Exploiting Substrate Recognition for Selective Inhibition of Protein Kinases

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Abstract: Protein kinases are potential targets of drugs to treat many human diseases. Intensive efforts have been made to develop protein kinase inhibitors, but a major challenge is achieving specificity. Exploiting regulatory elements outside the ATP binding pocket, such as the substrate binding site, may provide an alternative that allows generation of competitive inhibitors with improved selectivity. In-depth understanding of substrate recognition by protein kinase is essential for design and refinement of competitive inhibitors. Here we described strategies for specifically targeting protein kinases and highlight our current progress in the development of substrate competitive inhibitors for glycogen synthase kinase-3 (GSK-3).

Keywords: Protein kinase, GSK-3, substrate recognition, inhibitor design.

INTRODUCTION

Phosphorylation is a widespread type of post-translational modification of proteins; regulation based on phosphorylation state affects most – if not all – cellular processes including development and differentiation, metabolism, growth, and gene transcription. Phosphorylation is carried out by protein kinases, central players in signaling cascades. Deregulation and/or mutation of protein kinases directly results in many human diseases, and protein kinases are thus considered an important class of therapeutic drug targets. Selective inhibition of protein kinases has proven extremely challenging. Attention has recently focused on understanding the molecular recognition between kinases and their physiological partners such as substrates. It is anticipated that using advanced molecular and computational tools will facilitate rational design of small molecules that compete with substrate for selective binding to protein kinases.

PROTEIN KINASE STRUCTURE AND SUBSTRATE RECOGNITION

The several hundred members of the protein kinase super family account for nearly 2% of the genes in the human genome [1, 2]. Protein kinases share extensive sequence and structural homologies, especially within their catalytic domains [3-6]. Regions outside the catalytic domain usually serve as regulatory components that affect the catalytic activity directly or indirectly via docking into protein partners and/or altering complex formation. The catalytic domain is typically folded into a bi-lobal architecture that contains a smaller N-terminal lobe with five-stranded β-sheets and a glycine-rich loop (GXGXXG, also termed the **P-loop**) responsible for anchoring and positioning the ATP molecule that serves as the phosphate donor [3-6]. The larger and mostly helical C-terminal domain contains the catalytic loop and the 'activation loop' both important for kinase activity [3-6]. In many cases phosphorylation of the activation loop leads to an open and active conformation that enhances catalytic activity. The substrate binds within the cleft formed by the two lobes; the site to be phosphorylated is oriented toward the ATP binding pocket in the smaller lobe. Other parts of the substrate interact with specific pockets or subsites located within the two lobes, some of which are unique to individual kinases. These structural differences in the docking sites confer substrate specificity. Hence, understanding the molecular basis of substrate specificity may provide leads for design of selective inhibitors.

Molecular structures are an important source of information in understanding molecular recognition and may serve as templates for drug design. Three-dimensional structures of proteins kinases complexed with their substrates are not usually available. A number of high-resolution structures of protein kinases complexed with their substrates have been reported. These included structures of cAMP dependent kinase (PKA) complexed with its inhibitor PKI, protein kinase B (Akt) complexed with the N-terminal peptide of its substrate GSK-3β and phosphorylase kinase ,cyclic dependent kinase (CDK), proto-oncogene protein kinase (Pim1), and insulin receptor complexed with their respective peptide substrates [7-12]. These structures taught us that surface-surface contacts are major determinants in control of substrate recognition and specificity. Molecules that can mimic these interactions are a good choice in the substrate inhibitor-design approach.

It has been known for some time that substrate binding to an enzyme is a dynamic process and not a simple match of 'rigid' structures, as proposed in the 'key and lock' model. Rather, as initially suggested by Koshland, the 'induced fit theory' suggests that the substrate causes changes in the active site which brings the catalytic group into the proper orientation for catalysis; this results in an enthalpically driven binding process and stabilization of the enzyme-substrate complex [13]. Evidence accumulated in recent years indicates that protein structure is dynamic and that proteins have an inherent ability to shift among multiple conformations. The 'conformational selection theory' suggests that the substrate binds preferentially to the most favored conformation presented by the enzyme [14, 15]. This binding process stabilizes the enzyme-bound complex and, in contrast to the induced fit model, may also be driven by changes in entropy [14, 15]. Recent studies analyzing the substrate recognition process of PKA indicate that binding with substrate is entropically driven [16]. The presence of dynamics in the substrate and at the active site of PKA confer a conformational selection recognition [16]. However, the selected fit conformation process is not perfect, and a number of studies have shown that conformational selection is often followed by conformational adjustments [14, 15]. Hence, it appears that both mechanisms coexist and contribute to the substrate recognition process. This understanding of molecular recognition has encouraged the use of molecular dynamics (MD) simulations that provide physical insights into molecular functions that depend on conformational variability and take into account binding flexibility. Understanding the contribution of conformational entropy to the overall entropy binding may be significant for inhibitors design.

PROTEIN KINASE INHIBITORS AND THE PROBLEM OF SELECTIVITY

Most protein kinase inhibitors discovered to date are small molecules that compete with ATP for its binding site. These inhibitors, although often very effective, generally show limited specificity [17-19]. It is noteworthy, that some of these inhibitors have had

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successes in the clinic. These include two kinase inhibitors: the first FDA-approved tyrosine kinase inhibitor imatinib (sold as Gleevec) targets BCR-Abl and is used to treat chronic myelogenous leukemia (CML) [20], and sorafenib (marketed as Nexavar) targets serine/threonine kinase Raf-1and VEGFR and is used in treatment of primary kidney cancer [21]. Approaches that exploit subtle differences in the ATP binding site or binding to novel pockets adjacent the ATP binding site have been recently undertaken for design of more specific inhibitors with some success [22-24]. A different strategy exploits allosteric sites outside the ATP-binding site that are usually unique to a particular kinase. Detailed descriptions of these tactics in development and design of protein kinase inhibitors are found in [21, 25-27]. An alternate strategy for achieving selectivity is targeting the substrate binding site; this site differs substantially among kinases. Although, targeting this site has not been extensively exploited, it offers substantial opportunity for selectivity.

Substrate competitive inhibitors provide numerous advantages over the 'traditional' ATP-competitive inhibitors. They are by far more selective as the substrate recognition sites and types of interactions vary considerably among kinases, whereas the ATP-binding domain is structurally conserved. Long considered a disadvantage is the relatively weak binding affinity of substrate competitive inhibitors for their targets. It has increasingly been acknowledged, however, that strong and constitutive inhibition of protein kinases in biological systems can be harmful and often leads to adverse effects. The moderate inhibition of the kinase resulting from a substrate competitive inhibitor may provide sufficient effects with minimum damage. This property of substrate competitive inhibitors may be particularly advantageous during long-term treatment.

Substrate competitive inhibitors have been often overlooked in high-throughput screening efforts or ignored during the development processes due to their 'ineffective' inhibition properties. Efficacy of substrate competitive inhibitors is often underestimated by *in vitro* kinase assays, traditionally considered the 'gold standard' for selecting 'good inhibitors'. The *in vitro* kinase assays do not reflect the true potency of substrate competitive inhibitors as the substrate's concentration in these assays is far above that of its physiological concentration. Thus, substrate competitive inhibitors will show better efficacy in cells than in cell-free conditions. Overall, substrate competitive inhibitors hold promise in clinical applications and despite challenges in their discovery and development, the search for and the use of substrate competitive inhibitors is worthwhile.

The fact that many protein kinases can recognize peptides containing the local sequences surrounding the phosphorylation sites of their target substrates suggests that short peptides patterned after these recognition motifs may behave as competitive inhibitors.

The first discovered substrate competitive inhibitor was a natural 76-amino-acid protein, which was initially co-purified with the catalytic subunit of PKA [28]. This peptide kinase inhibitor or PKI contains the typical PKA recognition motif RRN**A**L with the phospho-acceptor serine replaced by alanine (underlined). Synthetic peptides derived from the "substrate-like" PKI sequence are PKA inhibitors [29, 30]. The crystal structure of the PKA/PKI complex further verified the important contribution of the surrounding residues in the inhibitory potency of the peptide [30].

Competitive inhibitors, called 'pseudosubstrates', are naturally occurring auto-inhibitory domains, located outside the catalytic domain of the kinase [31, 32]. Pseudosubstrates are characterized by their resemblance to sequences derived from the substrate phosphorylation sites and serve as competitive inhibitors by interacting with the substrate binding site [33-35]. In the PKC family, a pseudo-substrate sequence of about 13 amino acids is located in the N-terminal region of each PKC isoform [33]. Synthetic peptides derived from these sequences are highly selective inhibitors of their corresponding PKC isoforms as shown for PKCs α, β, η, θ, and ζ [33, 36-38]. Auto-inhibitory domains are found also in Ca^{2+}/CaM-dependent protein kinase II (CaMK-II), myosin light chain kinase (MLCK), twitchin, and phosphorylase kinase [33-35, 39]. In glycogen synthase kinase-3 (GSK-3), phosphorylation at serine 9 near its N-terminus mimics the pre-phosphorylation of its substrates and results in auto-inhibition of the catalytic activity. Short phosphorylated peptides derived from this pseudosubstrate region showed slight inhibition of the kinase [40, 41].

Synthetic peptides derived from the pseudosubstrate sequences are usually weak to moderate inhibitors (i.e., these peptides inhibit kinase activity in the micromolar range in cellular conditions), suggesting that copying the native sequence of the substrate (or pseudosubstrate) may not result in sufficient inhibition. This may not be surprising as the substrate's binding affinity to the kinase is relatively weak. It is possible that certain modifications inserted into the original peptide will enhance its binding affinity by generating additional (and different) contacts within the active site of the kinase. This approach should be successful since, unlike the substrate, the inhibitor does not require catalysis occur so exact positioning within the catalytic cleft is not necessary. Ideally, an inhibitor should mimic the substrate's binding and should also have novel contacts that enhance its binding affinity and or perturb the conformation necessary for catalysis in order to sequester the kinase in its inactive state.

Finally, different class substrate competitive inhibitors are inhibitors that compete with docking site located at the substrate. In this case the competitive inhibitors prevent interaction of the kinase with substrate. One example is the JIP peptide ("pepJIP1") derived from JIP1 (JNK interacting protein) scaffold protein that facilitates the assembling of JNK-cascade components [42-44]. This peptide, which copies the consensus sequence of the high affinity binding site, the site D-site, located within JIP1 and other JNK substrates, inhibits JNK *in vitro* and *in vivo* [44-46]. Recent work identified small molecules that mimic pepJIP1 activity, and behave as substrate competitive inhibitors that block JNK interaction with its substrates [47, 48]. Unlike the substrate competitive inhibitors that competes with substrate's binding within the catalytic core, this type of inhibitors prevent phosphorylation without grossly affecting the kinase activity. Therefore, inhibition of certain pathways may be separated functionally, namely, blocking phosphorylation of one substrate, but not necessarily affecting the others. This may be of advantage or disadvantage dependent on the desired inhibitory strategy.

GSK-3 AS A TARGET FOR THERAPEUTIC INTERVENTION

GSK-3 is a serine/threonine kinase expressed as two similar isozymes, α and β, that are abundant in most tissues [49]. GSK-3 was initially implicated in insulin signaling and metabolic regulation, but later studies identified additional roles in embryogenesis, cell survival, inflammation, and neuroplasticity [50-53]. Aberrant GSK-3 control has been linked with molecular pathology of several human diseases including diabetes and various neurodegenerative and psychiatric disorders [50-52, 54-57] Fig. (**1**). Increased activity of GSK-3 has been identified in diabetic tissues [58, 59], and inhibition of GSK-3 either by pharmacological inhibitors or genetic manipulation improves glucose homeostasis and insulin sensitivity in animal models [60-63]. Aberrant regulation of GSK-3 activity has also been associated with the pathogenesis of Alzheimer's disease, amyotrophic lateral sclerosis, schizophrenia, mood disorders, and spinal muscular atrophy [56, 64-72]. These observations provide the rationale for developing selective GSK-3 inhibitors. An open question is whether selectivity toward a particular GSK-3 isozyme will be required. At present, it seems that most GSK-3 inhibitors do not discriminate between the two isoforms, although their distinct functions suggest that an isozyme specific inhibitor

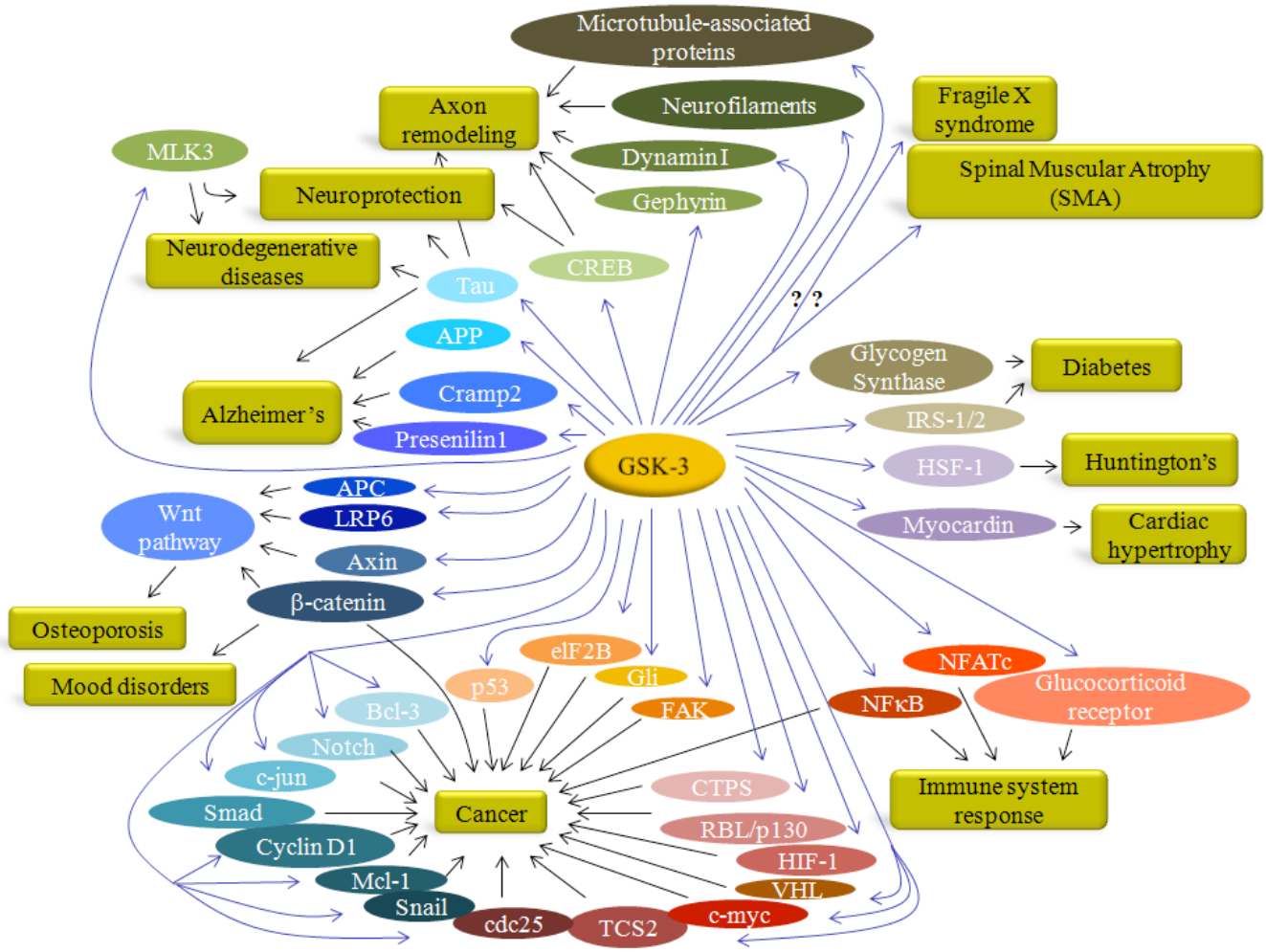

Fig. (1). GSK-3 in human disease. A schematic illustration of GSK-3 linked with pathological disorders and relevant substrate. Illustrated clockwise from the top **Fragile X syndrome** and **Spinal Muscular Atrophy (SMA)** linked with GSK-3 activity, but precise mechanisms are currently unknown. In **insulin resistance and diabetes** GSK-3 phosphorylates glycogen synthase (GS) and IRS1/2 (Insulin receptor substrate 1/2) inhibiting insulin signaling. Phosphorylation of HSF-1 (heat shock factor-1) by GSK-3 regulates heat shock proteins that prevent protein aggregation implicated in **Huntington's disease.** Phosphorylation of myocardin by GSK-3 is implicated in **Cardiac hypertrophy**. GSK-3 is involved in the **Immune system response** via phosphorylation of the glucocorticoid receptor, NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells), and promoting nuclear export of NFATc (Nuclear factor of activated T cells c). GSK-3 phosphorylates many factors and proteins involved in cancer including Gli, FAK (Focal adhesion kinase), CTPS, (cytidine triphosphate synthetase), RBL-2 (Retinoblastoma-like protein 2), HIF-1 (hypoxia-inducible factor-1), VHL (Von Hippel-Lindau protein), c-myc, TCS2 (Tuberous sclerosis 2), Mcl-1 **(**myeloid leukemia cell differentiation protein-1), Cyclin D1, Smad, c-Jun, Notch, Bcl-3 (B-cell lymphoma 3-encoded protein), elF2B (Eukaryotic initiation factor 2B), p53, snail, and b-catenin. In the **Wnt signaling** pathway GSK-3 phosphorylates LRP6 (low density lipoprotein related receptor protein), axin, APC (adenomatosis polyposis coli), and β-catenin. Wnt pathway is involved in **Mood disorders** and **Osteoporosis**. GSK-3 may contribute to Alzheimer's disease through phosphorylation of tau, CRMP-2 (collapsin response mediating protein-2), presenilin-1 and APP (amyloid precursor protein). Other neurodegenerative disorders or neuroprotection involves GSK-3 phosphorylation of MLK3 (Mixed lineage kinase-3) and CREB (cyclic AMP response element-binding protein). GSK-3 affects **Axon remodeling** via phosphorylation of microtubule associated proteins, neurofilaments, Dynamin I, and Gephyrin. Note that some substrates have overlapping functions in several diseases.

would be worthwhile [63, 73-77]. Development of such inhibitors will be based upon exploitation of the biochemical differences between the isozymes.

GSK-3 is distinguished from other protein kinases in many respects. One important feature directly related to the design of specific inhibitors is GSK-3's somewhat unique requirement for pre-phosphorylation. GSK-3 recognizes sequence motifs in the context of $S^1XXXS^2(p)$ where S^1 is the site phosphorylated by GSK-3 and $S^2(p)$ is the 'priming site' pre-phosphorylated by a different kinase [78, 79]. This requirement for pre-phosphorylation is very strict as replacement of $S^2(p)$ with a phospho-tyrosine residue or glutamic acid diminishes substrate phosphorylation by GSK-3 [80]. X-ray crystallographic studies of GSK-3β identified a pocket comprised of three basic residues, Arg 96, Lys 205, and Arg 189, that interacts with anions and presumably binds the phosphorylated moiety of the substrate [40, 81]. An additional element important for the kinase activity is the auto-phosphorylation of a tyrosine residue (Tyr 216 in GSK-3β, Tyr 279 in GSK-3-α) located within the activation loop that occurs in a chaperone-dependent manner [82-84]. In addition, the N-terminal region contains the highly conserved RPRTTSF motif that acts as a pseudosubstrate when phosphorylated [41, 53]. These three elements control the autonomic GSK-3 activity and should be exploited during inhibitor design.

GSK-3 SUBSTRATE COMPETITIVE INHIBITORS

The importance of GSK-3 as a therapeutic target highlighted the need for design and development of selective GSK-3 inhibitors. In the case of GSK-3, the use of substrate competitive inhibitors

may be particularly advantageous. GSK-3 is essential for the well-being of the cell and drastic inhibition of GSK-3 results in abnormality and damage. In addition, 'pathological' GSK-3 activity does not exceed 2 to 3 fold over 'normal' levels. Thus, a moderate-to-weak inhibition of the enzyme (about 50%) is actually desired for treating conditions associated with elevated levels of GSK-3 activity.

The fact that GSK-3 recognition of its substrate involves pre-phosphorylation supports the rational for using synthetic phosphorylated peptides as substrate competitive inhibitors [85]. A set of phosphorylated peptides patterned after known GSK-3 substrates were tested, and a peptide sequence derived from heat shock factor-1 (HSF-1) (^{1}KEAPPAPPQS(p)P^{11}) was selected as the most potent inhibitor (positions 3 and 6 in the HSF-1 substrate sequence were replaced by alanine). This peptide, L803, has an IC_{50} of 40 μM in an *in vitro* kinase assay [85]. Phosphorylated peptides derived from the N-terminal pseudosubstrate sequence of GSK-3β were very weak inhibitors [41, 86], indicating that copying 'natural' motifs does not necessarily provide sufficient inhibition. A cell-permeable version of L803 in which a myristic acid was attached to its N-terminus (termed "L803-mts") was generated [85]. L803-mts has high selectivity towards GSK-3, water soluble, and stable in serum, and shows low toxicity as determined by histopathology and single-dose, maximal-tolerated dose (MTD) analyses in mice ([61, 85] and unpublished results from our laboratory). L803-mts improves glucose intolerance and insulin sensitivity in diabetic mouse models [61, 87, 88]. In neurons, L803-mts provides neuroprotection against 6-hydroxydopamine and trisialoganglioside [89, 90], and L803-mts produced anti-depressive-like behavior in the mouse forced swimming test and after traumatic brain injury [91, 92]. Other studies found L803-mts a useful GSK-3 inhibitor in different experimental tests [93-97].

The results obtained with L803-mts were encouraging enough to support further development. A combined approach consisting of

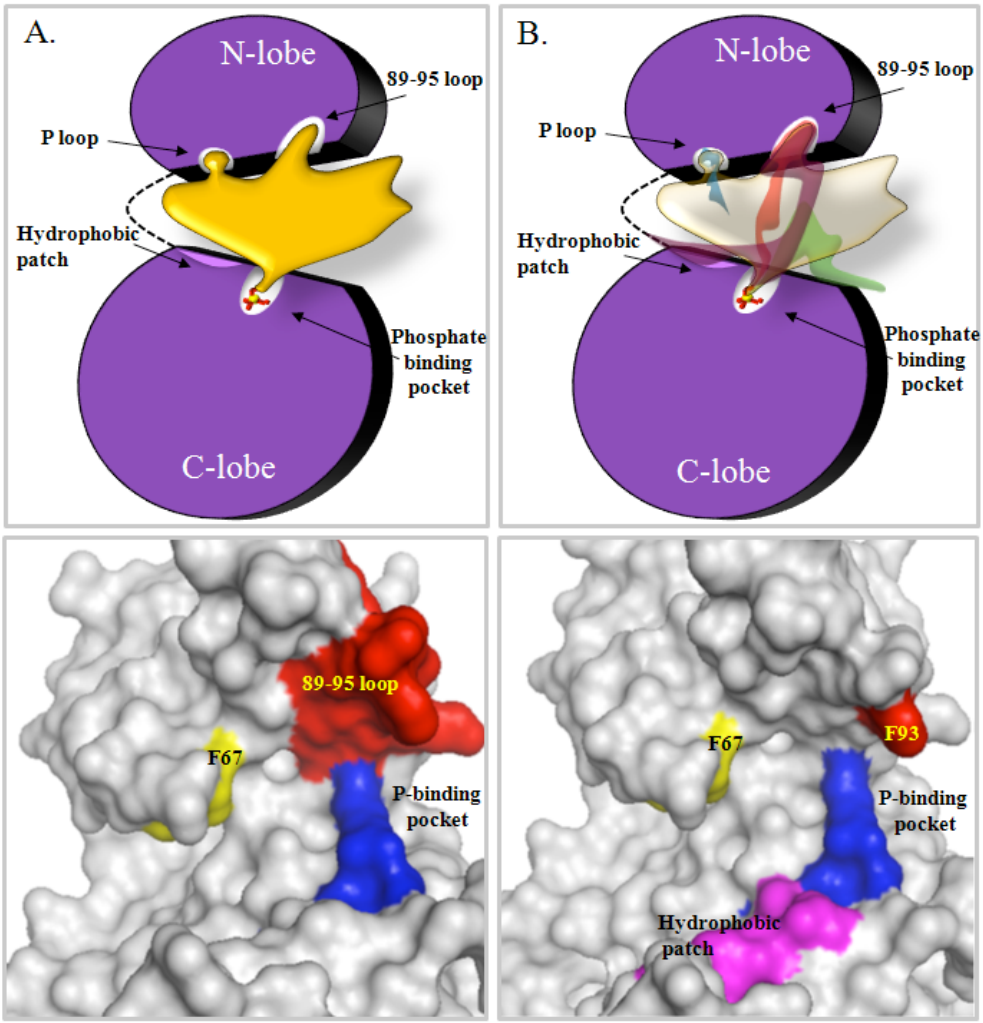

Fig. (2). GSK-3-interacting sites with substrates and competitive inhibitors. A) Schematic presentation of substrates' docking with GSK-3 (upper panel). The phosphorylated moiety of the substrate interacts with the phosphate binding pocket (Arg 96, Arg 180, and Lys 205). The serine to be phosphorylated is oriented toward the ATP binding site (P-loop). Other residues located within the consensus sequence interact with the cavity bordered by residues 89-95 ('89-95 loop'). The lower panel highlights these interacting sites at the surface of GSK-3b: Phosphate binding pocket (blue), F67 at the tip of the P-loop (yellow), 89-95 loop (red). **B**) Schematic presentation of potential competitive inhibitors interacting with GSK-3. The inhibitor does not require an exact positioning within the catalytic cleft. Ideally, an inhibitor should mimic the substrate's binding and should also have novel contacts that enhance its binding affinity (upper panel). The lower panel shows sites interacting with L803-mts at the surface of GSK-3b: Phosphate binding pocket (blue), F93 (red), hydrophobic patch (V214, I217 Y216) (magenta). F67 (yellow) indicates the ATP binding site. GSK-3 structure is based on PDB code 1gng and images were processed by PyMol software.

mutagenesis and computational protein-protein docking analyses was undertaken to explore the binding mode of GSK-3β with its substrates and L803-mts. These studies placed the substrates in contact with four residues within GSK-3β, Phe 67, Gln 89, Phe 93, and Asn 95 [80, 98] Fig. (**2**), and indicated that Asp 181 interacts with the N-terminal pseudosubstrate region [41]. Phe 67 is a conserved site within the protein kinase family, located at the tip of the P-loop. Mutation at this site completely impairs the enzyme activity [80]. Residues Gln 89, Phe 93, and Asn 95 reside in a promiscuous binding cavity delimited by Gln 89 and Asn 95 (termed the "89-95 loop"), which is spatially located near the ATP binding site and the phosphate binding pocket (consisting of Arg 96, Arg 189, and Lys 205) [98]. Recognition of substrate thus combines the promiscuity of the 89-95 binding loop, which allows binding of a broad selection of substrates, and the strict demand for primed phosphorylation. These together define specificity.

The studies with L803-mts indicated that the inhibitor has similar and non-overlapping interactions with GSK-3β as compared to the substrate Fig. (**2**). Like the substrate, L803-mts docks into the phosphate binding pocket via the phosphorylated serine moiety (position 10), but, unlike the substrate, L803-mts does not interact with Gln 89 or Asn 95. L803-mts forms tight contacts only with Phe 93 within the 89-95 loop; it also interacts with a hydrophobic surface (Val 214, Il 217, Tyr 216) located away from the ATP binding site [98] Fig. (**2**). Thus, unlike the substrate, L803-mts is not oriented toward the ATP binding pocket. L803-mts binding with GSK-3β is mainly governed by hydrophobic interactions, a conclusion deduced from alanine scanning mutational experiments [98]. To summarize, substrates and substrate competitive inhibitors interact with different geometries at the surface of the kinase, and these differences in binding modes likely enhance the inhibitory properties of the substrate competitive inhibitors.

These studies indicate that multiple avenues may be taken during optimization of substrate competitive inhibitors to tailor additional or different contacts with the kinase. As peptides are well suited to chemical modification these manipulations can be easily undertaken. The GSK-3β/L803 binding model predicted that peptide extension with an additional aromatic/hydrophobic residue would enhance contacts with GSK-3β via Phe 93. Accordingly, addition of a phenyl alanine to the C-terminus of L803-mts improved inhibition two fold [98]. The finding that L803mts binding with GSK-3β is mainly governed by hydrophobic interactions led us to predict that increasing the peptide's hydrophobicity would enhance inhibition. Replacement of the polar amino acid glutamine (at position 9) in L803-mts with alanine or proline indeed improved inhibition by 4 and 10 fold, respectively [98].

SUBSTRATE COMPETITIVE INHIBITORS OF OTHER PROTEIN KINASES

A series of substrate competitive peptide inhibitors of Akt was recently reported [99]. The peptides' design included a combination of two sequences: one derived from the Akt substrates FOXO 3 and the other derived from an optimal substrate sequence motif identified by peptide library screening [100]. The resulting hybrid peptides were more potent inhibitors than the original peptide sequence and inhibited cellular Akt in the micromolar range [99]. Another interesting example is the peptide derived from the PKC substrate MARCKS (myristoylated alanine-rich C kinase substrate) in which the four serine-phosphorylation sites were replaced by alanine [101]. The mutated peptide substrate inhibited PKC *in vitro* at nanomolar concentrations [101]. Unexpectedly, however, the kinetics of inhibition were not 'classically' competitive and suggested that the peptide probably binds at more than one site within the catalytic domain [101]. A small molecule substrate competitive inhibitor for IGF-1 receptor tyrosine kinase was identified by screening for molecules with ability to inhibit autophosphorylation [102]. The inhibitor termed "tyrphostin AG538" was competitive toward substrates and not ATP and inhibited cellular IGF-1 receptor at micromolar concentrations [102]. Another interesting series are peptides that compete with cyclin binding to CDKs [103]. Although not 'classical' substrate competitive inhibitors, these cyclin-groove inhibitory peptides prevent substrate binding to the kinase, as substrate binds after complex formation of CDK with cyclins [103, 104].

CONCLUSIONS

Substrate competitive inhibitors for protein kinases offer promising therapeutic opportunities with improved specificity and fewer off target side effects than the major type of inhibitors that compete with ATP. Development of substrate competitive inhibitors requires a sophisticated understanding of the kinase interaction with substrate. However, this knowledge of the molecular recognition of substrates is essential but not sufficient for design of an ideal inhibitor. Additional experimental and computational strategies are required to optimize the geometry of binding of the substrate competitive inhibitor to achieve high affinity binding and optimal inhibition as we have shown through our studies of substrate competitive inhibitors of GSK-3.

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