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Strategies for the Selective Regulation of Kinases with Allosteric Modulators: Exploiting Exclusive Structural Features

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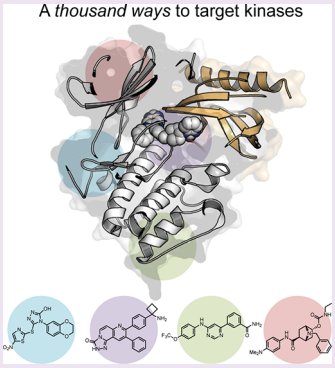
ABSTRACT: The modulation of kinase function has become an important goal in modern drug discovery and chemical biology research. In cancer-targeted therapies, kinase inhibitors have been experiencing an upsurge, which can be measured by the increasing number of kinase inhibitors approved by the FDA in recent years. However, lack of efficacy, limited selectivity, and the emergence of acquired drug resistance still represent major bottlenecks in the clinic and challenge inhibitor development. Most known kinase inhibitors target the active kinase and are ATP competitive. A second class of small organic molecules, which address remote sites of the kinase and stabilize enzymatically inactive conformations, is rapidly moving to the forefront of kinase inhibitor research. Such allosteric modulators bind to sites that are less conserved across the kinome and only accessible upon conformational changes. These molecules are therefore thought to provide various advantages such as higher selectivity and extended drug target residence times. This review highlights various strategies that have been developed to utilizing exclusive structural features of kinases and thereby modulating their activity allosterically.



The field of kinase research has witnessed a dramatic increase in the detailed understanding by which kinases function in the complexity of a cell and are regulated by upstream events. Kinases belong to the family of phosphate transferases and are responsible for phosphorylation reactions in cells, one of the most important classes of post-translational modifications of proteins. From a pharmacologic perspective, small-molecule kinase inhibitors are ideal reagents for perturbing and investigating kinase function at any stage of cellular development.[1] Variations in the kinase inhibitor concentration allow for graded phenotypes and a transient temporal control. Such features are essential for quantifying the flux of information that passes molecular networks and for elucidating network architecture and dynamics to, e.g., assess potentially druggable nodes beyond individual genes and proteins.[2]

Using ATP as a cofactor, kinases catalyze the transfer of the γ-phosphate group onto a substrate. Albeit diverse in their primary amino acid sequence, the 518 human kinases are remarkably similar in their three-dimensional structure. The pivotal feature of all kinases is the catalytically active kinase domain, consisting of an N-terminal lobe (N-lobe), usually containing an array of β-sheets, and a C-terminal lobe (C-lobe) dominated by α-helices, both connected *via* the flexible hinge region. The active site is located between these two lobes, containing the ATP-binding pocket, which contacts the hinge region, and the adjacent substrate-binding pocket (Figure 1a). Substrate-access to the active site is controlled by a flexible loop, the activation loop (A-loop), starting with the conserved amino acid sequence Asp-Phe-Gly (DFG). In the active state of a protein kinase, this DFG-motif occupies a hydrophobic back pocket adjacent to the ATP binding site next to the αC-helix of the N-lobe. In the inactive state, the DFG-motif can swing out of the hydrophobic back pocket resulting in an activation loop conformation, which partially blocks the ATP and substrate binding site.[3] However, not all kinases are known to be able to adopt such a DFG-out conformation, and the overall regulation of kinases is much more complex and requires additional regulatory elements.

The development of kinase inhibitors traditionally focuses on ATP-competitive small molecules. While inhibiting multiple kinase targets has been shown to have therapeutic value, selective inhibitors are particularly useful for understanding the function of a single kinase in complex cellular signaling.[4] Although a few examples of highly selective ATP-competitive kinase inhibitors exist and highlight the power of modern medicinal chemistry research, in the majority of cases, however, such Type I inhibitors are troubled by selectivity issues since the ATP binding pocket is highly conserved across the whole kinome (Figure 1b).[5] Emerging data suggests that moving away from classic ATP-competitive inhibitors and targeting alternative sites that, e.g., become accessible when the kinase adopts an inactive conformation can address the issue of kinase inhibitor selectivity and add additional pharmacological advantages such as extended drug target residence times. Targeting the DFG-out pocket, which is located adjacent to the ATP binding site and frequently referred to as an allosteric pocket, hydrophobic back pocket, or kinase-switch pocket, represents a prototypic approach along these lines (Figure 1c–e).

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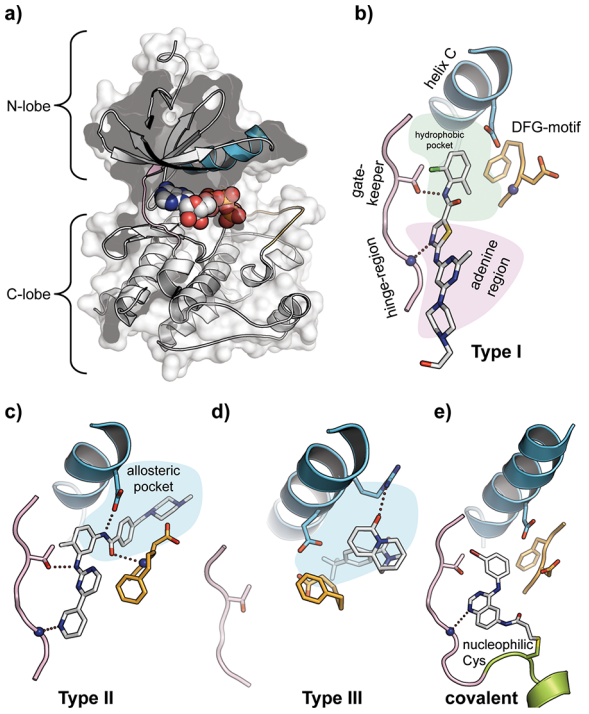

Figure 1. Targeting the kinase domain with different classes of kinase inhibitors. (a) Three-dimensional structure of a prototypical kinase domain with bound ATP (spheres). Key structural elements such as the αC-helix C (blue), the activation loop (orange), and the hinge region (pink) are highlighted. The hinge region connects the N- and C-lobes. (b) Type I inhibitors bind to the hinge region of the active kinase (DFG-in) and directly compete with ATP. (c) Type II inhibitors bind to and stabilize enzymatically inactive kinase conformations (DFG-out). (d) Type III inhibitors solely occupy the adjacent allosteric pocket or back pocket and keep the kinase in the enzymatically inactive DFG-out conformation. (e) Covalent inhibitors target activated Cys residues in the kinase.

By occupying this pocket, the conserved DFG motif is pushed toward the ATP binding site, thus blocking the access of ATP to the hinge region and keeping the activation loop in the inactive DFG-out conformation (Type II and III inhibitors). Finally, Type IV inhibitors bind at remote sites on the kinase and are capable to stabilize inactive conformations by addressing specific allosteric regulatory mechanisms.[6] Compounds with a purely allosteric mode of action offer distinctive advantages, which makes them particularly interesting for research. First, they do not have to compete with the high intracellular levels of ATP, thus allowing the identification of weak binders. Additionally, as allosteric binders do not need to interfere with interactions to native ligands, their binding can cause inhibition or amplification of kinase activity. Understanding the structural details of kinase conformational changes eventually allows for the rational design of innovative Type II, Type III, and Type IV inhibitors and next generation activators, to circumvent the current limitations. As regulation of activity can vary greatly among the different kinases, strategies to interfere with such mechanisms are equally diverse. Some general approaches for activation and inhibition have been employed such as regulating the cellular translocation of kinases, imitating native activators or preventing the formation of active or activated complexes (Table 1).

In this review, we will discuss some examples in more detail and examine how such regulatory mechanisms and structural characteristics of certain kinases have been exploited to be addressed by small molecules, which can allosterically modulate that kinase enzymatic activity, and explain the strategies that have been employed in their design. Such compounds offer the possibility of achieving high selectivity as these structural features and the related regulatory mechanisms are often unique and characteristic to a single kinase or kinase family.

AKT

The AGC kinase Akt, also known as Protein Kinase B (PKB), is among the best structurally understood protein kinases. First described in 1991, it was quickly identified as an oncogene, and current pharmaceutical research dedicates much effort to developing small molecule inhibitors for Akt.[7] The Ser/Thr-kinase Akt plays a pivotal role in signaling pathways responsible for cell survival, proliferation, and apoptosis, regulating these processes *via* its downstream targets such as FoxO1, MDM2, p21, p27, CREB, GSK-3, Bad, and procaspase-9.[8−10]

Hyperactive Akt signaling was shown to be capable of overriding apoptosis and thereby supporting increased proliferation of misregulated cells and, most importantly, rendering these cells resistant to treatment by radiotherapy as well as chemotherapy.[11,12] In addition to its kinase domain, Akt possesses a pleckstrin homology (PH) domain, that regulates its activity by binding to phosphatidylinositol lipids, which recruits Akt to the plasma membrane. In 2010, Wu et al. published the X-ray crystal structure (PDB entry 3O96) of full length Akt, allowing for great precise insights into how these domains interact with each other (Figure 2b).[13] The complex regulation mechanism of Akt is characterized by a multistep conformational change to gain its phosphorylation activity. In the inactive state (also known as "PH-in" or "closed" conformation), the PH domain forms tight interactions with the kinase domain *via* polar contacts,[13] and not only buries access to the ATP binding pocket but also displaces the αC-helix from the side of the allosteric pocket and locks the activation loop in an enzymatically inactive DFG-out conformation.

Akt signaling is initiated by external growth factors and subsequent activation of phosphoinositide 3-kinases (PI3Ks).[14] PI3K phosphorylates the second messenger PIP_2 to PIP_3, which binds to the PH domain of Akt. The PIP_3 binding pocket in Akt is located on the PH domain and forms electrostatic interactions with the negatively charged phosphate moieties of PIP_3.[15] Upon coordination to PIP_3, the overall positive charge of the PIP_3 binding pocket is reversed, causing an electrostatic repulsion of the PH domain toward the negatively charged residues on the kinase domain and, as a result, dissociates from it. The kinase changes into the "PH-out" or "open" conformation in which the activation loop now lies exposed (Figure 2a). Consecutively, Thr308 on the activation loop becomes accessible for phosphorylation by the phosphoinositide-dependent kinase-1 (PDK1).[16] This leads to an equilibrium shift of the activation loop from the inactive DFG-out to the active DFG-in conformation, thereby revealing the ATP binding pocket. Phosphorylation of Ser473 by PDK2 further increases Akt activity but is not sufficient to evoke kinase activity by itself.[17] It was proposed that Ser473 phosphorylation stabilizes an active conformation by inducing a 20° rotation of the N-lobe and the C-lobe against each other.[18] While the identity of PDK2 is still under debate, the

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Table 1. Overview of Representative Allosteric Kinase Modulators, Their Mode and Site of Action, and the PDB Entry Codes of the Respective X-ray Structures of Its Target Kinase; PDB Codes That Have an Asterisk (*) Show the Actual Allosteric Modulator Bound

Strategy	kinase	compound	mode of action	binding pocket	PDB
preventing formation of active or activated complexes	Abl	7c12	prevents adoption of active conformation by burying Ile164 on SH2 domain	kinase domain interaction site on N-lobe	3T04*
	CDK2	**13**	disrupts activation of CDK2 by cyclin A or E	cyclin groove	1JSU
	CDK2	ANS	displaces αC-helix, thereby disrupting the activation by cyclin A	pocket lined by αC-helix and β4 strand	3PXF*
	GSK-3β	VP0.7[117]	presumably locking the activation loop in an inactive comformation	docking experiments predict a pocket on top of C-lobe	1PYX
	IGF-1R	MSC1609119A-1	displaces the DFG-motif from the hydrophobic back pocket, arresting IGF-1R in the DFG-out conformation	novel pocket formed by C-lobe, A-loop, αC-helix, and hydrophobic back pocket	3LW0*
	MEK	PD-318088[118]	displaces αC-helix, disrupts A-loop conformation, prevents activation by autophosphorylation	hydrophobic back pocket	1S9J*
	mTOR	rapamycin	disrupts the binding of mTOR with raptor to form the active complex mTORC1	interface between FKBP12 and FRB domain of mTOR	3FAP
	PKC	PS171[119]	disrupts formation of αC-helix by mimicking interaction of C1 domain	PIF pocket	3PFQ
stabilizing inactive states or complexes	Abl	GNF-2	induces bending of αI-helix, allowing SH3 and SH2 domains to bind and restrict movement of the Abl kinase domain, adopting an autoinhibited state	myristate binding site	3K5V*
	Akt	MK-2206	fastens the PH and kinase domain together, preventing conformational change of Akt from closed inactive to open active	formed by interaction of PH and kinase domain	3O96*
	AMPK	Sanguinarine[120]	stabilizes active AMPK holoenzyme complex	cleft between β- and γ-subunit	2Y94
	JNK1	pepJIP1	induces inactive JNK1 conformation by inducing rotational misalignment of N- and C-lobes and destabilizing activation loop conformation, competes with JIP1 needed for activation	JIP1 binding pocket	1UKI
	PAK1	IPA-3[121]	interferes with the release from autoinhibited conformation by Cdc42	unknown allosteric site at regulatory domain	1F3M
blocking binding sites of native activators	Akt	PIA23	blocks the PIP_3 binding site, preventing opening of conformation and activation of Akt by PIP_3	PIP_3 binding pocket	1UNP
blocking sites of substrate recognition	Chk1	**10**	interferes with the substrate recognition	PDIG helix	3JVR*
	PDK1	**8**	prevents recognition of substrates	PIF pocket	3HRF
inducing degradation	Akt	SC66	prevents binding of PIP_3, induces ubiquitination of Akt and subsequent proteolysis	unknown site on PH domain	1UNP
occupying autoinhibitory binding sites	Abl	DPH	binds into the myristate pocket, pushes the αI-helix into linear conformation, preventing binding of the SH3 and SH2 domain, thereby forcing Abl to adopt an active state	myristate binding site	3PYY*
	AMPK	PT1[122]	disrupts binding interaction between autoinhibition domain and kinase domain, lifting autoinhibition state	novel binding pocket formed by N-lobe and autoinhibition domain	2Y94
stabilizing active conformations	AMPK	A-769662[123]	prevents deactivating dephosphorylation of Thr172 on AMPK	unknown site in γ subunit	2V8Q
	PDK1	PS48	transduces a stabilizing effect on the glycine-rich loop, activation loop, and αC-helix comprising the PDK1 ATP binding pocket	PIF pocket	3HRF*
	PKC	$\psi\beta$RACK[124]	stabilizes a transient, active PKC conformation by charge reversion	RACK recognition site	3PFQ

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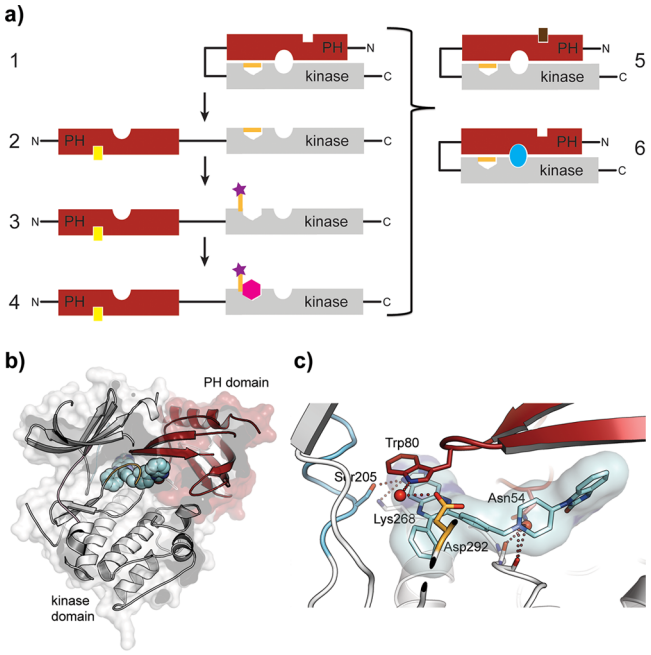

Figure 2. Allosteric activation of Akt. (a) Schematic representation of crucial steps involved in the Akt activation mechanism and resulting inhibition modes. In its basal state, Akt is in a closed conformation, with the PH domain obstructing the ATP binding pocket (1). PIP_3 (yellow) binding to the PH domain shifts the equilibrium toward the open conformation, revealing the activation loop (orange) (2). PDK1 phosphorylates (purple) Akt on Thr308 of the activation loop to shift equilibrium toward the active DFG-in conformation, enabling access to the ATP binding pocket (3). In the open DFG-in conformation, ATP (pink) can bind to Akt (4). PIP_3 antagonists (brown) occupy the PIP_3 binding pocket without effecting domain separation (5). MK-2206-type allosteric inhibitors (blue) bind into a hydrophobic pocket formed by residues of the PH domain–kinase domain interface, locking Akt in an inactive closed conformation (6). (b) X-ray crystal structure of full-length Akt in its closed conformation, cocrystallized with an interdomain inhibitor (PH domain red, kinase domain gray, inhibitor cyan, PDB entry 3O96). Access to the ATP binding pocket is blocked by the PH domain, which is held in place by interdomain interactions. Binding of PIP_3 into its pocket on the PH domain induces domain separation. (c) Allosteric inhibitor occupying unique binding pocket at the interface of the Akt PH and kinase domain. The inhibitor's quinoxaline core forms $\pi-\pi$-stacking interactions with Trp80 and hydrogen bonds to Asn54, Ser205, Lys268, Val271, and Tyr272.

rictor-mTOR complex (mTORC2) is considered the most likely candidate for PDK2.[19] In various cancers, the somatic Akt mutation E17K have been found. This charge reversal disrupts interactions between the PH and kinase domain and increases the affinity of Akt toward PIP_2, rendering Akt signaling independent from activation by PI3K.[20] In fact, these E17K mutants were found concentrated at the plasma membrane, exhibited higher activity in cells, and induced leukemia in mouse models. Extensive mutagenesis experiments showed that these phenotypes can be provoked by other mutations that interfere with these interdomain interactions.[21] Although much effort has been put into the development of ATP-competitive Akt inhibitors by pharmaceutical companies, their development have been discontinued due to toxicity issues.[22] Owing to its unique activation mechanism, different strategies for the inhibition of Akt are currently pursued. Initiated from a high-throughput screen in 2005, Merck & Co. reported on the discovery of a diphenylquinoxaline Type IV allosteric Akt inhibitor.[23] Further drug development resulted in the clinical candidate MK-2206 (**1**), which is currently in phase II trials (Figure 5).[24] A combined treatment of non-small cell lung cancer with MK-2206 and erlotinib showed promising effects in patient populations with erlotinib resistances.[25] Unlike previous ATP-competitive Akt inhibitors, this inhibitor family exhibited high selectivity against other closely related kinases of the AGC family such as PKA, PKC, SGK, PDK1, and S6K. Remarkably, these inhibitors only showed inhibitory activity against the full-length kinase and did not inhibit the Akt kinase domain alone.[26] SAR data, mutagenesis studies, and protein X-ray crystallography established that allosteric inhibitors of the MK-2206 type are sandwiched in between the kinase and PH domains, forming $\pi-\pi$-interactions and hydrogen bonds to both domains (Figure 2c). These interactions stabilize the closed conformation of the Akt, in which the ATP binding pocket is both inaccessible and disordered, preventing ATP and substrates from binding.[27,28]

Another strategy to inhibit Akt activity is to prevent its activation by PIP_3. A number of compounds such as sulfonamides,[29] DPIEL,[30] API-1,[31] PIA23 (**2**), (Figure 5)[32] and perifosine[33] have been shown to bind specifically to the PH domain of Akt with high affinity, mimicking PIP_3 without effecting an opening from the closed Akt conformation. All these inhibitors, however, suffer from low solubility, a high aggregation tendency, and adverse pharmacokinetics.[34]

Recently, Jo et al. have reported another allosteric Akt inhibitor with an entirely different mode of action.[35] Bisarylcyclohexanones such as SC66 (**3**), (Figure 5) were found to prevent activation of Akt by PIP_3 and its translocation to the cell membrane. Instead, SC66 primed Akt for ubiquitination followed by proteosomal degradation in the pericentrosomal region. This observation is in conflict with findings that ubiquitination of Lys63 can facilitate Akt activation in some cases of breast and colon cancer.[36] The exact binding mode of SC66 is unknown so far.

■ ABL

Another extensively investigated protein kinase is the Tyr-specific Abelson murine leukemia viral oncogene homologue (Abl, also known as JTK, p150, Abl1, or c-Abl). It is involved in signaling pathways regulating cell proliferation differentiation and apoptosis, and misregulation of Abl has been associated with tumorigenesis.[37,38] A prominent example of cancer induced by the misregulation of Abl is chronic myelogenous leukemia (CML), which is characterized by an increased growth of myeloid cells in the bone marrow.[39] It is caused by the translocation of a segment of the breakpoint cluster region (BCR) on chromosome 22 to the Abl gene on chromosome 9, yielding a new, fused BCR-Abl enzyme.[40] This fusion protein shows tyrosine kinase hyperactivity and is insensitive to native regulatory signals.

In addition to the catalytically active kinase domain, Abl possesses an N-terminal cap, two Src homology domains (SH3 and SH2), a DNA-binding domain and an actin-binding domain.[41] The N-terminal cap can be myristoylated, which plays a major role in the autoinhibition of the Abl phosphorylation activity (Figure 3a).[42] X-ray crystallography reveals that the myristate moiety is lodged in a deep pocket at the C-lobe of the kinase domain and causes a 90° bend of the αI-helix. This bending of residues 518–530 enables the SH3 and SH2 domains to approach and bind to the kinase domain

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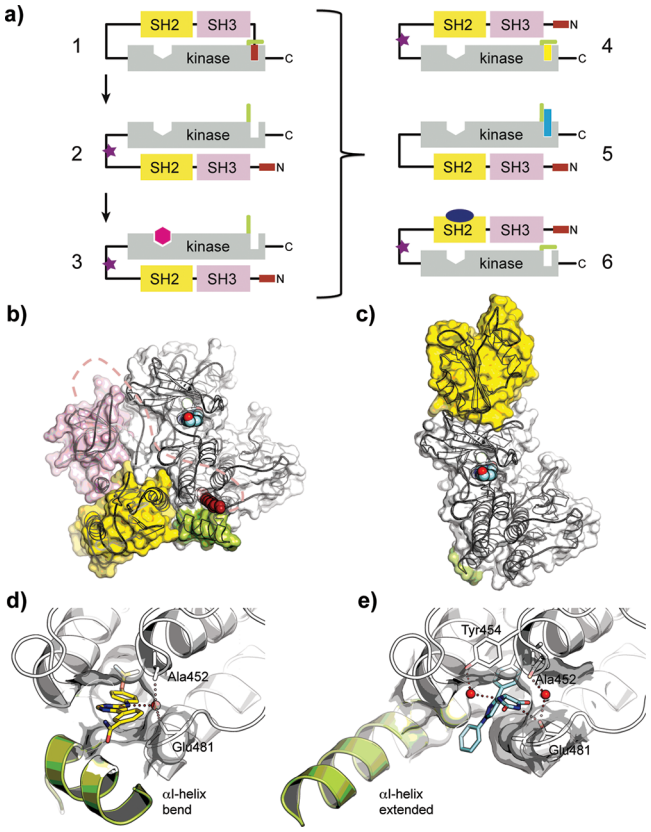

Figure 3. (a) Schematic representation of the regulation mechanism of Abl and different inhibition/activation modes. In its autoinhibited state, the myristoylated N-terminal cap (brown) resides in the myristate pocket. Bending of the αI-helix (light green) enables association of the SH3 and SH2 domains, locking it in an inactive conformation (1). Phosphorylation (violet) at Tyr245 by Src-family kinases translocates the regulatory domains on top of the kinase domain, allowing access to the ATP binding pocket (2). In the active conformation, ATP (pink) can bind to Abl after A-loop autophosphorylation (3). Myristate agonists like GNF-2 (yellow) bind into the myristate pocket, taking over the role of the myristoylated N-cap, e.g., in BCR-Abl (4). Myristate antagonists like DPH (cyan) bind into the myristate pocket but force αI-helix into linear conformation, preventing the association of the SH3 and SH2 domains and thereby acting as Abl activators (5). Interfering with binding of the regulatory domains to the N-lobe (e.g., with antibody 7c12, blue) prevents Abl from adopting an active conformation (6). (b) X-ray crystal structure of myristoylated Abl (PDB code 1OPL, chain A). The SH3 domain (pink) interacts with the N-lobe of the kinase domain (gray), the SH2 domain (yellow) with the C-lobe. The αI-helix (light green) is bent to accommodate the regulatory domains. (c) Abl in active conformation with the SH3 domain sitting on top of the N-lobe (PDB code 1OPL, chain B). (d) Allosteric inhibitor GNF-2 bound in the myristate pocket, inducing a bent αI-helix (PDB code 3K5V). (e) Allosteric activator DPH bound in the myristate pocket, forcing αI-helix into linear conformation (PDB code 3PYY).

(PDB entry 1OPL, Figure 3b).[43] Mutations of the residues connecting the SH3 and SH2 domains have shown that these two domains interact strongly with each other, thereby acting as a rigid clamp on the kinase domain.[44] Upon phosphorylation of Tyr245 by Src-family kinases, the SH domains adopt a different association pattern with the kinase domain, with the SH2 domain now sitting on top of the N-lobe, while the SH3 domain appears to be flexible in solution (Figure 3c).[45] In the constitutively active fusion protein BCR-Abl, the regulatory N-terminal cap has been replaced, rendering it incapable of myristoylation-induced autoinhibition.

In 2006, Adrián et al. reported on a series of *N*-phenylpyrimidinamines that specifically inhibited proliferation of BCR-Abl-transformed cell lines.[46] Biochemical experiments and X-ray cocrystal structures of Abl with the most potent inhibitor GNF-2 (**4**), (Figure 5) revealed the myristate pocket as its binding site (PDB entry 3K5V, Figure 3d).[47,48] GNF-2 binding induced Abl autoinhibition by bending of the αI-helix and resultant conformational fixation of the kinase domain by the SH3 and SH2 domains. In addition, GNF-2 improves potency of ATP-competitive inhibitors, indicating some form of cross-talk between myristate and ATP pocket.[49] In contrast, bulkier ligands in the myristate pocket were shown to have an activating effect on Abl. The phenylpyrazol-imidazolidine DPH (**5**), (Figure 5) increases the activity of Abl by up to 2-fold by enhancing autophosphorylation kinetics.[50] The X-ray crystal structure of Abl cocrystallized with DPH shows that its phenyl moiety sticks out of the myristate pocket, forcing the αI-helix into a linear conformation and hence prevents association of the SH domains to form the autoinhibited state (PDB entry 3PYY, Figure 3e). Recent studies in Fes and Abl demonstrated that binding of SH2 domains to the kinase domain contribute greatly to an increase in activity.[51] After extensive mutagenesis experiments and mouse xenografts, Ile164 was identified as a crucial amino acid for the interaction of SH2 to the kinase domain. Binding of the SH2-specific monobody **7c12** successfully prevents Abl phosphorylation and induces apoptosis in K562 CML cell lines. Both X-ray crystallography (PDB code 3T04) and ^{15}N HSQC NMR experiments showed that Ile164 was buried by **7c12**, thereby preventing the formation of interdomain protein−protein interactions between SH2 and kinase domain.[52] These findings illustrate potential in the development of small molecule inhibitors that interfere with the SH2−N-lobe interaction.

PDK1

The AGC-family member phosphoinositide-dependent kinase-1 (PDK1) is considered a master kinase and upstream activator of many other AGC kinases vital for growth factor and insulin signaling such as Akt, S6K, PKC, and SGK.[53] The Ser/Thr-kinase PDK1 was first purified and characterized by Alessi et al. in 1997[16] and later validated as a potential anticancer drug target.[54]

Like all AGC kinases, PDK1 features a catalytically active kinase domain with a hydrophobic groove on the N-lobe as well as a PH domain for the recognition of PIP_3 and recruitment to the plasma membrane.[55] However, in contrast to the other family members, PDK1 does not possess the C-terminal hydrophobic motif (HM), which binds intramolecularly to the hydrophobic groove.[56] Instead, the hydrophobic groove in PDK1 serves as a recognition site for the HMs of its substrate kinases.[53] This recruitment mechanism enables PDK1 to phosphorylate the activation loop of the substrate kinase and thereby activating its basal kinase activity.[57] After A-loop phosphorylation, the substrate HM is expelled from the PDK1 hydrophobic groove, complements with its own hydrophobic groove, and fully activates the substrate kinase. In 1999, Balendran et al. discovered that the C-terminus of the Protein Kinase C-related protein kinase-2 (PRK2) interacts with the hydrophobic groove of PDK1 and termed it the PDK1-interacting fragment (PIF).[58] Hence, the hydrophobic groove soon became known as the PIF binding pocket (Figure 4).

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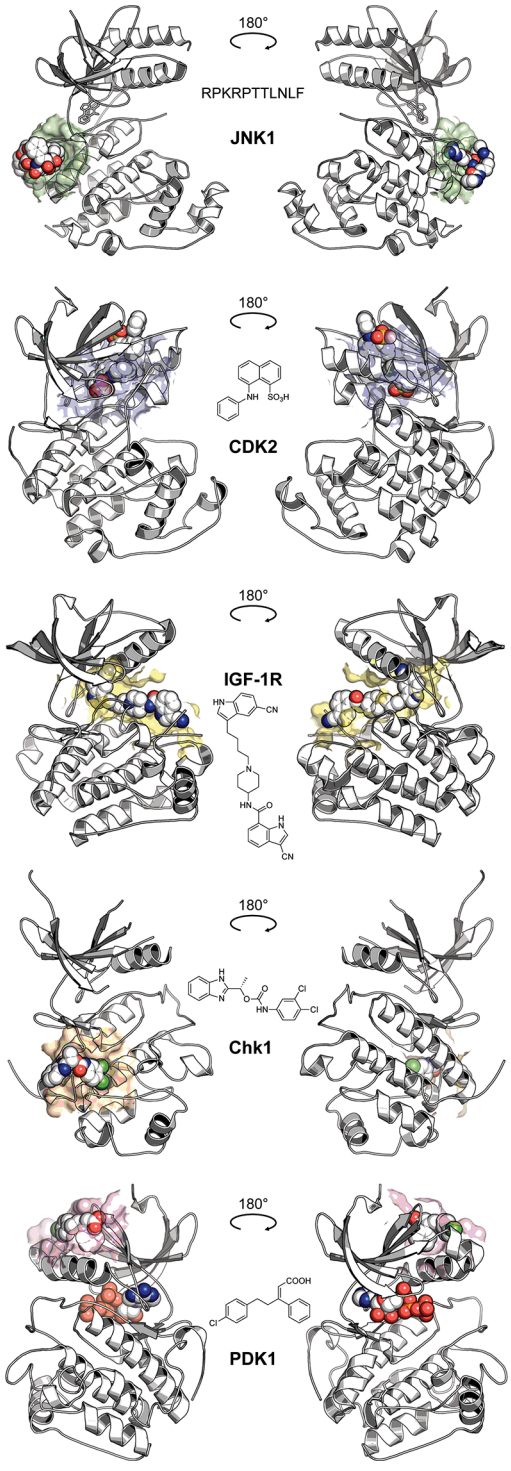

Figure 4. Location of several allosteric sites discussed in this review. pepJIP1 in the JIP binding pocket (green), cocrystallized with JNK1 (PDB code 1UKI). ANS at the cyclin binding site (blue), cocrystallized with CDK2 (PDB code 3PXF). MSC1609119A-1, which binds to a pocket reaching from the hydrophobic back pocket to the top of the C-lobe (yellow), cocrystallized with IGF-1R (PDB code 3LW0). **10** bound to the PDIG pocket (orange), cocrystallized with Chk1 (PDB code 3JVR). PS48 residing in the PIF pocket (pink), cocrystallized with PDK1 (PDB code 3HRF). Kinases are shown from the "front" (open ATP cavity, left image) and "back" (right image).

When treated with PIF, the activity of PDK1 increases dramatically. A synthetic 24-mer peptide derived from PIF (PIFtide) exhibited the same effects on PDK1 with a stronger activating effect than PIF itself. Several nonpeptidic small molecule PDK1 activators have been developed (e.g., PS48 **7**) (Figure 5).[59,60] Interestingly, the activity of other AGC kinases possessing similar hydrophobic grooves such as Akt, SGK, PKC, S6K, or PKA was not increased by such compounds demonstrating their high selectivity for PDK1. Most of these allosteric activators exhibit a negative charged moiety flanked by two aromatic rings arranged in an overall V-shape, imitating the phosphorylated Phe-Ser/Thr-Phe sequence within the hydrophobic motifs of PDK1 substrates.[61] The crystal structure of the PDK1-PS48 complex (PDB entry 3HRF) gave insights into the allosteric mode of action of these modulators.[62] The aromatic rings of PS48 are located in a position analogous to the phenylalanins of bound PIF peptides. Upon complexation, PS48 reduces the conformational plasticity of key structural elements of the kinase domain such as the αC-helix, the glycine-rich loop, and the activation loop, and thereby stabilizes the active conformation of PDK1.[63,64]

Bobkova et al. have reported the discovery of the alkaloid **8**, a selective non-ATP-competitive inhibitor of PDK1.[65] Docking experiments with published PDK1 structures suggest a binding mode similar to the activator PS48. However, lacking a negatively charged carboxylate group that mimics phosphorylated Ser/Thr residues of the native agonists, the alkaloid forms different interactions with the hydrophobic groove, which presumably turns an activator into an inhibitor.

JNK1

The Ser/Thr-specific c-Jun N-terminal kinases (JNKs), comprising the three isoforms JNK1, JNK2, and JNK3, are a subgroup of the MAP kinase family and are commonly known as stress activated kinases.[66] As such, they have been implicated in the regulation of inflammatory processes by activating the expression of cytokines such as eotaxin, IL-1β, and GM-CSF *via* their downstream targets, e.g., BAD, AP-1, ATF2, or p53.[67] Depending on the circumstances of their expression, JNKs can induce cell survival, proliferation, and differentiation as well as apoptosis.[68] To this date, their effect as either oncogene or tumor suppressor gene is still unclear and hence under debate.[69] Recently, the role of JNKs in the onset and progression of Alzheimer's disease (AD) has been investigated, as they are involved in the development and regeneration of brain tissue, neuroinflammation, and the stabilization of microtubuli in neuronal cells.[70] Aberrant levels of JNK expression have indeed been observed in many AD patients. Furthermore, JNK1 (also known as MAPK8) has been found to cause tau-induced degradation of neurons, a symptom characteristic for Alzheimer's disease.

Like many members of the MAP kinase family, the activity of JNKs is regulated by upstream kinases such as MKK4 or MKK7 *via* A-loop phosphorylation.[71] To achieve selectivity in the regulation of MAPKs, their activation is predominantly mediated by scaffolding proteins. Originally identified by Whitmarsh et al. in 1998 in yeast two-hybrid cells, the JNK interacting protein 1 (JIP1) was found to selectively bind to JNK1 and its upstream activators MKK7 and MLK3.[72] Bringing these enzymes in spatial proximity to each other, JIP1 forms a quaternary complex in which MLK3 can phosphorylate MKK7, which in turn activates JNK1. Although this demonstrated the physiological role of JIP1 in the activation of JNK1, overexpression of JIP1 has been found to inhibit JNK1 activity, possibly by retaining the JNK1-JIP1 complex in the cytosol.[73]

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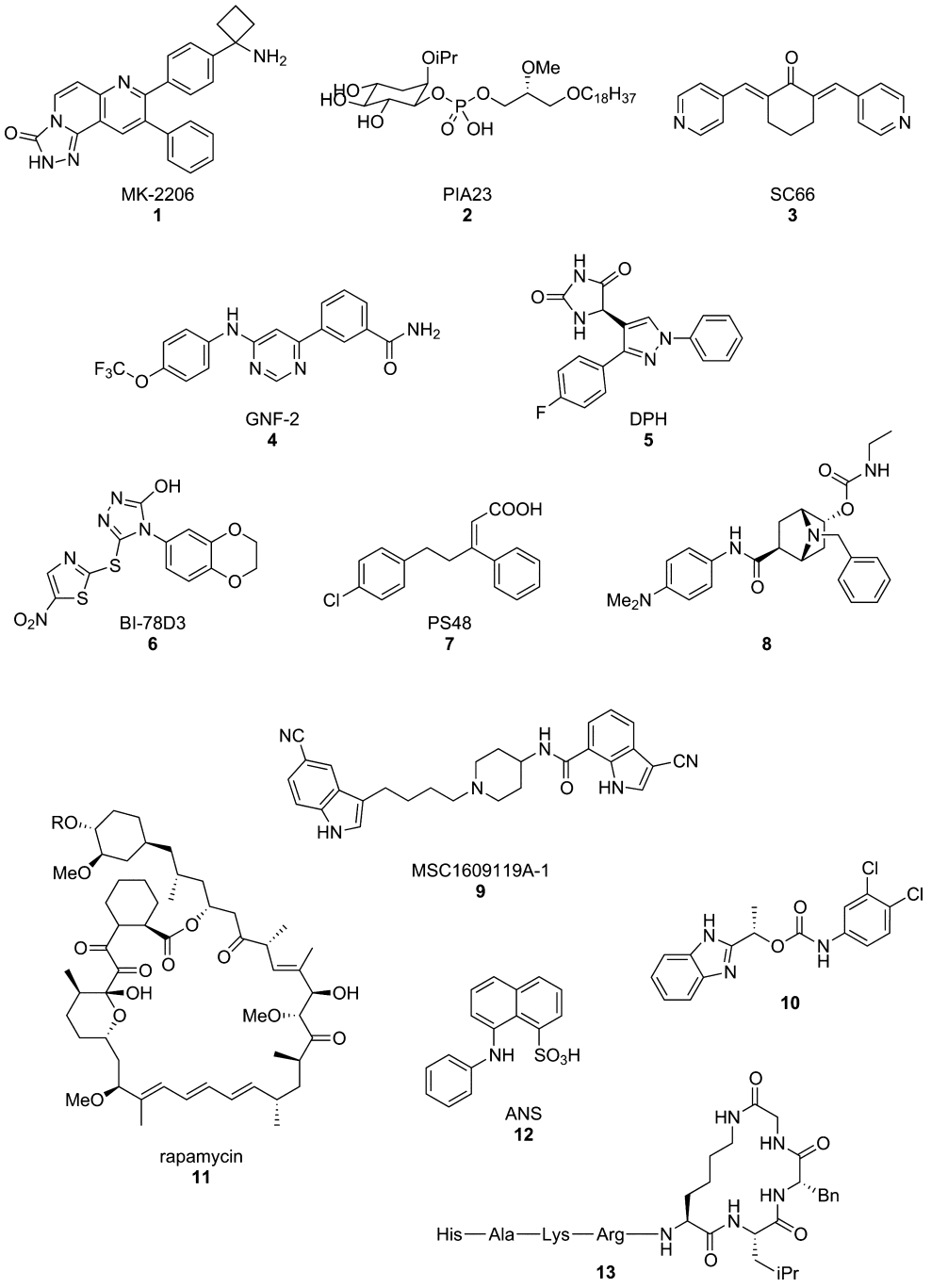

Figure 5. Selection of diverse allosteric kinase regulators discussed in this review. MK-2206: Akt inhibitor, stabilizes inactive closed conformation of Akt, currently in phase II clinical trials. PIA23: Akt inhibitor, PIP_3 antagonist that prevents PH domain dissociation-induced Akt activation. SC66: Akt inhibitor, primes kinase for ubiquitination and subsequent proteolysis. GNF-2: Abl inhibitor, myristoyl agonist, facilitates autoinhibition state of Abl by bending of αI-helix. DPH: Abl activator, forces αI-helix into linear conformation, prevents adoption of autoinhibited conformation. BI-78D3: JNK1 inhibitor, JIP1 antagonist, stabilizes the inactive JNK1 conformation. PS48: PDK1 activator, PIF agonist, stabilizes an active conformation of PDK1. **8**: PDK1 inhibitor, destabilizes active conformation of PDK1. MSC1609119A-1: IGF-1R inhibitor, arrests DFG motif in an inactive conformation, disrupts αC-helix of IGF-1R. Rapamycin: mTOR inhibitor, stabilizes mTOR-FKBP12 association, prevents formation of the active complex mTORC1. **10**: Chk1 inhibitor, interferes with the substrate recognition. ANS: CDK2 inhibitor, displaces αC-helix necessary for cyclin A binding. **13**: CDK2 inhibitor, peptidomimetic cyclin antagonist derived from cyclization of octapeptide HAKRKLFG.

In 2004, Heo et al. published the crystal structure of JNK1 in complex with pepJIP1, an 11-mer peptide consisting of the sequence that is essential for the interaction of JIP1 with JNK1 (PDB entry 1UKI, Figure 4).[74] The structure showed that pepJIP1 binds to the C-lobe of JNK1 and induces a rotation of the N-lobe against the C-lobe by 15°. This results in a less ordered activation loop, which might render it more accessible for the phosphorylation by MKK7. Recent molecular dynamics simulations suggest that the electrostatic contribution to the free energy of binding of the JNK1-pepJIP1 complex is decisive for its high selectivity.[75]

Exhibiting poor pharmacokinetic properties such as biodegradation, aggregation, cell permeability, or bioavailability, peptides are not considered ideal drug molecules. In

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consequence, a variety of small organic molecules have been developed to replace pepJIP1. Using a DELFIA (dissociation enhanced lanthanide fluoro-immuno assay) setup, Stebbins et al. have screened 30 000 compounds and have discovered a series of small molecules that are able to disrupt JNK1-pepJIP1 binding.[76] While some hits successfully competed with pepJIP1 binding, a few compounds such as BI-78D3 (**6**), (Figure 5) were also able to inhibit JNK1 phosphorylation activity with IC_{50} values in the low micromolar to high nanomolar range, both *in vitro* and *in vivo*. On the basis of their initial hit, the group synthesized additional derivatives to explore the SAR of these thiazole inhibitors.[77] As determined with an *in vitro* LanthaScreen kinase assay, these compounds showed a 100-fold lower inhibition of the close homologue p38α and no inhibition of mTOR and PI3K, suggesting the same high specificity of binding as the original JIP1 protein and pepJIP1 peptide. Analyzing the binding mode of their thiazoles predicted by their docking experiments, they proposed the development of bidentate inhibitors that bind to both the JIP1 and ATP binding site. In a separate screening initiative, Chen et al. identified pyrimidineamines that showed micromolar activities against JNK1 while exhibiting an even higher selectivity than pepJIP1.[78]

CHK1

The Checkpoint kinase 1 (Chk1) is a Ser/Thr-kinase and crucial transducer kinase in the cell-division cycle by regulating DNA damage response during mitosis. Chk1 activity has been identified at both the S and G2 checkpoints and is believed to facilitate the detection and repair of DNA damages at the G2/M checkpoint.[79] *Via* phosphorylation of its substrate Cdc25C phosphatase, Chk1 facilitates its association with 14-3-3 proteins, leading to an inhibition of Cdc25C activity. This in turn increases the formation of activated CDK-cyclin complexes by phosphorylation, which suppresses cell cycle progression. In contrast to healthy cells, many tumor cells show defects in their p53 DNA damage response pathway and are therefore sensitive to Chk1 inhibition.[80] A number of classic ATP-competitive Chk1 inhibitors have been developed both by academia and the pharmaceutical industry but suffer from low cellular potency.[81] However, various scaffolds are known that inhibit Chk1 in a non-ATP-competitive manner (e.g., quinazolinone **10**) (Figure 5).[82,83] Follow-up compound development and cocrystal structure analysis with Chk1 (PDB entries 3F9N, 3JVR, and 3JVS, Figure 4) confirmed the allosteric binding site of these inhibitors, forming hydrogen bonds and hydrophobic interactions to the protein surface adjacent to the αD-helix at the start of the C-lobe. In contrast to other kinases, the αD-helix of Chk1 contains a PDIG motif in which the N-terminal Pro induces a tight turn, resulting in the formation of a shallow, highly surface-exposed groove, which can be addressed by these types of inhibitors. This shallow binding pocket has been priorly described as a substrate recognition site, suggesting that these allosteric inhibitors might interfere with the substrate recognition of Chk1.[84]

IGF-1R

The insulin-like growth factor receptor (IGF-1R) is a receptor tyrosine kinase, consisting of two extracellular ligand-binding domains (α-subunits) and two cytoplasmic domains (β-subunits).[85] The α-subunits are covalently cross-linked *via* disulfide bridges and form a receptor complex that can bind to its native ligand IGF-1 and insulin. The β-subunits constitute of the transmembrane- and the kinase domain. Upon ligand binding, the IGF-1R tetramer undergoes conformational changes, which trigger autophosphorylation of its kinase domain, resulting in a gain of IGF-1R phosphorylation activity and the initiation of MAPK and PI-3K signaling pathways.[86,87] In numerous tumors including breast, lung, and prostate cancer, increased survival signals caused by elevated levels of IGF-1R have consequently led to inhibition of apoptosis.[88] Consistent with this, decreased IGF-1R expression levels have been shown to sensitize cells toward apoptotic signals.[89] However, the development of selective active site directed inhibitors has been difficult so far. The most successful approach toward IGF-1R selective inhibition could be achieved with monoclonal antibodies. Some of these antibodies such as figitumumab are currently in clinical trials for treatment of breast, prostate, adrenocortical carcinoma, and nonsmall cell lung cancer.[90,91]

Initiated from a high-throughput screening campaign, Heinrich et al. developed a series of indole-butyl-amines with anti IGF-1R activities at low- to submicromolar concentrations.[92] While such indole-alkyl-amines have been known to bind to subtype 1A serotonin receptors and serotonin reuptake transporters selectively, they do not exhibit any typical kinase inhibitor motifs.[93] The crystal structure of one of these derivatives MSC1609119A-1 (**9**), (Figure 5) in complex with IGF-1R (PDB entry 3LW0, Figure 4) revealed a novel, yet unprecedented binding mode, in which the inhibitor does not occupy the ATP binding site, but exclusively binds to the hydrophobic back pocket, displacing the DFG-motif and locking IGF-1R in an inactive DFG-out conformation. In addition, MSC1609119A-1 further extends into a shallow groove on the top of the C-lobe, forming interactions with the αC-helix, the activation loop, and several C-lobe residues *via* an extensive network of water-mediated hydrogen bonds. This unique binding mode presents an opportunity for the development of future allosteric inhibitors.

CDK2

Ser/Thr-specific cyclin-dependent kinases (CDKs) regulate important cell cycle processes, and their misregulation can lead to various diseases such as cancer and inflammation.[94] Notably, CDK2 plays an important role in the G1- to S-phase transition.[95] Loss of CDK2 activity in cell experiments resulted in an arrest in the G1 phase, potentially priming these cells for apoptosis.

The activity of CDKs is dependent on their binding interaction with cyclins, which represents a unique feature throughout the kinome. The various subtypes of cyclins (cyclin A, B, D, and E in humans) are regulatory proteins that are expressed at different stages in the cell cycle, each activating individual CDKs.[96] CDK2 forms active complexes with cyclins A and E. The crystal structure of the CDK2 in complex with cyclin A solved by Jeffrey et al. revealed the specific interactions between these two proteins (PDB entry 1FIN).[97] Cyclin A binds firmly and extensively to the N- and C-lobe of the kinase domain *via* hydrophobic and polar interactions. In particular, it binds to the activation loop and the so-called PSTAIRE-motif on the αC-helix, causing a 90° rotation of the helix, thereby restructuring the ATP binding pocket and stabilizing an active conformation. A number of cyclin-interacting proteins are known such as $p21^{WAF1}$, $p57^{Kip2}$, $p16^{INK4a}$, $p18^{INK6}$, or $p27^{Kip1}$, which function as native CDK inhibitors (CDKIs), representing important tumor suppressor proteins. The N-terminal inhib-

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itory domain of the cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$ or CDKN1B) was found to bind to the CDK2/cyclin A complex by interacting with the CDK2 N-lobe and the cyclin A cyclin box, preventing the activation of CDK2 by blocking the ATP binding pocket (PDB entry 1JSU, Figure 4).[98] Presumably, other CDKIs exhibit similar interactions with their respective target CDK/cyclin complex. On the basis of the cyclin binding motif of the cyclin-dependent kinase inhibitor 1 ($p21^{WAF1}$ or CDKN1A and CDKN1B), McInnes et al. have designed various peptides and peptidomimetics such as the 8-amino-acid peptide HSKRRLIF or the 5,8-*cyclo*-[*H*-His-Ala-Lys-Arg-Lys[5]-Leu-Phe-Gly[8]] (**13**), (Figure 5). These peptides showed high-affinity binding to the CDKI groove and potently inhibited the activity of the CDK2/cyclin E and CDK2/cyclin A complexes with IC_{50} values in the low micromolar range.[99] The same group later reported on the synthesis of even shorter peptidomimetics with nanomolar activities containing β-amino acids.[100] They concluded, that the increased flexibility of the additional methylene groups and spacing between the amino acid residues allowed a better fit to the cyclin binding groove. These findings clearly demonstrated the potential of potent and selective CDK2 inhibitors and present novel ways for the design of small organic molecules exhibiting similar interactions.

While a number of kinases can exhibit distinct A-loop conformations such as DFG-in and DFG-out, which usually are indicators for their state of activity, some of them, such as CDK2, are not known to be able to adopt a DFG-out conformation. Hence, the development of Type II and III inhibitors for CDK2 is difficult. In 2011, Betzi et al. reported on the discovery of 8-anilino-1-naphthalene sulfonate (ANS, **12**) (Figure 5) as a novel allosteric CDK2 inhibitor with a unique binding mode.[101] The inhibitory potency of ANS was determined to be competitive with neither ATP nor Type I CDK2 inhibitors such as JWS648, but with the CDK2 activator cyclin A. Subsequent structural investigations using X-ray crystallography revealed the binding mode of ANS to CDK2 and its precise mode of action (PDB code 3PXF). Still in a DFG-in conformation, two molecules of ANS are binding close to the DFG motif in a cavity formed by the αC-helix and the β-strands β3, β4, and β5 of the N-lobe. In order to accommodate the two inhibitor molecules, major reorganizations of the ATP binding pocket occur. Compared to the apo structure of CDK2, the hinge region and the activation loop display only minor distortions, while the αC-helix and the β strands β3, β4, and β5 are pushed apart, which causes the disruption of the recognition and binding site of cyclin A. Interestingly, a ternary crystal structure of CDK2 in complex with ANS and the Type I inhibitor JWS648 showed a nearly identical protein conformation and ANS binding, confirming the independence of ANS binding toward ATP and ATP-competitive inhibitors (PDB code 3PXZ). Taken together, these findings represent an opportunity for the development of new and selective allosteric CDK2 inhibitors by targeting this unique binding pocket. However, the authors concluded that inhibitors with an ANS-like binding mode must be highly potent to be effective, due to the usually very strong cyclin−CDK interactions. ANS itself, with a K_d of 37 μM, can easily be displaced by cyclin A, with a mediocre IC_{50} of 91 μM.

MTOR

The Ser/Thr-kinase mammalian target of rapamycin (mTOR) is a member of the phosphatidylinositol 3-kinase related kinases (PIKKs) and is involved in the PI3K/Akt/mTOR signaling pathway mentioned above. It can form two different complexes with different sets of proteins, the mTOR complex 1 (mTORC1) with raptor, mLST8, and PRAS40, and the mTOR complex 2 (mTORC2) with rictor, mSIN1, and mLST8, each with distinct biological functions.[102] While a great number of ATP-competitive mTOR inhibitors have been developed over the last years, they often show limited selectivity.[103]

The natural product rapamycin (**11**), (Figure 5), isolated from the bacterium *Streptomyces hygroscopicus*, inhibits mTORC1 in an allosteric fashion. Mediated by hydrophobic interactions with the 12 kDa FK506-binding protein (FKBP12), it forms a complex that is able to inhibit the activity of mTORC1 by binding to the C-terminus of mTOR, also termed the FKBP12-rapamycin binding (FRB) domain.[104] Interestingly, FKBP12 does not seem to interact with mTOR directly, the majority of contacts are formed by rapamycin. Probably, this binding relays conformational changes throughout mTOR and interferes with mTORC1 complex formation.[105] However, the exact inhibition mechanism remains unclear. Because of poor bioavailability and solubility of rapamycin, a couple of derivatives, also called rapalogs, have been developed and are now in clinical trials.[106] In renal cell carcinoma, everolimus has shown a favorable toxicity profile and good efficacy, whereas other important kinase inhibitors, for example, sunitinib or sorafenib, have failed.[107]

CONCLUSIONS

In the complexity of a living cell, kinase function is tightly regulated and often relies on small messenger molecules or signaling proteins to gain their full enzymatic activity. There exists a great variety in the complex regulation of protein kinase activity due to distinct structural features. These individual characteristics can be exploited and addressed by small organic molecules, e.g., to gain selectivity based on structural uniqueness. Although the search for novel chemical scaffolds that take advantage of such structural and regulatory exclusivity is moving to the forefront of kinase inhibitor research, efforts have been constrained by the lack of suitable HTS assay technologies that can reliably identify and discriminate stabilizers of enzymatically inactive kinase conformations (Types II−IV) from classic ATP competitive inhibitors (Type I). Recently, however, a range of direct binding assays were reported that enable the rapid and robust identification of ligands that bind to and stabilize specific kinase conformations.[108−111] One of these approaches is FLiK (fluorescent labels in kinases), which reports on conformational changes induced by the binding of specific types of ligands and does not require kinase activity.[112,113] To date, FLiK has been used to successfully monitor conformational changes associated with the slow binding of DFG-out binders as well as a method for identifying more selective allosteric ligands (Type IV), including assays for the MAP insert pocket of p38α as well as the myristate pocket of Abl kinase.[114,115] However, next generation technologies are needed that will allow for the identification of ligands that modulate more complex regulatory mechanisms such as interdomain communications at the full-length kinase level and the dynamic association with adaptor proteins.[116]

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The authors declare no competing financial interest.

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KEYWORDS

Allosteric regulation: Allosteric regulation of protein function is a key principle often found in nature. From a pharmacological perspective, the interference with allosteric regulatory processes provides various advantages such as access to higher compound selectivity, extended drug target residence times, and overcoming of mutation associated drug resistance and is therefore highly desired in targeted approaches; **Interdomain interactions**: The function of protein kinases is often modulated *via* regulatory domains, which are part of the full length kinase and interact with the catalytic kinase domain; **Conformational changes**: Conformational changes are essential for all biological processes and often used to switch protein function on or off. *Via* conformational changes, the access to binding sites is controlled and often found in kinases; **Structural flexibility**: It is important to understand that proteins are flexible. Structural flexibility is often found in protein kinases as part of the mechanisms by which their enzymatic activity is regulated. Unique structural features are known for a variety of kinases including Akt, Abl, CDK2, Chk1, IGF-1R, JNK1, mTOR, and PDK1. Such unique features can be exploited in the design and development of selective kinase inhibitors; **Inactive conformations**: Kinases can adopt inactive conformations. The stabilization of inactive conformations by small organic molecules is a highly desired task in the development of innovative chemical perturbators; **Regulatory domains**: Most kinases are regulated by a variety of regulatory domains; **Inhibitor**: Small molecule inhibitors enable chemical biology studies in complex cellular systems. In a simple experiment, perturbed states can be compared from unperturbed states; **Binding site**: Binding sites differ in their characteristics; **Protein−ligand interactions**: The understanding of protein−ligand interactions at the molecular and atomic levels is key for the design and development of compounds with optimized properties such as potency and selectivity

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