molecular glues [243], PROTACS also differ. Unlike the molecular glues which consist of single small molecule, a PROTAC is a linkage of two. PROTACs are heterobifunctional degraders that enter cells and reduce their targeted proteins through the ubiquitination system. Allosteric PROTAC consists of a molecule (the warhead) that recruits an E3 ligase. The molecule is also linked to a high affinity allosteric inhibitor of the target protein. The inhibitor and the warhead molecules are covalently joined by a chemically suitable linker such that the target protein and the ubiquitination complex are brought into proximity. The IMiD molecular glue ligands above provide a good example [236], with the drug property downside of the construct. PROTACs have been exploited in mediated ternary complex formation (e.g., [244–247]) and their cooperativity have been delineated as well [248,249]. The challenge in designing them is in tuning the affinity and cooperativity of binding at both ends [248]. Especially challenging is the linker [250]. Its length and flexibility, essential to induce a ternary complex with the two proteins at the appropriate distance and orientation, require an optimal conformational bias. Differential allosteric PROTAC substrate specificity has been shown to be dictated by orientation of recruited E3 ligase [251], and the impact of linker length on the activity of PROTACs has also been investigated [252,253]. A PROTAC database has also been developed [254]. A list of selected degraders in, and approaching, the clinic has been compiled [233].

Recently, the first allosteric PROTACs were constructed. Abl1 contains an allosteric myristoyl binding site [255]. Multiple allosteric drugs have been designed to bind in this pocket [256,257]. The PROTACs were designed to covalently link to the allosteric inhibitor (GNF-5, an analog of GNF-2 [256]) (Figure 7) to degrade the Bcr-Abl1 mutant protein (with the T315I mutation). Since GNF-5 acts together with competitive inhibitors, such as imatinib, the allosteric PROTACs can collaborate with the competitive inhibitors trampling the oncogenic recalcitrant Bcr-Abl mutant, permitting a reduced drug dose and lesser side effects. GNF-5–PROTAC also acts on Bcr-Abl1 lacking mutations, permitting broad therapeutic stem cell applications [258].

Bifunctional molecules developed for targeted protein degradation through ubiquitination [243] are new potential allosteric drugs. In principle, this technology can bring any two proteins together through small molecules that target each partner. Details, outlines, and examples of the development of small molecule degraders that work by stabilizing and increasing the affinity of the ternary complexes and cases that have been shown to work have recently been reviewed in-depth, including an induced cooperativity in the catalytic degradation profiles [259]. The potential and innovation of such applications is only beginning to be apparent. One recent example has been discussed [238]. In another, G protein-coupled receptor (GPCR) example [235], it has been employed to promote the association of protein complexes, an emerging therapeutic strategy. The authors discovered a GPCR ligand that stabilizes an active state conformation by cooperatively binding both

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the receptor and orthosteric ligand. Drug resistance could arise through mutations in the degradation pathway, suggesting combination strategies to slow it. Additional innovative allosteric drug approaches have also been reviewed (e.g., [18,260]).

In another innovative and promising feat [261], a chemical biology approach developed a bitopic ligand for the RORγt nuclear receptor (NR), which concomitantly exploited two binding pockets. Three candidates were obtained, yielding an orthosteric and allosteric RORγt pharmacophore covalently linked via a polyethylene glycol (PEG) linker. Covalent occlusion of the RORγt ligand binding pocket was further shown to permit targeting of an allosteric site [262]. Here too, the linker's length influences the RORγt binding mode. Bitopic ligands can powerfully improve the affinity and (or) selectivity profiles [263]. Apart from identification of the allosteric pocket, the challenge is in the properties of the linker. Currently, linkers are mostly synthetic polymers such as polyethylene glycols, making an engineered conformational bias a formidable task.

Signal transduction

Molecular events, such as activation, are governed by the conformational behavior of the protein [1,2,6,8,10,32,92,264–276]. To be effective, the allosteric signal – physiologic or oncogenic – needs to propagate downstream. For that it needs to be sufficiently strong but not too strong. We dubbed productive signal transduction "*signaling by-the-numbers*". *Signaling by the numbers does not imply a stronger functional effect* [277], but rather, as setting the threshold for the signal for propagating downstream through the pathway to the cell cycle to activate (repress) transcription, or exit via physiologic senescence, premature developmental senescence, or oncogene induced senescence (OIS). Activity of a single protein (node) in the pathway cannot serve as the threshold for passing from one node to the other. To calculate the threshold, three quantities are needed: the expression level of the protein, how much is located (recruited) to the right place, and the population activated by the upstream signaling node. Cell type [278], cell state [279], timing window [280–283] in cell development, and chromatin modeling, all play a role [284–289].

Conclusions: future of studies of allostery

Where will studies of allostery go? If we can venture to predict, we see them focusing on translation, including the mechanisms of activating allosteric mutations, identification of druggable allosteric sites, and innovative and productive allosteric drugs. The advantages of allosteric drugs are well-established, especially, the higher specificity which is coupled with reduced side effects. Besides increasing the drug repertoire, efforts will focus on higher affinity, and potency. Exploiting the already available repertoire, which has already undergone clinical trials and is in use, will save development time and cost. Covalent linkage of such drugs via appropriate linkers to warheads that recruit degraders, as in the case of Bcr-Abl1's PROTAC, or to orthosteric drugs, can accomplish such a goal. In a way, this is analogous to repurposing drugs that are already in the clinic with documented safety profiles. The challenge is in the construction of linkers (or spacers) with appropriate length and conformational bias such that the orthosteric and allosteric pharmacophores yield bitopic ligands that achieve improved affinity and selectivity. The search is also on for

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allosteric drugs at newly discovered pockets or covalently linked to residues other than Cys, such as Tyr [290]. Innovative allosteric drugs may also be engineered to mimic allosteric rescue mutations [194].

Over 55 years have elapsed since Monod, Wyman, and Changeux have proposed the transformational two-state concerted model (MWC model) [291]. In the late 1990's, we proposed the conformational selection and population shift *versus* induced fit model to explain how biological functions are achieved through allostery [6,55,292]. Going forward, the challenge is in identifying rare allosteric activating mutations, their mechanisms, and innovative allosteric pharmacology.

In addition, we foresee conceiving ways of defining and measuring signal transduction, initiated by allosteric events, and propagating downstream, a formidable but achievable aim [65]. As we emphasized recently in "allostery, and how to define and measure signal transduction", such measurements are vastly important in setting thresholds for 'actionable' signals, which would permit assessing and predicting oncogenic signaling in tumor development and drug resistance. On their own, activating mutations may or may not influence the cell cycle and the level of expression. We have been trying to contribute our share toward these aims [65,157].

Acknowledgements

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261201500003I. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This Research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

Abbreviations

MD	molecular dynamics
NMR	nuclear magnetic resonance
AKT	protein kinase B
mTOR	mammalian target of rapamycin
PI3K	phosphoinositide 3-kinase
SHP2	SH2 domain-containing phosphatase 2
NEK	NIMA-related kinase
MARK	MAP/microtubule affinity-regulating kinase
KA1	kinase-associated-1
PTEN	phosphatase and tensin homolog
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate

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ABD	adaptor binding domain
RTK	receptor tyrosine kinase
MAPK	mitogen-activated protein kinase
CR	conserved region
RBD	Ras binding domain
CRD	cysteine rich domain
MEK	mitogen-activated protein kinase kinase
A-loop	activation loop
PBM	PIP_2-binding motif
CTT	C-terminal tail
ASD	autism spectrum disorder
GAP	GTPase-activating protein
EGFR	epidermal growth factor receptor
PDGFR	platelet-derived growth factor receptor
ALL	acute lymphoblastic leukemia
CML	chronic myeloid leukemia
CML-N	neutrophilic-chronic myeloid leukemia
TKI	tyrosine kinase inhibitor
RORγ	RAR-related orphan receptor γ
PROTAC	proteolysis targeting chimera
IMiD	immunomodulatory imide drug
CRBN	cereblon
Sig1R	sigma-1 receptor
CDK	cyclin-dependent kinase
GPCR	G protein-coupled receptor

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- We overview the principles of allosteric activating mutations and allosteric drugs
- Examples of activating mutations include the Ras signaling network and Abl kinase
- We overview innovative allosteric drug concepts, underscoring the challenge
- The review links allostery on the molecular level and productive cell signaling
- From the cellular standpoint, we propose a signaling by-the-numbers lens

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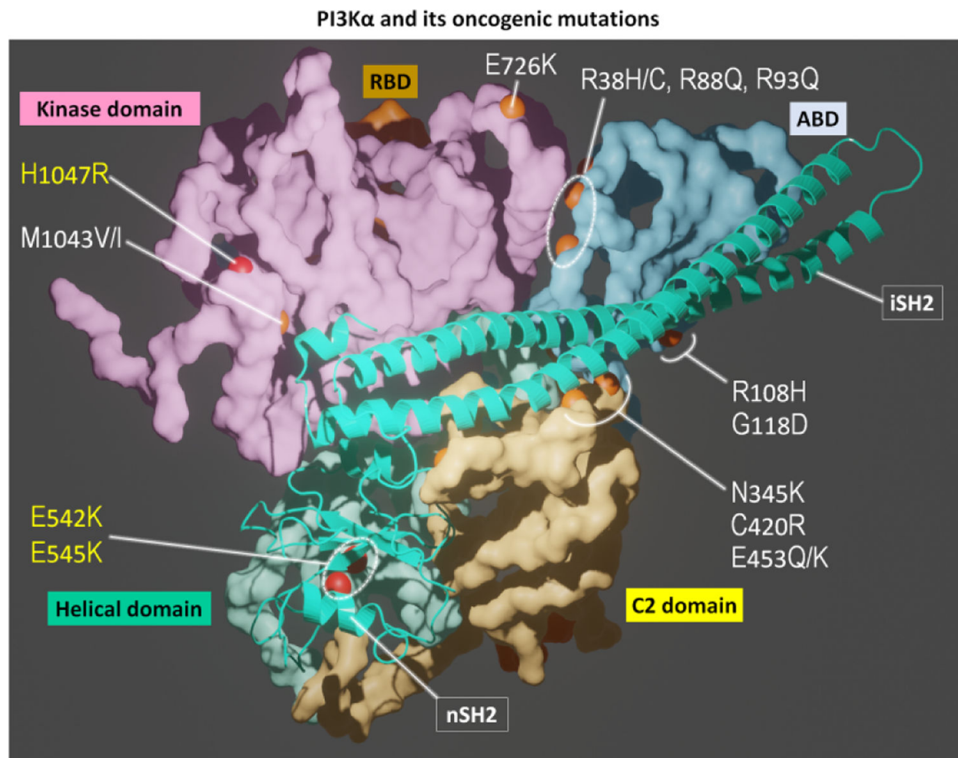

Figure 1.

PI3Kα and its oncogenic mutations. PI3Kα is an obligate dimer with catalytic p110α (surface representation) and regulatory p85α (cartoon representation) subunits. p110α contains ABD (cyan), RBD (orange), C2 domain (yellow), helical domain (light green), and kinase domain (pink). The iSH2 and nSH2 domains in p85α interact with p110α subunit. PI3Kα contains hotspot mutations (H1047R in the kinase domain; E542K and E545K in the helical domain, yellow letter) and weak mutations (M1043V/I and E726K in the kinase domain; R38H/C, R88Q, R93Q, R108H, G118D in the ABD; N345K, C420R, E453Q/K in the C2 domain, white letter). These mutations can collaborate to transform cells more potently.

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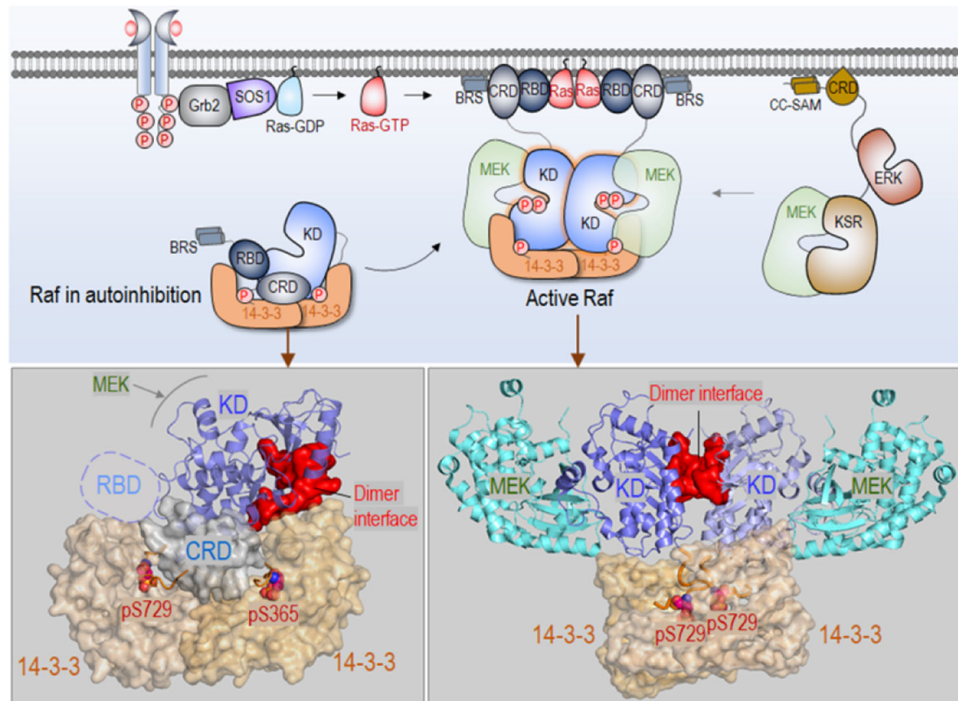

Figure 2.

Autoinhibited and activated Raf in the Ras/Raf/MEK/ERK pathway. Here, examples are shown for B-Raf (*top panel*). In the cytosol, Raf monomer is autoinhibited via the interaction with 14-3-3 proteins. Activated Ras by Son of sevenless 1 (SOS1) recruits Raf to the membrane, releasing autoinhibition. Raf is activated through side-by-side dimerization of the kinase domain. Active Raf dimer phosphorylates and activates MEK, and subsequently phosphorylates and activates ERK, leading to cell proliferation. Kinase suppressor of Ras (KSR) can act as a scaffolding protein, promoting the signaling. In Raf, KD denotes the kinase domain and BRS domain denotes B-Raf specific domain. In KSR, CC-SAM denotes coiled coil sterile α motif. The crystal structure of autoinhibited B-Raf interacting with 14-3-3 proteins (PDB: 6NYB) (*bottom left*). Two phosphorylated sites, pS729 and pS365, have strong interactions with 14-3-3 proteins. The dimer interface of the kinase domain (red surface) is blocked by 14–3-3 protein. The crystal structure of active Raf dimer in complex with 14-3-3 proteins (PDB ID: 6Q0J) (*bottom right*). pS729 interacts with 14-3-3 protein, and the MEK proteins are loaded to the kinase domain for phosphorylation.

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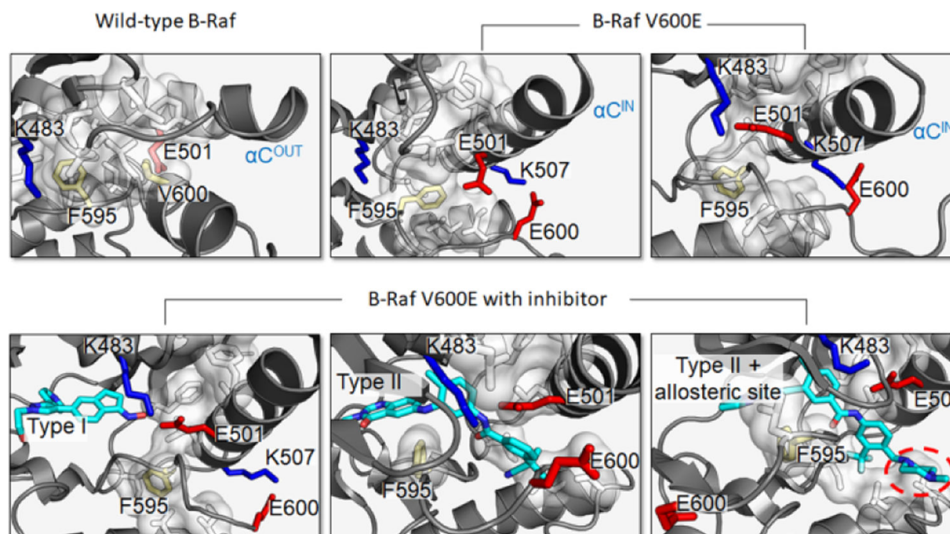

Figure 3.

B-Raf V600E destabilizes the inactive state and stabilizes the active state. Inactive wild-type B-Raf contains a large hydrophobic pocket (white) that includes Phe595 and Val600 (yellow), which prevents the formation of the Lys483–Glu501 salt bridge and maintains an outward αC-helix (*top left*). The V600E mutation disrupts the hydrophobic pocket and causes the activation loop of B-Raf to extend and forms the Lys507–GluE600 salt bridge. However, in a low population state, Phe595 can orient to prevent the Lys483–Glu501 salt bridge formation, which keeps the αC-helix from moving fully inward (*top middle*). B-Raf V600E with proper orientation of Phe595 to allow both Lys507–GluE600 and Lys483–Glu501 salt bridge formation, stabilizing the active state (*top right*). B-Raf V600E maintains an active configuration in the presence of Type I inhibitor GDC0879 (*bottom left*). Type II inhibitor derived from diarylthiazole allosterically inhibits B-Raf V600E by stabilizing the “DFG out” orientation (*bottom middle*). Type II inhibitor Ponatinib extends to an allosteric site (red circle), stabilizing the “DFG out” orientation and displacing the activation loop and E600 from its typical conformation (*bottom right*). Inhibitors are colored cyan. Cartoons depict the crystal structures of B-Raf V600E with inhibitor (PDB: 4MNF, 4CQE, 6P3D).

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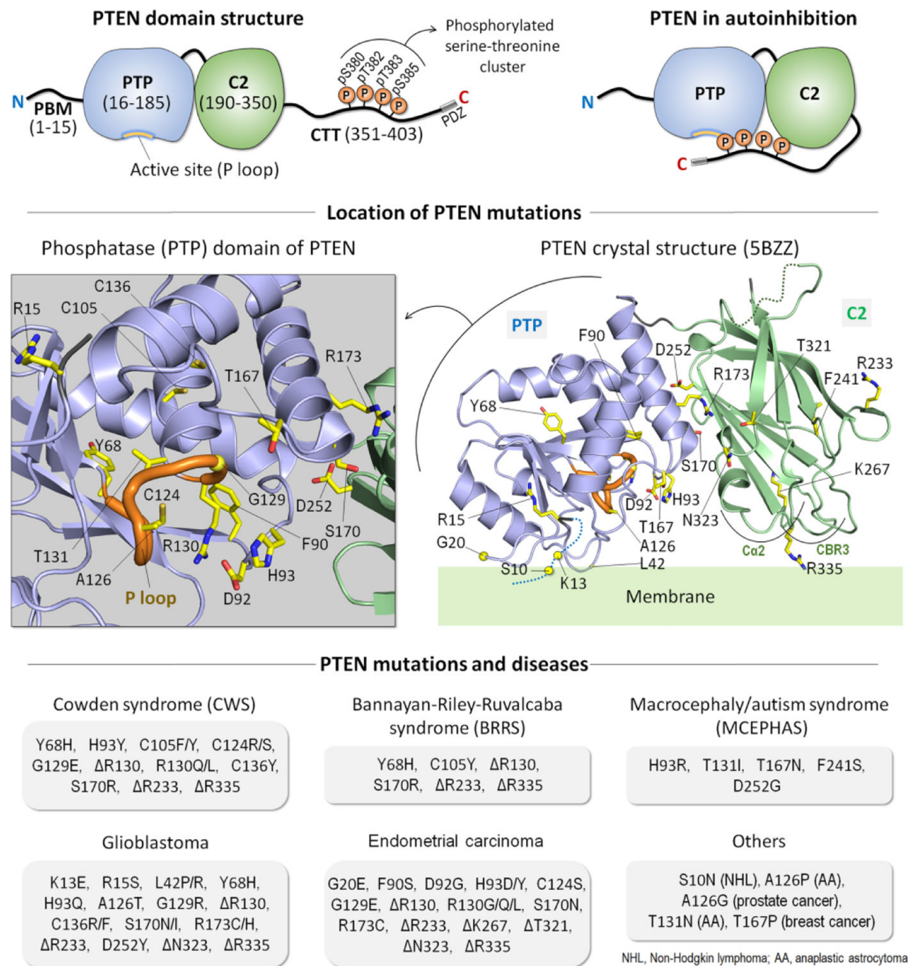

Figure 4.

PTEN structure and mutations. Domain structure of phosphatase and tensin homolog (PTEN) (*top left*). It contains the N-terminal PIP_2-binding motif (PBM), the phosphatase (PTP) domain, the C2 domain, and the C-terminal tail (CTT). The three residues, ^{401}TKV^{403}, at the C-terminus serve as the PDZ binding motif at the membrane.

In the cytosol, PTEN is autoinhibited by its CTT with phosphorylation on a serine-threonine cluster (pS380, pT382, pT383, and pS385) yielding a closed conformation (*top right*). Dephosphorylation on the CTT relieves the autoinhibition, promoting membrane localization. The locations of mutations mapped on the crystal structure of PTEN (PDB: 5BZZ) (*middle right*) and a highlight of the active site (P loop) in the PTP domain (*middle left*). Types of the mutations and their associated deceases are summarized (*bottom*). These include germline mutations of the neurodevelopmental disorders (e.g., Cowden syndrome (CWS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), and macrocephaly/autism syndrome (MCEPHAS)) and somatic mutations of the tumors (e.g., glioblastoma, endometrial carcinoma, prostate cancer, breast cancer, non-Hodgkin lymphoma, and anaplastic astrocytoma).

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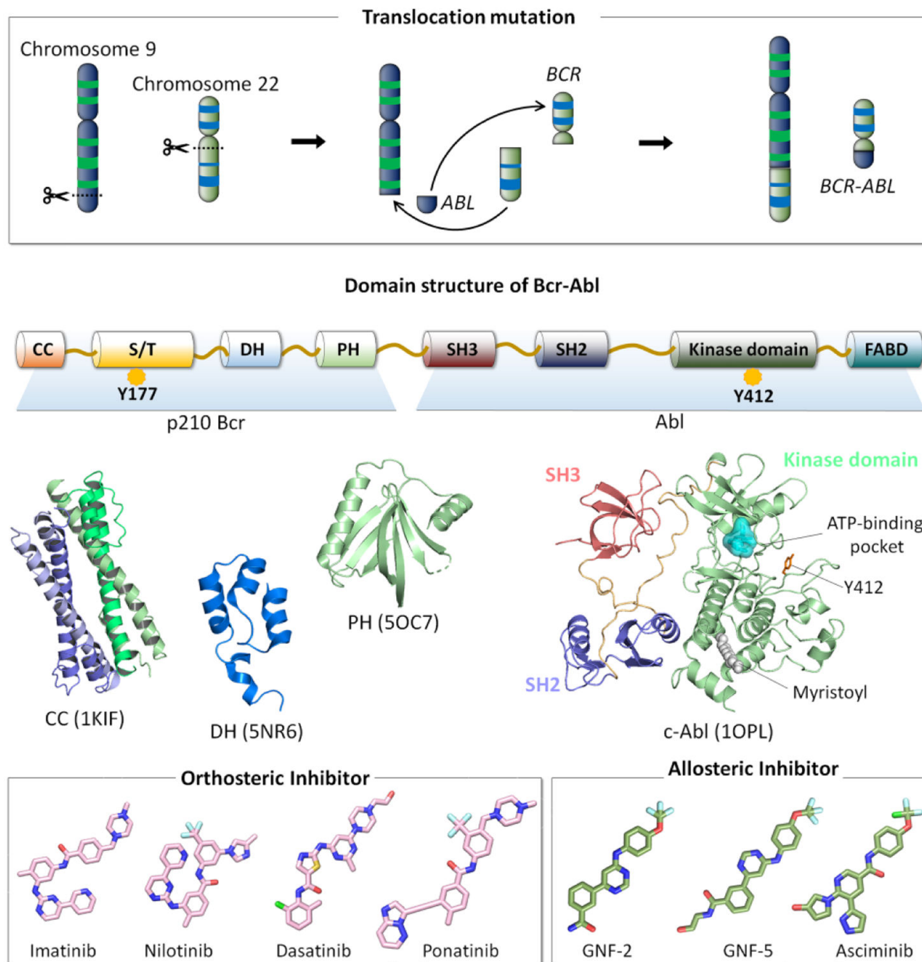

Figure 5.

Chromosome 9 consists of the *ABL* gene and chromosome 22 contains the *BCR* gene. In *BCR-ABL*-induced leukemia oncogenesis, the translocation mutation between chromosome 9 and chromosome 22 leads to the generation of the fusion *BCR-ABL* gene, which encodes the Bcr-Abl oncoprotein. p210 Bcr-Abl protein is the hallmark of chronic myeloid leukemia (CML). p210 Bcr region possesses the coiled coil (CC) domain, the serine/threonine (S/T) domain, the Dbl homology (DH) domain, and the pleckstrin homology (PH) domain. Phosphorylation of Tyr177 in the S/T domain enables to Bcr-Abl recruitment of Grb2 through its SH2 interaction, which activate Ras/MAPK pathway. Abl region contains the SH3 domain, the SH2 domain, the kinase domain, and the FABD (F-actin-binding domain). Phosphorylation of Tyr412 (numbered by Abl 1b isoform) in the activation loop activates Abl kinase. In Abl kinase domain, orthosteric inhibitors (e.g., imatinib, nilotinib, dasatinib, and ponatinib) bind to the ATP-binding pocket in the N-lobe of the kinase domain, while allosteric inhibitors (e.g., GNF-2, GNF-5, and asciminib) prefer to occupy the myristoyl-binding pocket in the C-lobe of the kinase domain.

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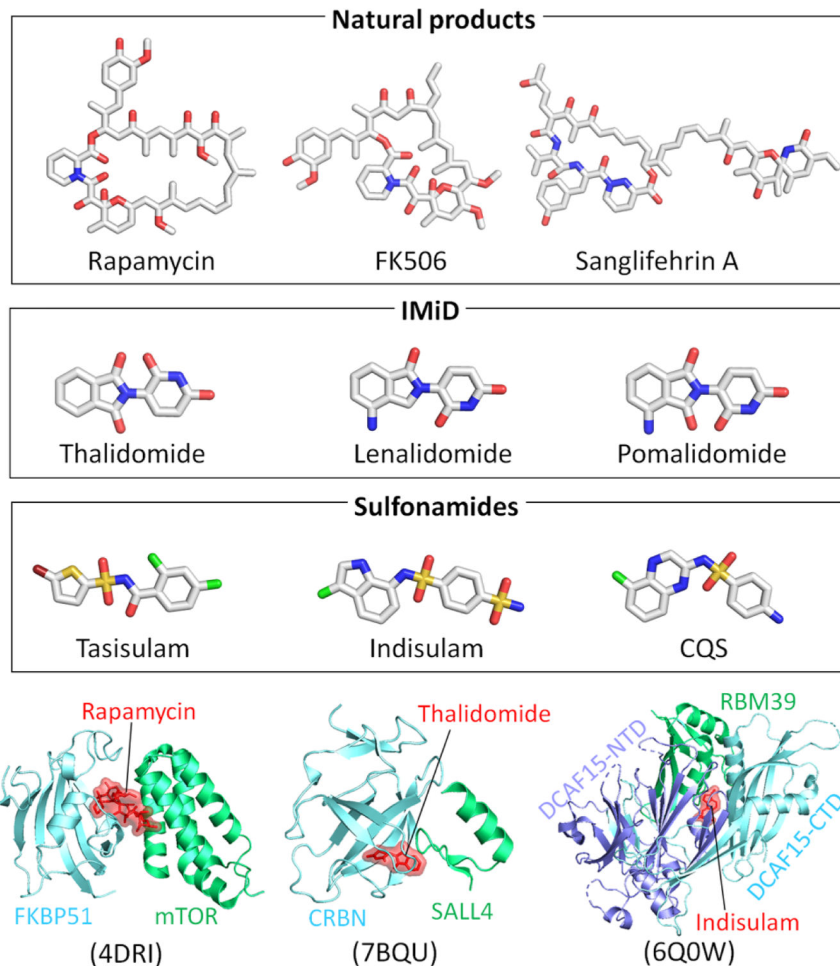

Figure 6.

Structures of different types of molecular glues, including natural products (rapamycin, FK506, and sanglifehrin A), IMiD (thalidomide, lenalidomide, and pomalidomide), and sulfonamides (tasisulam, indisulam, and CQS). Examples of the molecular glues in the intermolecular interface of protein complexes for rapamycin (PDB: 4DRI), thalidomide (PDB: 7BQU), and indisulam (PDB: 6Q0W).

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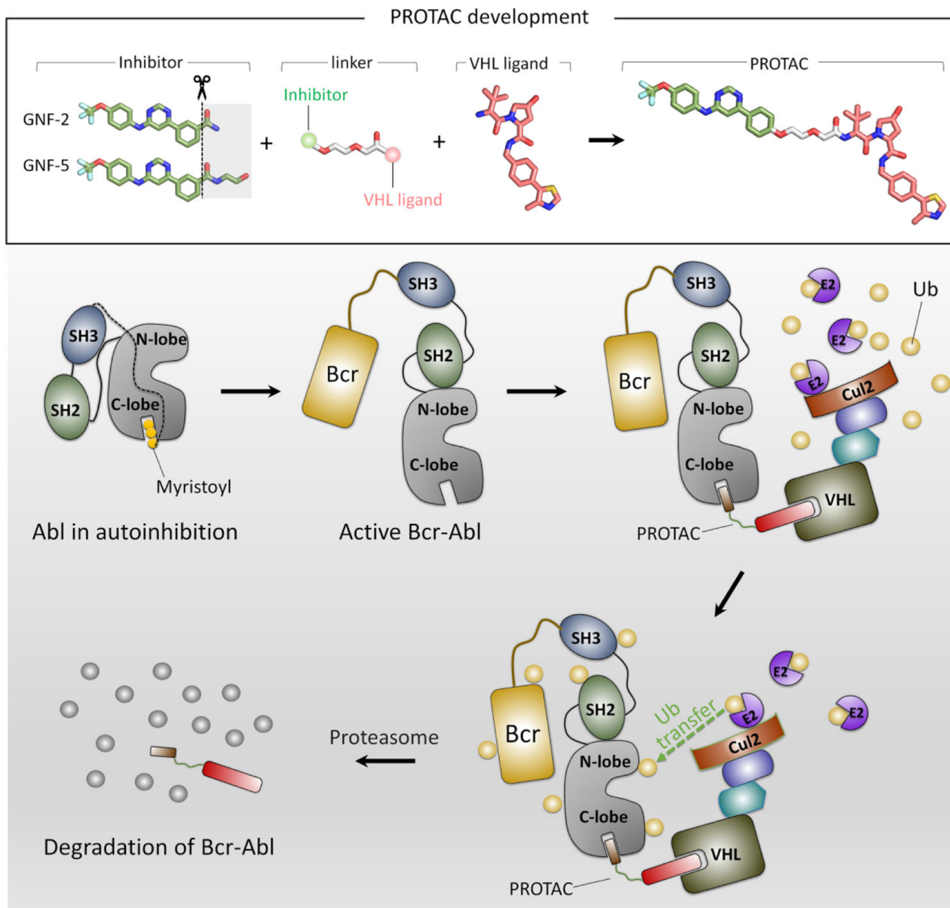

Figure 7.
GNF-5-PROTAC is formed by a GNF-5 inhibitor and a warhead molecule which are connected by a linker. Release of the myristoyl group from the C-lobe of Abl kinase domain activates Abl protein, leading to a vacant myristoyl-binding pocket. GNF-5, an allosteric inhibitor, can bind to the myristoyl-binding pocket of Abl. Of the heterobifunctional GNF-5-PROTAC molecule, GNF5 is responsible for the binding of Abl, while the other end recruits E3 ligase. This forms a Abl/GNF-5-PROTAC/E3 ternary complex. Then, this complex is brought into the ubiquitin system. The E3 ligase binds to E2 enzyme, regulating the transfer of ubiquitin protein to Abl from E2 enzyme. The Abl protein is degraded by proteasome accompanied with the dissociation of Abl/GNF-5-PROTAC/E3 ternary complex.