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Commentary

Non-ATP competitive protein kinase inhibitors as anti-tumor therapeutics

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ABSTRACT

Alternative approaches for inhibitor development in targeting sites other than the ATP cleft are increasingly being pursued in the search for new therapeutics based on inhibition of protein kinases. While recently approved kinase inhibitor drugs offer benefit in cancer treatment, further advances are required to affect tumor selective cell killing, avoid off-target related toxicities and improve survival rates. Protein–protein interactions involved in kinase regulation and substrate recognition as well as exploiting allosteric pockets, offer the potential for selectivity and avoid decreased efficacy as a result of competition with high intracellular ATP concentrations. We discuss several preliminary examples where regulatory and substrate binding sites present potential druggable interfaces. These include the cell cycle targets which are the cyclin-dependent and polo-like kinases among several others.

1. Introduction

Protein kinases play important roles in regulating cellular functions including proliferation and apoptosis, and the deregulation of kinase activity is implicated in a number of diseases. Pharmacological inhibitors of protein kinases are of great interest since these represent the second largest group of drug targets after the G-protein-coupled receptors [1]. The majority of inhibitors to date have targeted the ATP binding pocket with several potent and selective inhibitors having been successfully developed and seen clinical success. Since the first small molecule protein kinase inhibitor **1** (Fig. 1; imatinib and Gleevec®) was approved for the treatment of chronic myelogenous leukemia in 2001 [2,3], several more potent inhibitors have followed and are in clinical use. While agents currently used are effective, a source for potential problems in kinase inhibitor development is that the ATP binding pocket is highly conserved across the protein kinase family. Therefore, it is difficult to identify specific kinase inhibitors that can discriminate among the more than 500 protein kinases that have been identified in the human genome [4] as well as the many other proteins that utilize ATP as a cofactor. Additionally, kinase inhibitors must compete with intracellular ATP concentrations as high as 10 mM [5]. Non-specific inhibitors could be expected to exhibit undesirable toxicities that would limit their usefulness as drugs. Indeed such issues have slowed development of many compounds in preclinical and clinical development. For example, no cyclin-dependent kinase inhibitors have yet to be approved as drugs due to side effects and toxicity. Lack of selectivity would be an especially important consideration for non-oncology indications where use of drugs for chronic

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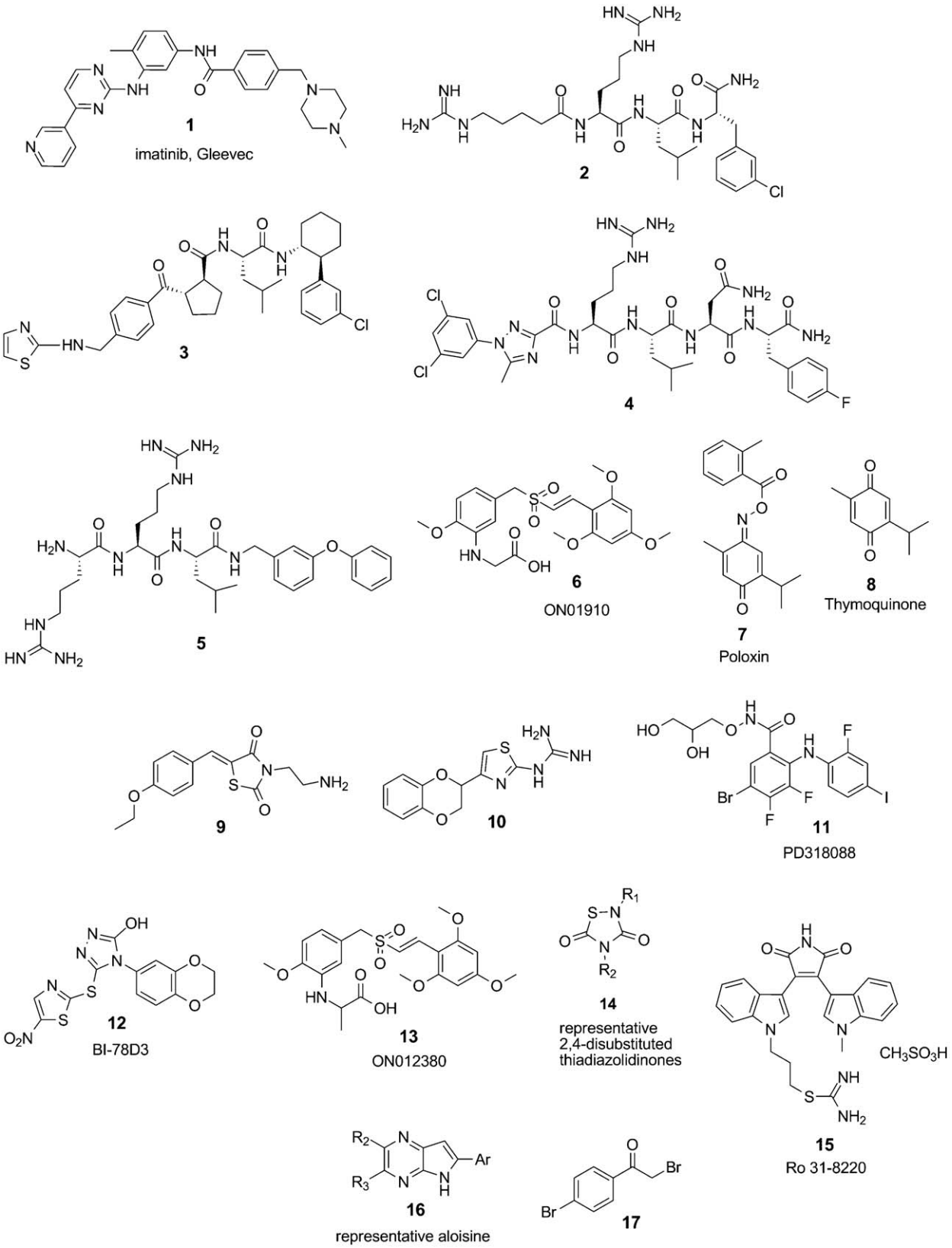

Fig. 1 – Chemical structures of described non-ATP competitive inhibitors.

conditions such as diabetes would necessitate low toxicity profiles. Yet another problem anticipated with ATP competitive protein kinase inhibitors is drug resistance as a result of mutations in the ATP binding site. This has been observed in patients undergoing treatment with imatinib [6].

While approved kinase inhibitor drugs offer benefit in cancer treatment, further advances are required to affect tumor selective cell killing, avoid off-target related toxicities and improve survival rates. In contrast, alternative approaches for inhibitor development in targeting sites other than the ATP cleft are increasingly being pursued in the search for new therapeutics targeting protein kinases. Inhibitors of kinase substrate and regulatory binding sites have emerged as attractive alternative targets for the development of drug-like protein kinase inhibitors. Substrate docking sites and the corresponding binding interactions typically differ significantly from kinase to kinase. Therefore, opportunities are available to develop non-ATP competitive protein kinase inhibitors with high selectivity for use as potential anti-tumor therapeutics and in other indications. Several preliminary examples where regulatory and substrate binding sites present potential druggable interfaces are discussed. The discussion is primarily focused on compounds that do not interact with the ATP site, i.e. as a whole does not discuss so-called allosteric compounds that inhibit kinase activity by inducing an inactive conformation of ATP binding cleft.

1.1. *Cyclin-dependent kinases*

The cyclin-dependent kinase (CDKs) family of protein kinases contains members that are involved in the regulation of the cell cycle and have been targeted as potential molecular targets for anti-cancer therapy. CDKs phosphorylate proteins on serine and threonine amino acid residues and are, therefore, classified as serine/threonine kinases. The catalytic subunit is activated by association with a cyclin and subsequent phosphorylation by the CDK activating kinase thus forming a cyclin-dependent kinase complex. CDKs are important in the regulation of the cell cycle because of the role they play in controlling cell cycle checkpoints. In this manner, key CDK/cyclin complexes are active during the G1/S (CDK4/cyclin D1, CDK6/cyclin D1 and CDK2/cyclin E), S (CDK2/cyclin A) and G2/M (CDK1/cyclin B) phases of the mammalian cell cycle.

A necessary factor in the activity of CDK/cyclin complexes is that many of the substrates involved in the regulation of the cell cycle must undergo a recruitment and recognition step before phosphorylation can occur. In this step, substrates interact with a shallow hydrophobic groove on the surface of the cyclin regulatory subunits termed the ‘cyclin groove’. This interaction is thought to bring protein segments into close enough proximity for phosphorylation to occur as well as increase the local macromolecular substrate concentration to a level high enough for catalysis to occur [7]. While traditional CDK inhibition blocks ATP binding, blocking of the recruitment site should promote CDK-specific inhibition due to the fact that each subset of kinases requires a particular substrate for docking.

The short peptide sequence responsible for substrate recognition and recruitment by cyclin complexes in CDK2, 4, and 6 is referred to as the cyclin binding motif (CBM). The cyclin groove recruitment site is comprised of three sub-sites that form interactions with substrates: a shallow hydrophobic pocket, an adjacent site providing polar and ionic contacts with basic residues of the peptide, and a secondary hydrophobic pocket that has been exploited in peptidomimetic design (Fig. 2). Information has recently increased on the composition of the CBM through the research of several different groups [8–11]. This research led to the identification of CDK2/cyclin binding sites in the natural inhibitory proteins

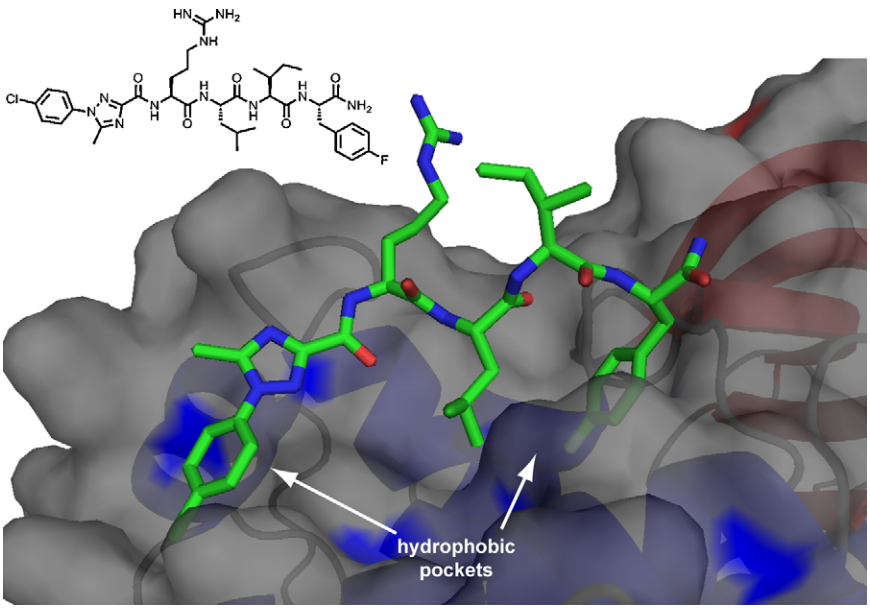

Fig. 2 – X-ray crystal structure of N-terminally capped peptide bound to cyclin A. The phenyltriazole partial ligand alternative interacts with a secondary hydrophobic pocket in the cyclin, replacing the critical functionality of Arg1 in the RRLIF p21 derived sequence.

$p21^{WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$ based on sequence similarity. Related experiments displayed dose-dependent inhibition of the enzymatic activity of the CDK complex using synthetic peptides derived from these sequences [7]. An additional study demonstrated the importance of the CBM interactions with the cyclin subunit by looking at the hydrophobic residues of the cyclin groove. When these residues were altered, no binding was observed between the cyclin subunit and the CBM-containing proteins [12]. Aligned with the results from these and similar studies, this conclusion helped to verify that CDK substrates must first be recruited to the complex before phosphorylation can take place and that CBMs are used by natural inhibitory proteins for blocking CDK function and inducing cell cycle checkpoint effects. This approach to drug discovery has been validated through the use of cell-permeable peptides derived from $p27^{WAF}$ and E2F1 and shown to act in a synthetically lethal fashion in tumor cells where E2F transcriptional activity is excessive [13]. Since the CDK substrate phosphorylation is susceptible to inhibition by small synthetic peptides, this protein–protein interaction may be an appropriate target for small drug-like molecules for the treatment of cancer.

One such effort involved in the syntheses of potent small molecule inhibitors of the CDK2/cyclin A recruitment site [14]. Transcription factor E2F, a substrate for the CDK2/cyclin A complex, is phosphorylated prior to S phase exit during the cell cycle. Since E2F is deregulated in tumor cells and results in cell death in combination with CDK2/cyclin A inactivation [15], the inhibition of this specific phosphorylation can block S-phase exit and lead cancer cells into apoptosis. Endogenous substrates and inhibitors of the CDK2/cyclin A recruitment site share the sequence RXL and require a second C-terminal hydrophobic amino acid either directly after the leucine or separated by an additional residue (p27-RNLF and E2F1-RRLDL) [14]. Using the octapeptide PVKRRLFG as a starting point (IC_{50} = 12 nM) and the retinoblastoma protein (Rb) as a substrate, structure activity relationships for a series of octapeptides were studied to determine the contribution of each amino acid side chain. Since the most significant loss in activity was observed when leucine, phenylalanine, or arginine was modified, the most necessary residues were shown to be those consistent with the consensus RXL sequence. The additional amino acids showed little to no effect on binding [14].

Substitution and truncation studies with the octapeptide lead resulted in the identification of a smaller, though less potent, tetrapeptide lead. Truncation of the peptide from the N-terminus to the consensus sequence caused at least a 120-fold reduction in potency, while truncation from the C-terminus was well tolerated. Side-chain optimization and rigidification recouped the loss in binding affinity and uncovered neutral small molecules to replace guanidine functions from the important arginine residues. Additionally, a variety of natural and unnatural amino acids were used to study binding. While most resulted in a loss of activity, substitution about the aryl ring of phenylalanine proved successful. The optimized compound **2** with an IC_{50} of 3 μM was determined to be a more appropriate starting point for medicinal chemistry than the parent peptides previously reported due to its lower molecular weight and smaller size [14].

Lead peptide **2** was subsequently modified in an effort to increase binding energy through optimization of favorable interactions with the protein surface. SAR studies determined that reduced conformational freedom at both the N- and C-terminuses led to improved binding affinity. Consequently, an optimized compound was developed that is 500 times more potent than the lead peptide. In addition, the compound contains fewer rotatable bonds and is neutral at physiological pH. Leading to this compound, the flexible alkyl chain of the lead peptide's N-terminal arginine mimetic was replaced by an aryl group while the C-terminus was constricted using a *trans*-2-arylcyclohexyl. The *trans* stereochemistry is the same as that of the full length peptide and was necessary for potency. The compound was improved by replacing the guanidine functionality with a weakly basic aminothiazole that could be protonated after nearing the anionic region of the protein surface. Finally, the compound was improved further by performing a one-carbon homologation on the internal alanine to α-aminobutyric acid to yield a highly potent inhibitor. Introduction of a proline mimetic resulted in a more drug-like compound (**3**, 40 nM) containing only one natural amino acid and two amide bonds and had a slight reduction in *in vitro* potency as a compromise for lower peptidic character.

In a separate report, a drug discovery strategy was applied to identify cyclin-dependent protein–protein interactions [16]. In the REPLACE (replacement with partial ligand alternatives through computational enrichment) strategy, non-peptidic surrogates for specific determinants of known peptide ligands are identified *in silico* by using a core peptide-bound protein structure as a design anchor. The methodology accommodates the protein–protein interactions, or PPIs, which are usually difficult to target by conventional methods. The segments of a protein are replaced iteratively by small molecules and are chemically joined to the peptide. In this example, the charged and non-drug-like N-terminal arginine of p21 (a natural CDK inhibitory protein) peptides, was replaced with phenyltriazole substructure while resulting in a twofold potency increase (to 2.1 μM). This group interacts with the secondary hydrophobic sub-site of the cyclin groove while an identified biphenyl ether system replaced the interactions of the C-terminal phenylalanine residues that makes critical contacts with the primary lipophilic pocket (compounds **4** and **5** respectively). Through this process, it was demonstrated that critical residues of a cyclin groove inhibitory peptide could be bypassed through ligation of small drug-like molecules while simultaneously increasing both drug-likeness and potency.

In addition, the all D-amino acid hexapeptide RWIMYF-NH_2, named as NBI1, that inhibits kinase activity of the CDK2-cyclin A with an IC_{50} of 1.1 μM through selective binding to cyclin A has been identified [17]. In order to determine the mechanism of the compound, an analysis of the steady-state kinetics of CDK2-cyclin A inhibition by NBI1 was performed. Phosphorylation of a histone H1-derived peptide by CDK2-cyclin A was measured in the presence of increasing amounts of ATP and in the presence of 5 M NBI1. A double reciprocal plot showed that NBI1 responds as a non-ATP competitive inhibitor. The ATP competitive inhibitor olomoucine was used as a control during this assay. Unlike many existing CDK inhibitors, NBI1 interferes with the formation of the CDK2-cyclin A complex.

NBI1 is also attractive in that a cell-permeable derivative of the compound induces apoptosis and inhibits proliferation of tumor cell lines. The all D configuration of the peptide suggests that it would be pharmacologically stable, however cellular penetration will likely remain an issue. Nonetheless, this study represents proof of concept that alternative druggable sites are available for the development of non-ATP competitive CDK inhibitors.

1.2. *Polo-like kinases*

Polo-like kinases (PLKs) regulate numerous cell cycle events including the onset of mitosis, DNA-damage checkpoint activation, regulation of the anaphase promoting complex, and centrosome duplication and maturation [18–21]. Four distinct PLKs have been identified to date in mammals: PLK1, PLK2 (aka SNK), PLK3 (aka PRK and FNK), and PLK4 (aka FNK) [22]. The four are structurally homologous with two conserved regions—an N-terminal catalytic kinase domain and a C-terminal region which is comprised of ''polo boxes.'' Of these, PLK1 is the best characterized kinase and fulfills the most roles. Initiation of mitosis requires activation of the complex between the cyclin-dependent kinase CDK1 and B-type cyclins, while PLK1 has been shown to activate this complex indirectly through the activation of CDC25c (Figs. 3 and 4). PLK1 is overexpressed in human tumors [23,24] and specific antisense oligonucleotides were shown to induce growth inhibition in cancer cells both *in vitro* and *in vivo*, demonstrating a rationale for inhibitor study [25,26].

A recent example involving a potential kinase substrate recruitment site was demonstrated using a proteomics and structural study of the validated anti-tumor drug target PLK1 [27]. The approach identified phosphopeptide binding domains that modulate kinase-dependent signaling pathways. Domains that bind to proteins phosphorylated by a kinase were isolated by using a library of partially degenerate phosphopeptides biased toward a particular phosphorylation motif. When this approach was applied, the polo box domain (PBD) of PLK1 was discovered to contain a specific binding domain involved in substrate interactions and in sub-cellular localization of the kinase. The binding motif can be found in known PLK1 substrates such as Cdc25C, a key substrate which regulates the activation of CDK1/cyclin B and crystal structures of peptides based on this motif and in complex with the PBD have been solved (Fig. 2) [27,28]. Subsequently, the motif was shown to interfere with PBD-substrate binding and localization of the PBD to centrosomes indicating that PLK1 can localize to specific sites within cells in response to phosphorylation. These results help to establish the PLK1 kinase domain as a molecular target for anti-tumor drug development.

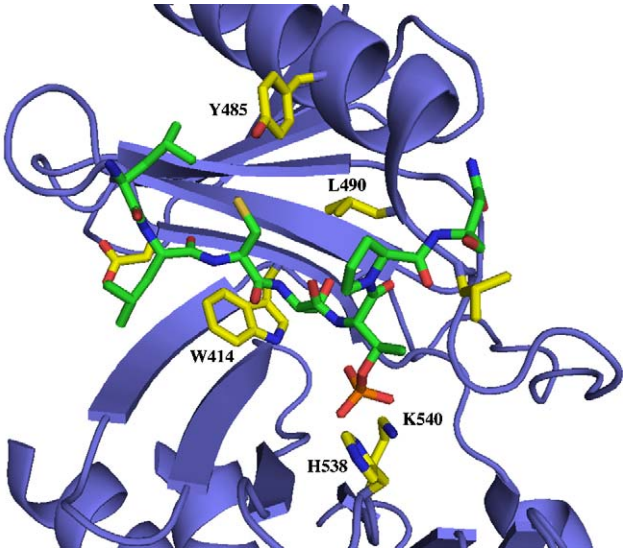

Fig. 3 – Interactions of the phosphorylated CDC25c derived peptide bound to the polo-box domain of Plk1. Interactions with Trp414, His538 and Lys540 of Plk1 among other residues are critical for binding of this peptide and its derivatives.

A recent study reports progress toward a small molecule inhibitor of PLK1 and highlights the role of PLK in cell cycle progression and cancer [29]. PLK1 is overexpressed in many tumor cells, and those patients whose tumors display higher levels of PLK1 have lower survival rates than those with low levels. The study introduces ON01910 (compound **6**), a small molecule inhibitor of PLK1 activity with an IC_{50} of 9–10 nM which induces mitotic arrest of tumor cells characterized by spindle abnormalities leading to their apoptosis. A non-ATP competitive inhibitor, ON01910 competed for the substrate binding site of the enzyme. No hematotoxicity, liver damage, or neurotoxicity was reported *in vivo*, and the compound was a potent inhibitor of tumor growth in a variety of xenograft nude mouse models. The compound was also found to be highly effective in combination with conventional chemotherapy. The mechanism of inhibition was determined by performing a steady-state analysis that looked at the effects of increasing concentrations of ATP on the compound. Analyses showed that the velocity of substrate phosphorylation was unaffected by increasing ATP concentrations, and IC_{50} values for ON01910 remained between 9 and 10 nM. The results support the conclusion that ON01910 is not an ATP competitive inhibitor although its interaction with the PBD has not yet been confirmed.

Since the polo box domain is unique to the PLKs and necessary for their functions, the PBD provides a prime opportunity to inhibit a serine/threonine kinase without targeting its ATP binding site. Reindl and co-workers [30] explored inhibiting the PLK1 PDB using cell-permeable small molecules which prevent it from binding to its intracellular anchoring sites and substrates. Using a fluorescence polarization assay, the group screened 22,461 small molecules with the potential to interfere with the PLK1 PDB. This library screening led to the identification of compound **7**, poloxin (apparent IC_{50} of 4.8 ± 1.3 μM), as an inhibitor of PLK1 PDB [30]. Poloxin is a synthetic derivative of the natural product thymoquinone **8**, which is the bioactive constituent of the volatile oil of black seed (*Nigella sativa*). This natural product is well known for its anti-inflammatory, anti-oxidant, and antineoplastic properties [31]. Thymoquinone's antineoplastic activity appears to be specific to cancer cells, yet direct molecular targets to explain the antineoplastic activity have not been identified. Thymoquinone (apparent IC_{50} of 1.14 ± 0.04 μM) was found to inhibit PLK1 PDB more potently than does poloxin [30]. However,

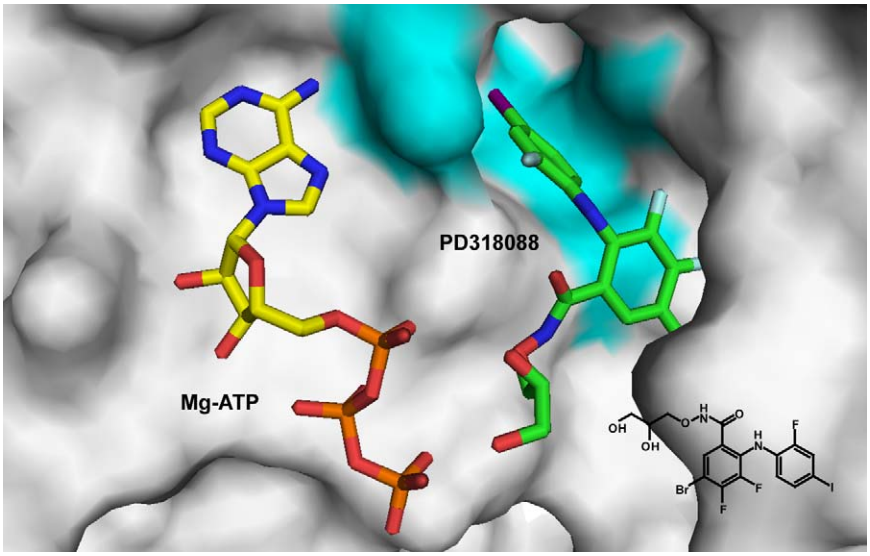

Fig. 4 – PD318088, a MEK1 inhibitor, binds to a novel allosteric pocket adjacent to the ATP binding site, therefore allowing Mg-ATP and this inhibitor to bind to the kinase simultaneously. This dual binding locks the binding pocket in an inactive conformation.

thymoquinone also affected other subtypes of phosphothreonine/phosphoserine binding domains and was, therefore, found to be less specific. Therefore, it is suggested that the PLK1 PDB is a direct molecular target of thymoquinone that could explain its antineoplastic activity. The specificity profiles of agents such as poloxin that target the unique PBD are potentially easier to analyze than ATP competitive kinase inhibitors; consequently, these agents could be important in the development of anti-cancer therapeutics based on PLK1 inhibition. The redox potential of these compounds may limit their useful as drugs however.

1.3. *Mitogen-activated protein kinases*

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that respond to extracellular stimuli and regulate cellular activities including gene expression, differentiation, mitosis, and apoptosis. The three major mammalian subfamilies of MAPKs—extracellular signal-related kinase (ERK), JNK, and p38—each has different biological functions. However, all phosphorylate substrates containing the minimal consensus sequence Ser/Thr–Pro [32].

The MAPKs play roles in a variety of therapeutic conditions. For example, one particular MAP kinase, the p38 Ser/Thr MAP kinase, is reported to be necessary for the signal transduction cascade leading to the production of proinflammatory cytokines such as TNF and IL-1 [33]. Therefore, blocking p38 is proposed to yield anti-inflammatory agents. Additionally, the ERK mitogen-activated protein kinase pathway is commonly activated in human cancers. Raf kinases phosphorylate MEK1 and 2. These dual specific kinases then phosphorylate and activate the mitogen-activated kinases ERK1 and ERK2. Oncogenic mutations in Ras effectors, along with additional upstream signaling events, result in the ERK MAPK pathway being one of the most commonly activated in human cancers [34]. The ERK MAPK signaling downstream of Ras has been implicated in multiple studies of metastasis [34]. Though the mechanism has not yet been fully determined, several possible modes of action have been proposed to explain the role of the ERK MAPK pathway in metastasis. One plausible proposal is that the pathway promotes invasion and metastasis by favoring epithelial mesenchymal transition in tumor cells. Alternatively, it is proposed that ERK MAPKs promote metastasis through the upregulation of matrix metalloprotease expression and extracellular matrix remodeling. Likely, however, is that the regulation of cell motility and migration represents the means by which the ERK MAPK pathway is related to metastasis. Therapeutic intervention is important in this area, because metastasis that allows cancer cells to reach distant parts of the body causes approximately 90% of human cancer deaths [34].

The mitogen-activated protein kinase c-JUN kinase (JNK) binds its substrates outside of the active site and, therefore, involves substrate sequences that are distant from the site of phosphorylation [35,36]. Since the time of this initial discovery, research in the area has reiterated the fact that many if not all MAP kinases use docking sites to bind substrates. Additionally, research has shown that a similarity between these docking site interactions exists for the MAP kinases [32].

Among these, the extracellular signal-regulated kinase ERK2 substrate docking site has been specifically targeted. In one instance, *in silico* screening was used to identify 80 candidate compounds for testing as inhibitors of the growth factor stimulated phosphorylation of ERK substrates, the downstream kinase Rsk1, and the transcription factor Elk-1 from a pool of over 800,000 [37]. Two respectably potent compounds **9** and **10** were identified from this study with K_d values of 5 and 16 μM respectively. As predicted from its interaction with the ERK substrate docking site, one of the key inhibitor compounds did not prevent phosphorylation of docking domain-independent ERK substrates. Co-crystallization of ERK2 with the inhibitor can confirm the mode of interaction with the substrate docking site and allow for direct inhibitor optimization [38].

MAPK/ERK kinases (MEKs) are dual specificity kinases that phosphorylate MAPK. Certain protein kinase inhibitors, including the MEKs, can induce conformational changes in the structure of ATP's binding region. Consequently, these changes can be used to indentify potential selective therapeutics [39]. Small molecule inhibitors have been identified that bind to and stabilize inactive conformations of this protein kinase. One such example, PD318088 (compound **11**), is a highly selective MEK inhibitor that exploits unique features of the active site adjacent to the ATP binding cleft. This compound was crystallized in complex with MEK and shown to induce an inactive kinase conformation (PDB 1S9J and 1S9I) [40]. PD318088 therefore exploits this pocket through allosteric inhibition (PDB ID: 1S9J) by binding concurrently with Mg-ATP and presents a unique mechanism of inactivation where the kinase is inactivated while not precluding binding of the ATP substrate. As a result of this novel mechanism, these analogues show marked selectivity and potency profiles due to the fact that this allosteric pocket in which the inhibitor series binds is unique in sequence to MEK1 and 2 compared to other MAP kinases and other protein kinases in general.

The JNKs interact with, phosphorylate and enhance activity of a range of transcription factors including c-Jun, p53, and Elk-1. Consequently, these stress and cytokine-activated kinases appear to hold a critical role in many apoptotic and inflammatory disorders. Since protein substrate docking domain interactions are required for their activity, it has been reasoned that non-ATP competitive inhibitors could be useful in the inhibition of JNKs. Small molecule inhibitors aimed at selectively inhibiting phosphorylation of JNK substrates are of great interest due to the wide range of therapeutic applications being considered for JNK inhibitors [38].

JNK activation by extracellular stimuli such as stress or cytokines leads to multiple pathways including the phosphorylation of transcription factors, insulin receptor signaling, and mRNA stabilization [41–44]. These pathways are related to the pathogenesis of diseases such as diabetes, cancer, atherosclerosis, stroke, and Alzheimer's and Parkinson's diseases. Therefore, JNKs are attractive targets for a range of new drug treatments [45].

JNK-interacting protein-1 (JIP1) is a scaffolding protein that enhances JNK signaling through its ability to create a proximity effect between JNK and upstream kinases [44]. A minimal peptide region of JIP1, consisting of a single D-domain, has been determined to be an inhibitor of JNK activity [46,47]. A peptide that corresponds to the D-domain of JIP1 (pepJIP1) inhibits JNK activity *in vitro* toward recombinant c-Jun Elk, and ATF2 while selectively exhibiting little inhibition of the closely related Erk and p38 MAPKs [48]. With the JIP site of JNK likely to be a druggable target, small molecule libraries were screened in hopes of disrupting the interaction between pepJIP1 and JNK1 [49]. Of the small molecules that were discovered to be substrate competitive inhibitors of JNK, BI-78D3 (**12**) was found to dose-dependently inhibit the phosphorylation of JNK substrates both *in vitro* and *in cell* [49]. In mouse models of type 2 diabetes, BI-78D3 blocks JNK-dependent Con A-induced liver damage as well as restores insulin sensitivity. Though the results with BI-78D3 were shown for the treatment of diabetes, its initial success serves as a proof of principle for targeting substrate specific docking sites rather than the conserved ATP binding sites, and the exploration can be expanded to other disease states including cancer.

1.4. *Bcr-Abl kinase*

In an effort to affect the T3151 mutant Bcr-Abl, regions outside of the ATP binding site of the enzyme were targeted. Such compounds have the potential overcome the effects of mutations that make chronic myelogenous leukemia (CML) cells resistant to the current CML therapeutic standard imatinib and therefore have significant potential in creating future therapeutics against resistant tumors. The result of these efforts was the development of the non-ATP competitive inhibitor **13**, ON012380 [50]. ON012380 can specifically inhibit Bcr-Abl and induce the cell death of Ph+ CML cells expressing the T315I isoform at a concentration of $<$10 nM. ON012380 was found to induce the apoptosis of known imatinib resistant mutants at concentrations of $<$10 nM *in vitro*. The potential of this compound as an anti-tumor therapeutic was assessed through *in vivo* studies indicating that i.p. injections as 100 mg/kg resulted in significant growth inhibition of T315I leukemias grown in nude mice. ON012380 was well tolerated by rodents as no hematoxicity was observed despite daily dosing over 3 weeks with a concentration $>$100 mg/kg [51].

Upon identification of ON012380, *in vitro* studies of purified recombinant Bcr-Abl preparations showed that the compound exhibited strong inhibition of Bcr-Abl kinase activity as evidenced by the inhibition of Bcr-Abl autophosphorylation and Crk phosphorylation, which was used as a substrate. Binding assays performed with the recombinant protein showed the IC_{50} of ON012380 to be 9 nM. The same assays performed with imatinib gave an IC_{50} of 100 nM, which indicates that ON012380 is 10-fold more active than imatinib in Bcr-Abl kinase inhibition assays [50]. When Bcr-Abl kinase assays were performed with T315I recombinant protein, ON012380 resulted in an IC_{50} value of 1.38 nM (imatinib was essentially inactive), thus validating the hypothesis that non-ATP competitive inhibition is an effective strategy for targeting imatinib resistant mutants [50]. When the two were used simultaneously in an assay, they acted synergistically to inhibit Bcr-Abl, suggesting that they bind to the Bcr-Abl at different locations and confirming the non-ATP competitive behavior of ONO12380. Additionally, its biological mechanism of action was determined by performing a steady-state analysis that looked at the effects of increasing concentrations of ATP or the substrate protein Crk on the compound. Analyses showed that the velocity of substrate phosphorylation was unaffected by increasing ATP concentrations and both K_m and IC_{50} values for ON012380 remained unchanged. The results support the conclusion that ON012380 is not an ATP competitive inhibitor. When the same experiments were performed with imatinib, opposite results were obtained and IC_{50} values increased with increasing concentrations of ATP which suggests competition between ATP and the inhibitor.

1.5. *Glycogen synthase kinase 3-β*

GSK3-β is a protein kinase that participates in the wnt signaling pathway, a complex network of proteins most well

known for their roles in embryogenesis and cancer, through the phosphorylation of axin and β-catenin. FRAT proteins block recruitment of these substrates to GSK3-β by binding to a specific site of the kinase [39]. FRATtide, a peptide derived from a GSK-3 binding protein, binds to GSK-3 and blocks its interaction with axin. Consequently, the axin-dependent phosphorylation of β-catenin is inhibited as well [52]. The crystal structure of GSK3-β/FRATtide (PDB ID: 1GNG) shows the peptide FRATtide forming a helix-turn-helix motif in binding to the C-terminal lobe of the kinase domain. The activation loop adopts a conformation similar to that of the phosphorylated and active forms of the related kinases CDK2 and ERK2 [52]. The binding site for this peptide is near to the substrate binding channel of GSK3.

The interaction of GSK3-β and its substrate appears to be well positioned as a target for small molecule inhibitor development. The peptide–protein interface is small, the structure of the GSK3-β/FRATtide docking has been identified, and substrates are known to bind in this region. In fact, 2,4-disubstituted thiadiazolidinones (TDZDs) **14** appeared as the first ATP non-competitive GSK3 inhibitors [53]. They are specific to only glycogen synthase kinases. While the TDZD efforts focus on neurodegenerative diseases involving the Tau protein, other chronic disorders such as cancer can be affected by these inhibitors. In a 2008 follow-up study that aimed to created potent derivatives of the initial TDZD non-competitive inhibitors, a kinetic analysis was performed to verify the non-competitive action of these compounds [54]. These analyses were carried out using combinations of six ATP concentrations ranging from 6.5 to 100 μM and two concentrations for both the TDZD compounds and a known ATP competitive inhibitor Ro 31-8220 (compound **15**) [55]. Double reciprocal plotting of the data indicated that the TDZD compounds were, in fact, ATP non-competitive inhibitors while highlighting the ATP competitive binding mode of Ro 31-8220.

Because the majority of GSK3-β inhibitors reported thus far are ATP competitive, their specificity is limited. For example, the aloisine family of ATP competitive GSK3-β inhibitors (compound **16**) is active on CDK1/cyclinB, CDK2/cyclinA–E, CDK5/p25, and GSK3-α-β [56]. Also a recent study examining specificity of a number of widely investigated kinase inhibitors, four of five GSK3-β compounds studied were found to be non-specific [57]. For this reason, efforts have been undertaken to produce selective, non-ATP competitive inhibitors. A series of inhibitors of this kinase that were based on α-halomethyl ketones have been successfully created [58]. The compounds show IC_{50} values as low as 0.5 μM (compound **17**), though their druggability needs to be improved [58].

1.6. Other kinases

3-Phosphoinositide-dependent kinase-1: Like that of the mitogen-activated kinases, the 3-phosphoinositide-dependent kinase-1 has an important role in the regulation of cell survival and proliferation. PDK1 has been shown to regulate several kinases including protein kinase B and also to promote MAPK activation in a MEK-dependent manner [59]. Recently, specific low molecular weight compounds which target the HM/PIF-pocket of PDK1 have been shown to be allosteric activators. The mechanism of action for these compounds was determined by studying mutagenesis of the binding site, compound SAR analysis, interaction-displacement studies, and isothermal titration calorimetry experiments [60]. This further demonstrates the need for inhibitor development focused on protein–protein interaction.

Janus kinases (JAKs) are key signal transducers for a variety of cytokines, growth factors, and interferons. JAKs relay signals received from extracellular stimuli and induce a signaling cascade. The cascade consists of autophosphorylation of the receptor followed by phosphorylation of JAK. Next, signal transduction and activation of transcription proteins (STATs) are recruited by JAK and/or the receptor. STAT translocates to the nucleus where it binds to the enhancer regions of DNA for transcription of cytokine-responsive genes. Together, JAKs/STATs integrate the signal transduction of cytokines in haematopoietic cells, lymphocytes, and other bone-derived mammalian cells [61]. In recent years, elevated levels of JAK/STAT have been identified in cancer cells. High-throughput screening methods using ELISA and virtual screening have been used to help identify possible therapeutic candidates [61]. Of the potential candidates, some interacted in a non-ATP competitive fashion including AS701173, which is one such example showing an IC50 of 0.03 μM [62]. The inhibitory activity of AS701173 has been shown to be independent of ATP concentrations within the ranges used in the MEK1 assay.

Protein kinase C iota (PKCι): Protein kinase C isoenzymes (PKCs) are a family of serine/threonine kinases involved in diverse intracellular pathways. PKCs are tied to numerous biological functions including proliferation, cell cycle control, and differentiation [63]. Therefore, mutations in PKC can lead to diseases including cancer. PKCs are divided into three main classes (classical, novel, and atypical) that are quite structurally and functionally similar in their catalytic domain which contains the ATP binding site. Differences in the N-terminal regulatory domain, however, allow for isoenzyme-specific regulation and function.

While the classical and novel PKCs are activated by second messengers such as phorbol esters, diacylglycerol, calcium, or phosphatidylserine, atypical PKCs (aPKCs) require only phosphatidylserine for activation [63]. The activity of aPKCs can be regulated by specific protein–protein interactions that are mediated through the PB1 domain (Phox-Bem1 domain) contained within the N-terminal regulatory domain. The PB1 is a structurally conserved domain that mediates interactions between PB1 domain-containing proteins [64]. PB1 domain-containing proteins, including MEK5 (mitogen-activated protein kinase/extracellular-signal-regulated kinase), bind aPKCs via PB1–PB1 domain interactions.

Recently, the atypical PKCι was the first PKC isoenzyme shown to be a human oncogene [65]. Overexpression of PKCι is seen in non-small cell lung cancer (NSCLC) cell lines, primary NSCLC tumors [65], and ovarian cancer [66,67]. Proteins containing PB1 domains interact with the PB1 domain of PKCι and play a key role in oncogenic activity. For instance, ectopic expression of the PKCι PB1 domain blocks anchorage-independent growth in NSCLC cell lines which indicates that the PB1 domain of PKCι is required for its oncogenic activity [68]. Par6 is a PB1 domain protein that links aPKCs to the downstream effector molecule Rac1.

Given the role of the PB1 domain of PKCι in oncogenic signaling, small molecules that target this domain were pursued. A fluorescence resonance energy transfer-based assay was developed to identify inhibitors of PB1–PB1 interactions between PKCι and Par6 [63]. Several high affinity compounds were discovered including the aurothioglucose (ATG) and aurothiomalate (ATM) compounds. Both compounds disrupt PKCι-dependent signaling to Rac1 and inhibit transformed growth of NSCLC cells *in vitro* and tumor formation *in vivo* [69].

2. Future developments

The ability to selectively target a specific protein kinase implicated in tumorigenesis while not affecting those involved in normal physiological processes remains key to optimizing protein kinase inhibitors as therapeutics. While it is possible that multi-targeted kinase inhibitors may be required to prevent the bypass of redundant pathways, in practice it is challenging to develop these drugs by design. In addition, selective kinase inhibitors in oncology can act in a synthetic lethal manner, inducing cell death in the context of another cellular defect while reducing the likelihood for off-target side effects. Therefore, alternative approaches for inhibitor development in targeting sites other than the ATP cleft are being increasingly pursued. The described protein–protein interactions involved in kinase regulation and substrate recognition offer the potential for this selectivity and in addition avoid decreased efficacy as a result of competition with high intracellular ATP concentrations. Recent advances with cyclin-dependent kinases are an example of the movement toward developing non-ATP competitive inhibitors where blocking of the recruitment site promotes CDK-specific inhibition. Likewise, inhibitors of the polo box domain of polo-like kinases target a specific binding domain involved in substrate recognition and sub-cellular localization of the kinase. Mitogen-activated protein kinases have been included in the research efforts for non-ATP competitive inhibitors due to the fact that all MAP kinases use docking sites to bind substrates. GSK3-β is of interest because the structure of the GSK3-β/FRATtide docking has been identified, and initial successes in the development of inhibitors binding to sites other than the ATP cleft have been obtained. Recent results showing that non-ATP competitive inhibitors of Bcr-Abl are effective against imatinib resistant mutants further validate the strategy of targeting regions outside of the ATP cleft. 3-Phosphoinositide-dependent kinase-1, the janus kinases and PKCι have also shown promise as targets for non-ATP competitive protein kinase inhibitors. These results have significant potential for future generations of protein kinase inhibitors as anti-tumor therapeutic agents.

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