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Review

Substrate Competitive GSK-3 Inhibitors – strategy and Implications

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ABSTRACT

Glycogen synthase kinase-3 (GSK-3) is a highly conserved protein serine/threonine kinase ubiquitously distributed in eukaryotes as a constitutively active enzyme. Abnormally high GSK-3 activity has been implicated in several pathological disorders, including diabetes and neuron degenerative and affective disorders. This led to the hypothesis that inhibition of GSK-3 may have therapeutic benefit. Most GSK-3 inhibitors developed so far compete with ATP and often show limited specificity. Our goal is to develop inhibitors that compete with GSK-3 substrates, as this type of inhibitor is more specific and may be useful for clinical applications. We have employed computational, biochemical, and molecular analyses to gain in-depth understanding of GSK-3's substrate recognition. Here we argue that GSK-3 is a promising drug discovery target and describe the strategy and practice for developing specific substrate-competitive inhibitors of GSK-3.

1. Introduction

Aberrant protein kinase signaling has been implicated in many human diseases. Protein kinases have thus become important targets for design and development of therapeutic agents. Identification and development of compounds that manipulate cellular activity of various kinases is a major focus of pharmaceutical research. Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase expressed as two similar isoforms, α and β, that are abundant in most tissues [1]. GSK-3 was initially implicated in insulin signaling and metabolic regulation, but later studies identified additional roles in embryogenesis, mitotic regulation, inflammation, and neuroplasticity [2–5]. Unlike many other protein kinases, which are typically activated by extracellular signaling, GSK-3 is constitutively active in resting conditions and undergoes rapid inhibition by variety of stimuli. This is achieved by phosphorylation of a serine residue located at the N-terminus of the enzyme that is catalyzed by various upstream protein kinases mainly from the AGC family [2,6,7]. GSK-3 plays a central part in many cellular functions, contributing to the regulation of apoptosis, cell cycle, cell polarity, and gene expression [2,4,8–10]. Phosphorylation of GSK-3 downstream targets typically results in attenuation of the signaling pathway and/or inhibition of the substrate's activity. For example, GSK-3 phosphorylates and inhibits glycogen synthase [11,12] and it inhibits insulin signaling via the phosphorylation of the insulin receptor substrate proteins IRS-1 and IRS-2 [13,14]. GSK-3 is also a key suppressor of the canonical Wnt signaling pathway that controls cell fate determination and cell renewal [15,16,17]. Phosphorylation of β-catenin, a key target in Wnt pathways, by GSK-3, enhances its proteosomal degradation and suppresses its transcriptional activity [18,19]. Phosphorylation of additional transcription factors such as cAMP response element binding protein (CREB), nuclear factor of activated T cells (NFATc), heat shock factor-1 (HSF-1), and nuclear factor κB (NFκB) by GSK-3 inhibits their transcriptional activity [20–23]. It is not surprising that abnormally elevated activity of GSK-3 is found in several pathological disorders. The mechanisms by which GSK-3 contributes to human disease are summarized in Fig. 1.

The involvement of GSK-3 in 'unrelated' fields appears extraordinary. However, accumulated data now indicate that metabolic dysregulation contributes to the pathophysiology of neurological and psychiatric diseases. For example, unbalanced nutrient intake or dysregulation in insulin signaling appears to influence the risk of clinical dementia and Alzheimer's disease [24,25]. It is also noteworthy that the contribution of GSK-3 to inflammatory processes may underlie mechanisms coupling GSK-3 with diabetes, obesity, neurodegenerative disorders, and cancer [4]. GSK-3 thus became a prime target in drug discovery and development of GSK-3 inhibitors is of major interest in pharmaceutical research.

Two different genes encode GSK-3 isoforms α and β that have very similar catalytic domains but significantly differ in their N- and C-termini. The α and β isoforms split from a common precursor approximately at the emergence of vertebrates, suggesting that at least one of the isoforms took on a new function tied to the emergent vertebrate system (SP, unpublished data). Recent studies have addressed the possible differences between the two GSK-3 isoforms

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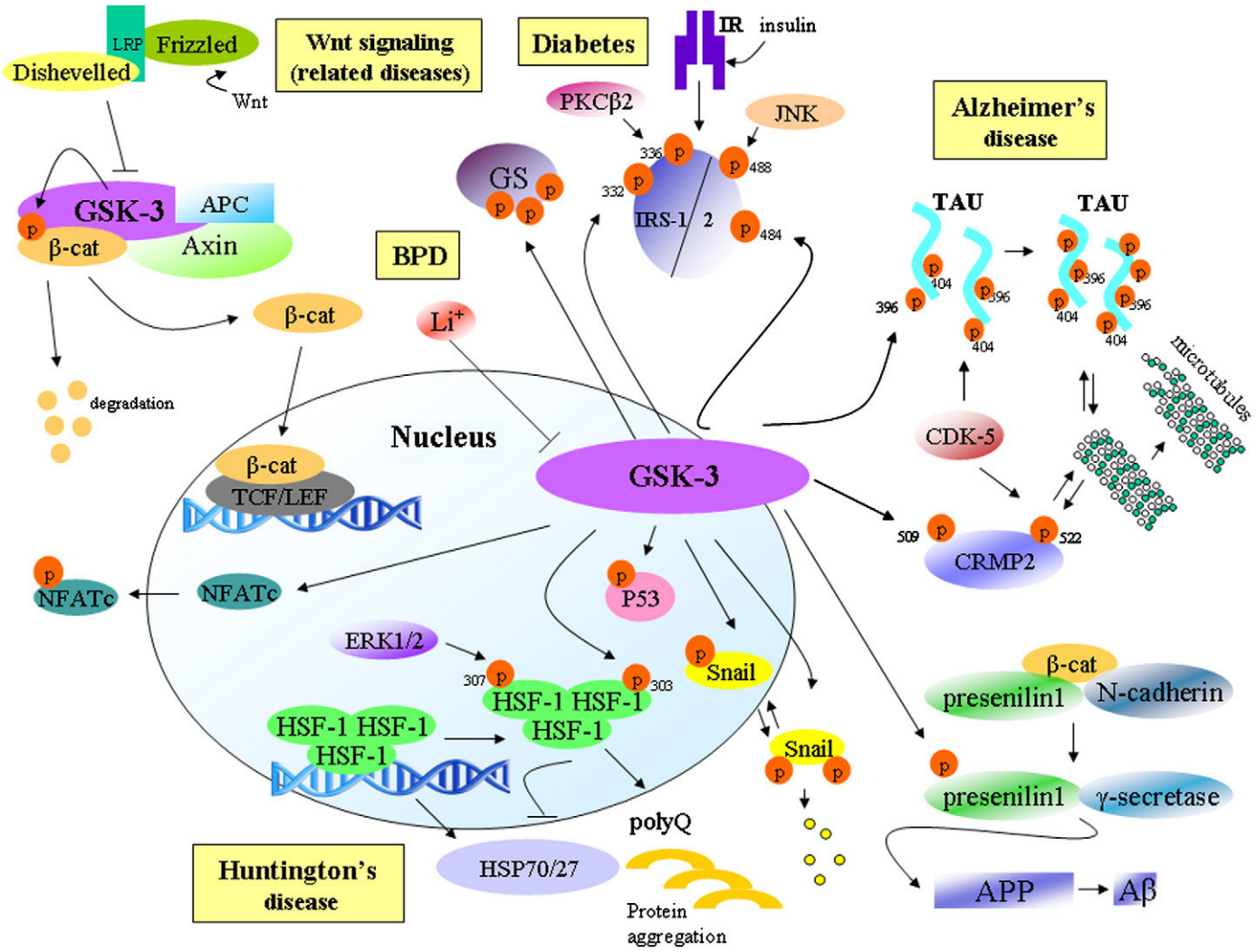

Fig. 1. GSK-3 in human disease. Illustrated clockwise from the top are signaling paradigms in which GSK-3 contributes to pathological disorders. Diabetes: GSK-3 phosphorylates and inhibits two important targets of insulin signaling, glycogen synthase (GS) and IRS proteins (IRS-1 and IRS-2); the priming kinases are CK-2, PKCβII and JNK, respectively. This, in turn, inhibits glycogen synthesis and produces negative feedback regulation of insulin signaling contributing to insulin resistance and type 2 diabetes. Alzheimer's disease: GSK-3 may contribute to neuropathology of Alzheimer's disease through several routes. GSK-3 phosphorylates the microtubule-associated protein, tau, (priming kinase CDK-5). Hyperphosphorylated tau has reduced affinity to microtubules; this, in turn, enhances microtubule instability and tau-protein aggregation that may eventually proceed to neurofibrillary tangle formation. GSK-3 also phosphorylates collapsin response mediating protein-2 (CRMP-2, priming kinase CDK-5), affecting microtubule stability and synaptic plasticity. GSK-3-mediated phosphorylation of presenilin-1 reduces its complex formation with N-cadherin and β-catenin. This in turn enhances γ-secretase-mediated amyloid precursor protein (APP) cleavage and Aβ peptide generation. Huntington's disease: GSK-3 phosphorylation of HSF-1 (presented as a trimer in its active form) by GSK-3 (priming kinase ERK1/2) inhibits its transcriptional activity and reduces the cellular amount of the heat-inducible chaperones HSP70/HSP27. This in turn increases ployQ protein aggregation typical of the neuropathology of Huntington's disease. Wnt signaling: Wnt signaling has been linked with cancer, osteoporosis and neurodegenerative disorders. GSK-3 is inhibited by Wnt. GSK-3 is associated with a multiprotein complex that includes axin adenomatosis polyposis coli (APC) and β-catenin. Phosphorylation of β-catenin by GSK-3 targets it for proteosomal degradation. Wnt activates the receptor Frizzled and coreceptor LRP5/6 and inhibits GSK-3 through disheveled and Frat 1. Unphosphorylated β-catenin accumulates and translocates to the nucleus, where it transactivates genes regulated by TCF/LEF transcription factors. Bipolar disorder (BDP): The mood stabilizer lithium, frequently used in bipolar disorder therapy, is an effective GSK-3 inhibitor. This implicates GSK-3 activity in the underlying causes of this disease. Also shown are the effects of GSK-3 on several transcription factors. GSK-3 enhances nuclear export of NFATc, mediates the activity of the tumor suppressor gene p53, and controls snail degradation as well as subcellular localization.

[26–30], yet their precise roles in different tissues are not fully understood. At present it seems that most GSK-3 inhibitors do not discriminate between the two isoforms. Development of isoforms-specific inhibitors is desired but will be a challenging task.

2. GSK-3 substrate competitive peptide inhibitor L803-mts

Most of the protein kinase inhibitors developed so far are ATP competitors. This type of molecule competes for the ATP binding site of the kinase and often shows off-target effects due to serious limitations in their specificity. The low specificity of these inhibitors is due to the fact that the ATP binding site is highly conserved among diverse protein kinases. Non-ATP competitive inhibitors, on the other hand, such as substrate competitive inhibitors, are expected to be more specific as the substrate binding sites have a certain degree of variability among the various protein kinases. Substrate competitive inhibitors usually make a weak binding interaction with the enzyme (as does the substrate); this may be considered a disadvantage. It may also explain why this type of inhibitors has been overlooked in high-throughput screening efforts to select protein kinase inhibitors. Yet, chemical modifications can improve the specific binding affinity and the *in vivo* efficacy of substrate competitive inhibitors [31].

Another problem concerns the *in vitro* assays used to test and evaluate given protein kinase inhibitor candidates. As the substrate's concentration in these assays is far above that of its physiological concentration, *in vitro* kinase assays do not reflect the true potency of

substrate competitive inhibitors. Thus, in many cases, substrate competitive inhibitors will show better efficacy in cells than in cell-free conditions and we recommend that searches for this type of inhibitor be undertaken in cellular conditions. Overall, despite the difficulties in discovery and development, the search for substrate competitive inhibitors is worthwhile.

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 causes damage. This is demonstrated by the fact that GSK-3-knockout mice die late in gestation [32]. 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.

Understanding the mode of interaction of GSK-3 with its substrates is a central theme in the design and development of substrate competitive inhibitors. It has been known for many years that GSK-3 substrate recognition is unique as, unlike many other protein kinases, it requires prephosphorylation [11,12]. GSK-3 substrates have recognition motifs of $S^1XXXS^2(p)$, where S^1 is the site phosphorylated by GSK-3 and $S^2(p)$ is the 'priming site' prephosphorylated by a different kinase [11,12]. Apparently, the GSK-3 requirement for prephosphorylation is very strict. Lack of prephosphorylation and/or replacement of $S^2(p)$ with a phosphor-tyrosine residue or glutamic acid diminishes substrate phospho-tyrosine by GSK-3 [33]. The three-dimensional structure of GSK-3β in crystals provided new insights into this phenomenon. The catalytic domain possesses a positive binding pocket (Arg 96, Arg 180, and Lys 205) that interacts with negatively charged groups such as phosphate and sulfate ions [34,35]. Mutation of Arg 96, Arg 180, and Lys 205 prevented GSK-3 phosphorylation of its substrates [34,36].

The absolute requirement for substrate prephosphorylation raised the possibility that short phosphorylated peptides might serve as selective substrate competitive inhibitors. A set of phosphorylated peptides patterned after known GSK-3 substrates was generated and shown to inhibit GSK-3 *in vitro* in the micromolar range (100–300 μM) [37]. Peptide L803 was found to be the best inhibitory sequence. L803 is derived from heat shock factor-1 (HSF-1). L803 has the sequence KEAPPAPPQS(p)P) and contains two modified residues; the GSK-3 phosphorylation site and the glutamic acid upstream to the P1 site were both replaced by alanine [37]. In an advanced version of L803, a myristic acid was attached to the N-terminus of the peptide, now termed L803-mts, to enhance cell permeability [37]. L803-mts was very specific for GSK-3 and did not inhibit a wide repertoire of protein kinases [37] (HE-F, unpublished results). It was stable in serum and showed low toxicity as determined by histopathology analysis and single-dose, maximal-tolerated dose (MTD) tests (HE-F, unpublished results). The ability of L803-mts to reduce phosphorylation of physiological targets of GSK-3 such as glycogen synthase, IRS-1, and β-catenin further provided a proof of concept [37] (HE-F, unpublished results).

The *in vivo* impact of L803-mts has been tested in diabetes and CNS models. Insulin resistant and obese ob/ob mice were injected intraperitoneally (i.p.) daily with L803-mts for 3 weeks. L803-mts reduced plasma glucose levels and improved glucose tolerance; it did not change body weight or food consumption compared to controls [38]. Detailed investigation of the insulin-sensitive tissues indicated that L803-mts suppresses hepatic gluconeogenesis, increases glucose transporter-4 (GLUT4) in the skeletal muscle and increases glycogen content in both tissue [38]. Similar results were reported in a different model of high-fat diet-induced diabetes [39]. Together, the studies suggested that inhibition of GSK-3 by L803-mts reduces hyperglycemia and reverses the diabetic phenotype.

GSK-3 was implicated in various aspects of the nervous system, including cell survival, cell polarity, and synaptic plasticity [9,40–42]. The finding that the mood stabilizer lithium is an effective GSK-3 inhibitor further implicated GSK-3 in cognition and behavior [43]. We evaluated the impact of L803-mts on depressive behavior using the forced swim test (FST), a common protocol used to assess activity of antidepressants [44]. In this test, the animal is subjected to a swimming trial and the immobility time, namely the time it spends without moving, is measured. The immobility time reflects the depressive degree of the animal [44]. Administration of L803-mts significantly reduced the mouse-immobility time in the FST; in addition, L803-mts increased β-catenin levels in the mouse hippocampus [45]. These results indicated that inhibition of GSK-3 can promote antidepressive like activity; they further provided proof of concept for the *in vivo* inhibitory capacity of L803-mts in the brain. The important role of GSK-3 in behavior was also demonstrated using heterozygote $GSK3\beta^{+/-}$ mice and using wild-type mice treated with the GSK-3 inhibitor AR-A014418 [46,47]. In another model system of traumatic brain injury (TBI), mice developed depressive behavior after the insult and this was abolished by pretreatment with L803-mts [48]. L803-mts has also been tested in models of Parkinson's disease, axon growth, and schizophrenia with positive results [49–51].

3. GSK-3-substrate recognition – computational and molecular analyses

Design of substrate competitive inhibitors is mainly based on our understanding of the kinase/substrate binding mode. The prerequisite of pre-phosphorylation of GSK-3′s substrates suggests that phosphorylation is an important element in substrate recognition. Still, it is likely that the GSK-3 catalytic core makes specific interactions with other regions of the substrate. The availability of the 3D structures of GSK-3β and a phosphorylated peptide derived from its substrate CREB (pCREB) enabled computational attack of this problem. Protein–protein docking analysis of pCREB with GSK-3β identified new docking sites, Phe 67, Gln 89, and Asn 95, which are spatially close to the active site and which interact with the substrate's core (i.e., $S^1XXXS^2(p)$). Specifically, Arg 135 of pCREB forms hydrogen bonds with Gln 89 and Asn95 of GSK-3β and Tyr 134 of pCREB interacts with Phe 67 of GSK-3β [33]. In addition, and as expected, the priming site, Ser 133, interacted with the phosphate binding pocket of GSK-3β (i.e., Arg 96, Arg 180, Lys 205). Mutations of Gln 89, Asn 95, or Phe 67 reduced the ability of GSK-3β to phosphorylate its substrates [33]. Mutation of both Gln 89 and Asn 95 severely impaired GSK-3 phosphorylation of peptide substrates derived from CREB, IRS-1, and glycogen synthase (Fig. 2A). The polar natures of Gln 89 and Asn 95 allow interaction with a wide variety of polar/charged groups. This may explain the broad specificity of GSK-3. Indeed, we could identify a polar or charged amino acid located at position +2 downstream to the phosphorylated priming site ($S^2(p)$) in a number of substrates (Fig. 2B). We suggest that like CREB/Arg 135, these residues interact with Gln 89 and Asn 95. The preferential conservation of Gln 89 and Asn 95 across the GSK-3 family [33] also implies a special role for these residues in substrate recognition. Phe 67, on the other hand, is located in the conserved P-loop, and is more generally conserved across protein kinases. Mutation of Phe 67, however, did not impair the catalytic activity of GSK-3 *per se* [33], suggesting that this residue is indeed important for substrate recognition.

GSK-3 is inhibited by serine phosphorylation at a residue its N-terminus (Ser 9 in GSK-3β and Ser 21 in GSK-3α) [52–54]. This raised the possibility that the N-terminal tail may function as a pseudo-substrate by mimicking the pre-phosphorylated substrate. The interaction mode of the pseudo-substrate with the catalytic core may thus represent another model for substrate recognition. Bioinformatic analysis of pseudo-substrate sequences in GSK-3β and α indicated that this region is conserved across the GSK-3 proteins from complex metazoans and simple creatures such as choanoflagellates and sponges [55]. Strikingly, Arg 4 and Arg 6 were absolutely

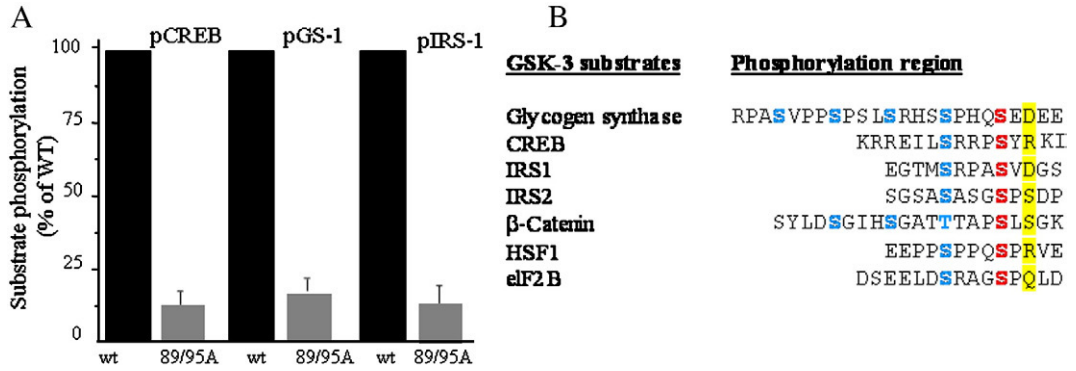

Fig. 2. Gln 89 and Asn 95 are important for substrate recognition. (A) Wild-type (WT) GSK-3 and the double-mutant Q89/N95A, in which Gln 89 and Asn 95 were both mutated to alanine (Q89/N95A), were subjected to *in vitro* kinase assays using different peptide substrates: pCREB (ILSRRPS(p)YR), pGS-1, (YRRAAVPPSPSLSRHSSPSQS(p)EDEEE) and pIRS-1 (RREGGMSRPAS(p)VDG), where S(p) is a phospho-serine. Reactions were spotted on p81 paper and counted for radioactivity. Phosphorylation of substrates is presented as the percentage of WT-GSK-3 activity (set to 100%). Results are the mean of two or three independent experiments ± SEM. *$p<0.01$ for Q89/N95A mutant vs. WT. (B) Recognition motifs of GSK-3-substrates. Priming site is marked by red; GSK-3 phosphorylation site is marked by blue, polar residues located +2 to the priming site are marked yellow. These residues likely interact with Gln89/Asn95.

conserved in all GSK-3 pseudo-substrate regions identified [55]. Mutations of Arg 4 or Arg 6 to alanine enhanced 'basal' GSK-3β kinase activity and impaired its ability to autophosphorylate at Ser 9. In addition, and unlike WT-GSK-3β, these mutations prevented auto-inhibition even in the presence of phosphorylated Ser 9. This indicates that Arg 4 and Arg 6 facilitate the interaction of the pseudo-substrate with the catalytic core [55]. It also appears that Gln 89 and Asn 95 are involved in the pseudo-substrate interaction, since mutations at Gln 89 and Asn 95 prevented autoinhibition [55]. Moreover, computational docking identified specific interactions between Arg 6 and Gln 89/Asn 95. Arg 4 on the other hand, interacts with Asp 181 (Fig. 3), another highly conserved residue among all protein kinases that functions as a proton acceptor during catalysis [56]. Hence, the interaction of the pseudo-substrate with Gln 89 and Asn 95 competes with substrate binding, while its interaction with Asp 181 prevents phosphorylation catalysis. This model further supports a central role for Gln 89 and Asn 95 in GSK-3 substrate recognition.

4. Other GSK-3 inhibitors

The wide interest in GSK-3 as a prime therapeutic target accelerated the search and progression in developing pharmacological inhibitors. The reported GSK-3 inhibitors are of diverse chemotypes and mechanism of action. Most of them, however, are ATP-competitive inhibitors. The maleimides derivatives, SB-216763 and SB-41528, developed by GlaxoSmithKline were shown to be potent GSK-3α/β inhibitors *in vitro* and in cellular systems [57]. A different class of inhibitors developed by Chiron is the aminopyrimidines in which the most potent inhibitors CHIR 98023, CHIR99021 were shown to produce antidiabetic effects in animal models [58,59]. The amino thiazole AR-A014418 is another ATP competitive inhibitor developed by AstraZeneca [60]. AR-A014418 was implicated in neuron cell survival [60] and was shown to produce antidepressive like activity in FST [46]. Other GSK-3 inhibitors of different structures such as pyrroloazepine, benzazepione, bis-indole and pyrazolopyridine were reported [61–64]. Small heterocyclic molecules thiadiazolidinones (TDZD) inhibitors belong to a different class of non-ATP competitive inhibitors [65]. The compounds TDZD-8 and NP00111 developed by Norscia are potent selective GSK-3 inhibitors [65,66], they show good oral bioavailability and blood–brain barrier penetration [9]. A special interest was focused on their potential therapeutic use in the treatment of Alzheimer's disease [9,67]. The specificity of all these inhibitors is always a concern, yet, the lead compounds reported showed high selectivity toward GSK-3 when tested against number of other protein kinases [60,68]. Detailed description of GSK-3 inhibitors is also described elsewhere [9,69,70].

5. Conclusions

Development of selective GSK-3 inhibitors is a focus of many drug discovery programs. Low molecular weight compounds that bind GSK-3 with high affinity are usually ATP competitive inhibitors. In many cases, such compounds fail in the preclinical or clinical stages due to limited specificity and unfavorable off-target effects. Substrate competitive inhibitors, on the other hand, are more specific. Yet, these inhibitors perform moderately in *in vitro* inhibition assays and their discovery and design are challenging. The moderate activity of substrate competitive inhibitors of GSK-3 may be of advantage, as GSK-3 is essential for life and its 'pathological' activity is only 2- to 3-fold above its normal value. Computational, molecular, and biochemical analyses have contributed to our understanding of the geometry and key sequence features of binding of substrate to GSK-3. This in

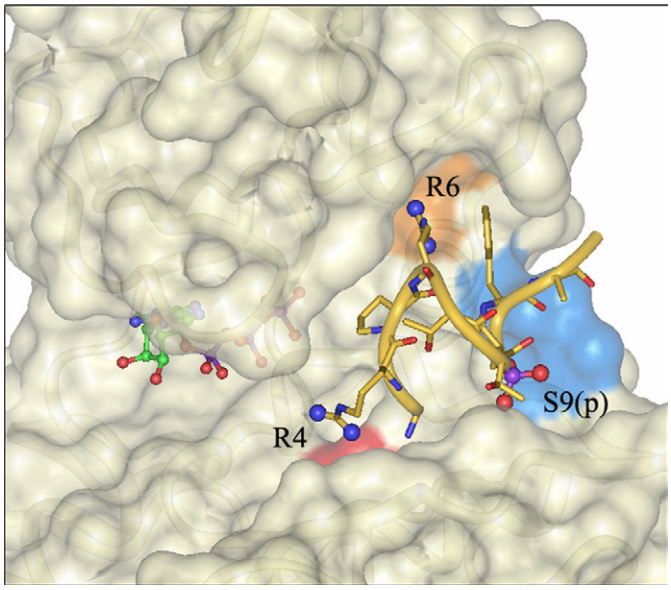

Fig. 3. A docking model of the pseudo-substrate peptide interactions with GSK-3β. GSK-3β is depicted by the gray surface ribbon. The position of the primed phosphate binding site is indicated in blue, the positions of Q89 and N95 are shown in orange and the position of D181 in red. The N-terminal region of the pseudo-substrate peptide (3-GRPRTTSpFA-11) is shown as a golden ribbon; in the several minimizations. The side chains of interacting residues are shown with the oxygen atoms colored in red, nitrogen atoms in blue, and phosphorous in purple. The binding position of ATP is indicated although this molecule was not included in the energy minimization.

turn has provided new templates for drug design of specific substrate competitive inhibitors.

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