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Review

GSK-3 inhibition: Achieving moderate efficacy with high selectivity ☆

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ABSTRACT

Inhibiting glycogen synthase kinase-3 (GSK-3) activity has become an attractive approach for treatment of neurodegenerative and psychiatric disorders. Diverse GSK-3 inhibitors have been reported and used in cellular and *in vivo* models. A major challenge, however, is achieving selectivity. In addition, it is increasingly recognized that a moderate inhibition of a cellular target, particularly for long-term treatment, provides more favorable outcome than complete inhibition. Substrate competitive inhibitors can fulfill the requirement for selectivity and allow fine tuning of the degree of inhibition. Here we describe the therapeutic potential of GSK-3 inhibitors and highlight our progress in the development of substrate competitive inhibitors. This article is part of a Special Issue entitled: Inhibitors of Protein Kinases (2012).

1. GSK-3 in the CNS

The first indication that glycogen synthase kinase-3 (GSK3) plays a role in the central nervous system (CNS) emerged from the demonstration that the 'classical' mood stabilizer lithium inhibits GSK-3 [1,2]. This unexpected finding made GSK-3 the subject of considerable research in the psychiatric arena; however, as alterations in mood behavior are tightly coupled with neurological disorders, efforts soon shifted toward attempts to understand GSK-3 signaling in the CNS. Numerous studies have now implicated GSK-3 in control of various neuronal functions and have demonstrated that aberrant regulation of GSK-3 is involved in the etiology of neurodegenerative diseases, such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, and Alzheimer's disease (AD), as well as in brain aging [3–8]. Transgenic mice expressing elevated GSK-3 activity display memory deficits, reduced brain size and alterations in mood behavior and social interactions [9–12]. These animals also developed characteristics of AD such as hyper-phosphorylation of the microtubule associated protein tau and beta-amyloidal aggregates [13–16]. Pharmacological inhibitors or selective GSK-3 knockout models also demonstrated the impact of this kinase on brain morphology, neuronal plasticity and behavior [17–23].

Abnormal regulation of GSK-3 activity is reported in patients with AD, ALS, major depression, schizophrenia and bipolar disorder [12,24–31]. The mechanisms linking GSK-3 with pathogenesis likely involve regulation of targets that are directly or indirectly controlled by GSK-3. These include the microtubule associated protein tau, presenilins, amyloid precursor protein, collapsin response mediator proteins, components of the Wnt signaling pathway, β-catenin, and heat shock proteins [3–6] (Table 1). It is also noteworthy that GSK-3 contributes to inflammatory processes that have been recently recognized as important elements in neurodegenerative disorders. GSK-3 likely activates a variety of immune response targets, such as toll-like receptors, transcription factor NF-κB, cyclic-AMP response element binding protein, and proteins involved in cytokine production [32,33] (Table 1). Finally, GSK-3 has recently emerged as a negative regulator of lysosomes, which results with reduction in clearance efficiency of intracellular neurotoxic aggregates, such as Aβ depositions in the AD brain [34,35]. Thus, 'normalization' of GSK-3 activity emerges as a promising therapy for treatment of neurodegenerative and behavior disorders. Indeed, inhibition of GSK-3 results in beneficial outcomes in multiple *in vivo* models (Table 1).

Thus, 'normalization' of GSK-3 activity emerges as a promising therapy for treatment of neurodegenerative and behavior disorders.

The number of small molecule GSK-3 inhibitors is continuously rising and many have been tested in animals. These studies have provided additional support for specific roles of GSK-3 in neuronal functions under both normal and pathological conditions. Inhibition of GSK-3 has profound effect on neuroprotection, self-renewal and pluripotency of stem cells, axon fate determination, and mood behavior (reviewed in [36,37]). The reported GSK-3 inhibitors are of diverse chemotypes and mechanisms of action and include inhibitors isolated from natural sources, cations, synthetic small-molecules, and peptides [36]. Many of these molecules compete with ATP for binding to the ATP binding site in GSK-3 and inhibit catalysis of phosphorylation. The limited specificity of the ATP competitive inhibitors is a concern; the ATP binding site is highly conserved among protein kinases [38,39]. It is thus not surprising that many of these GSK-3 inhibitors

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Table 1
Neurotherapeutic effects of GSK-3 inhibition in *in vivo* animal models or patients.

Neuropathology	Biological activity of GSK-3 inhibition	Animal models	Reference
Alzheimer's disease (AD)	• Ameliorates Aβ pathology • Reduces tau phosphorylation • Improves learning and memory • Attenuates inflammation • Neuroprotective activity	• FTDP-17 tau transgenic mice • APP/PS1 mice • hAPP mice • Rats injected with preformed β-amyloid • hTau (T44) mice • 3xTg mice • GSK-3β mice • Postnatal rats	[16,66–73]
Amyotrophic lateral sclerosis (ALS)	• Attenuates motor neuron death • Induces clearance of protein aggregates • Improves learning and memory	• Human ALS patients • G93A-SOD1 mice	[74–76]
Bipolar disorder (BD) and Depression	• Anti-depressive activity • Neuroprotective activity • Reduces the prevalence of AD • Mood stabilizer (Lithium salts)	• Human BD patients • "Clock mutant" mice- hyperactivity induced by mphetamine/chlorodiazepoxide/methamphetamine	[16,77,78]
Cerebral ischemia (CI)	• Attenuates neuronal death • Reduces infraction size • Reduces inflammation • Neuroprotective activity • Improves behavioral functions	• Ischemic stroke rat • Rats subjected to permanent middle cerebral artery occlusion • Rats with hypoxia–ischemia brain injury	[76,79–81]
Epilepsy	• Reduces the development of hippocampal sclerosis (lithium salts)	• Rats injected with kainite	[82]
Fragile X syndrome (FXS)	• Reduces audiogenic seizures frequency • Improves learning and memory • Improves behavioral functions • Neuroprotective activity	• Mice with mutated Fmr1 protein • Drosophila with mutated FMR1 protein	[83–85]
Huntington's disease (HD)	• Improves motor performance • Reduces polyglutamine toxicity • Neuroprotective activity	• R6/2 mice (expressing mutated huntingtin) • Drosophila expressing mutated huntingtin	[86,87]
Neuropathic pain	• Anti hyperalgesic activity	• Mice with partial ligation of the sciatic nerve (PSNL)	[88]
Parkinson's disease (PD)	• Promotes dopamine neurons differentiation	• Mice treated with neurotoxin MPTP	[89]
Schizophrenia	• Neuroprotective activity	• Mice treated with stereotaxic injection	[90]
Spinal muscular atrophy (SMA)	• Maintains motor neuronal survival	• Fibroblasts derived from SMA patients	[91]
Spinocerebellar Ataxia type 1 (SCA1)	• Improves motor coordination • Improves learning and memory	• SCA1(154Q/2Q) mice	[72]
Traumatic brain injury (TBI)	• Anti-depressive activity	• Traumatic brain injury mice	[62]

failed in the pre-clinical or early clinical testing due to severe toxic effects. The challenge of specificity hence requires different thinking in the design and development of protein kinase inhibitors.

Mammalian GSK-3 is expressed as two isozymes: GSK-3α and GSK-3β that have the same catalytic domain but different N- and C-termini. There are certain physiological differences between the GSK-3 isozymes, although they share many redundant functions [5,10,11,17,23,40–44]. Inhibition of GSK-3α has more potent effects than inhibition of GSK-3β in pathological models of AD [14,45]. The underlying mechanisms that allow distinctive functions probably involve differential subcellular localization or interaction with different protein partners. Most characterized GSK-3 inhibitors do not discriminate between the two isoforms. Recently novel compounds with high selectivity for GSK-3α were reported [46]; their physiological impact remains to be elucidated. Obviously, our understanding of the distinct physiological functions of GSK-3 isozymes has important implications for drug discovery.

2. Inhibition of GSK-3 — the substrate competitive approach

A strategy for achieving selectivity when targeting protein kinases is to target regions that are characteristic of a specific subfamily of the protein kinases, such as the substrate binding site. Although targeting of the substrate binding site has not been extensively exploited, it offers substantial opportunity for selectivity. Long considered a disadvantage is the relatively weak binding affinity of substrate competitive inhibitors to their targets. It is now recognized, however, that strong and constitutive inhibition of protein kinases in biological systems often leads to adverse effects. Moderate inhibition of the kinase may provide sufficient desired effects with minimum damage and will likely be particularly advantageous during long-term treatment. In the case of GSK-3, the use of substrate competitive inhibitors may be particularly beneficial. GSK-3 is essential for the well-being of the cell and drastic inhibition of GSK-3 causes damage. This is demonstrated by the fact that GSK-3-knockout mice die late in gestation [40]. In addition, 'pathological' GSK-3 activity does not exceed 2 to 3 fold over 'normal' levels. Thus, moderate-to-weak inhibition of the enzyme (about 50%) is actually desired for treating conditions associated with elevated levels of GSK-3 activity. Substrate competitive inhibitors offer a unique opportunity to achieve high selectivity and low toxicity.

3. Design and development of substrate competitive inhibitors

GSK-3 differs from other protein kinases in many respects. One important feature directly related to the design of specific inhibitors is GSK-3's 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 [47,48]. This requirement for pre-phosphorylation is very strict as replacement of $S^2(p)$ with a phospho-tyrosine residue or glutamic acid significantly diminishes substrate phosphorylation by GSK-3 [49]. Crystallographic studies of GSK-3β identified a pocket delimited by three basic residues, Arg 96, Lys 205, and Arg 180 (human GSK-3β numbering), that interacts with anions and presumably binds the phosphorylated moiety of the substrate [50,51]. 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 [52–54]. In addition, the N-terminal region contains the highly conserved RPRTTSF motif that acts as a pseudosubstrate when phosphorylated [55,56]. These three elements control autonomic GSK-3 activity and should be exploited during inhibitor design.

The fact that GSK-3 recognition of its substrate involves pre-phosphorylation supports the rational for using synthetic phosphorylated peptides as substrate competitive inhibitors [57]. Phosphorylated peptides derived from the N-terminal pseudosubstrate sequence of GSK-3β were very weak inhibitors of GSK-3 (IC_{50}s in the range of mM, unpublished results from our laboratory and [56,58]). On contrast, a peptide derived from heat shock factor-1 (HSF-1), ^{1}KEAPPAPPQS(p)P^{11} (termed L803), was found to be a potent inhibitor in an *in vitro* kinase assay [57]. A cell-permeable version of this peptide with myristic acid attached to its N-terminus (L803-mts) was generated [57]. L803-mts is highly selective toward 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 [57,59]. L803-mts has biological activity in neuronal cells and *in vivo* systems. It provides neuroprotection in cultured neuronal cells exposed to the Parkinson 'inducer' 6-hydroxydopamine and to trisialoganglioside [60,61]. Mice treated with L803-mts show that the compound has anti-depressive-like activity in the forced swimming tests [18] and after traumatic brain injury (TBI) [62]. Recently, L803-mts was shown to ameliorate intra-neuronal amyloid beta peptide loads and improve cognitive deficits in an Alzheimer's mouse model [35]. L803-mts has lower toxicity in neurons than other GSK-3 inhibitors [63].

The results obtained with L803-mts were encouraging enough to support further development. A structure-based design approach was undertaken combining mutagenesis and computational docking analyses. These studies suggested that substrates make important contacts with four residues within GSK-3β: Phe 67, Gln 89, Phe 93, and Asn 95 [49,64] (Fig. 1A). Phe 67 resides in the P-loop and is a conserved site within the protein kinase family. As expected, mutation at this site completely impairs the enzyme activity [49]. Residues Gln 89, Phe 93, and Asn 95 reside in the "89–95 loop" that is highly conserved in GSK-3s of vertebrates [64], and which, together with the P-loop, delimits a promiscuous binding cavity for GSK-3 substrates (Fig. 1A). While Gln 89 and Asn 95 are located at the bottom of the cavity, Phe 93 is highly exposed and located opposite the phosphate binding pocket [64]. Recognition of substrate thus combines the promiscuity of the 89–95 binding loop, which allows interaction with a broad selection of substrates, with the strict demand for primed phosphorylation. These features of the protein together define substrate specificity.

The studies with L803-mts indicated that the inhibitor has similar but non-overlapping interactions with GSK-3β as compared to a natural substrate [63] (Fig. 1B). 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 a tight contact with Phe 93 within the 89-95 loop and also interacts with a "hydrophobic patch" (Val 214, Ile 217, and Tyr 216) located in the C-terminal lobe of the kinase, facing the ATP binding site [64] (Fig. 1B). We concluded that substrates and substrate competitive inhibitors interact with different geometries in the substrate binding trough of the kinase. The different binding modes likely enhance the inhibitory properties of the substrate competitive inhibitors. For example, in aqueous surroundings, hydrophobic interactions are energetically more favorable than polar and charged interactions, and therefore binding of the inhibitor to the hydrophobic patch might hamper its dissociation.

4. Refinement of a substrate competitive inhibitor

The finding that the binding geometry of L803-mts with GSK-3β is directed by hydrophobic interactions led us to predict that increasing the peptide's hydrophobicity would enhance inhibition. Indeed, replacement of the polar amino acid glutamine (at position 9) in L803-mts with alanine or proline improved inhibition by 4 and 10 fold, respectively [64]. In an attempt to further understand the binding mode of L803, we mapped preferred binding sites of amino acid side chains on the surface of GSK-3, using the ANCHORSmap procedure [65]. The low free energy anchoring spots in the substrate binding region are shown in Fig. 1A. This analysis indicated that the positive cavity strongly prefers a negative anchor (Glu), whereas the cavity near loop 89–95 is promiscuous and binds a variety of amino acids (Arg, Lys, His, Gln, Leu, Met, Phe, Trp, and Tyr). The computational study also suggested that GSK-3β residue Phe 93 would provide an interaction site for an additional residue of the inhibitor (Fig. 1A, C). Experimentally, addition of a phenylalanine residue to the C-terminus of L803-mts (L803F-mts) improved inhibition two fold [64].

5. Conclusions

Substrate competitive inhibitors of protein kinases hold tremendous promise as therapies for various diseases. These inhibitors provide numerous advantages over the 'traditional' ATP competitive inhibitors, mainly in selectivity, but also in their moderate levels of

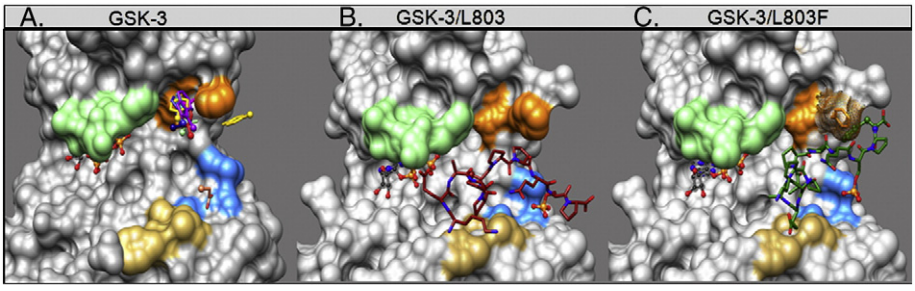

Fig. 1. Structural features of the substrate binding site of GSK-3 and the binding modes of the inhibitor L803 and the 'refined' inhibitor L803F. The surface of GSK-3 is shown in light gray with the P-loop highlighted in green, the substrate binding residues Q89, N95, and F93 (protruding) in orange, the positive binding cavity in blue, and the hydrophobic patch in beige. (A) Anchoring spots in the substrate binding region of GSK-3. The positive cavity binds a negative anchor (Glu); the cavity delimited by Q89, N95, and F93 and the P-loop binds a variety of amino acids (see text). Note the additional Phe binding site (yellow) near GSK-3 Phe 93. (B) Binding of L803, which interacts with the positive cavity, Phe 93, and the hydrophobic patch. (C) Binding of L803F. The surface of GSK-3 Phe 93 was made transparent to show the location of the C-terminal Phe of L803F.

inhibition. These properties appear to be important as we seek to treat diseases resulting from up-regulation of GSK-3 activity. Mild inhibition of GSK-3 is favored because this decreases the exacerbated GSK-3 function in the tissue affected with minimum deleterious effects on healthy tissues. We have learned that design of substrate competitive inhibitors cannot rely entirely on analyses of enzyme/substrate interactions. It appears that the binding geometries of substrates and substrate-competitive inhibitors differ and therefore the binding mode of the competitive inhibitor should be analyzed in order to design more potent inhibitors. Combined experimental and computational strategies have been used to design effective GSK-3 inhibitors that mimic substrate binding but employ different binding modes.

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