Inhibitors of glycogen synthase kinase-3: future therapy for unmet medical needs?

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Glycogen synthase kinase-3 (GSK-3) has recently emerged, in the field of medicinal chemistry, as one of the most attractive therapeutic targets for the development of selective inhibitors as promising new drugs for numerous serious pathologies, including Alzheimer’s disease, stroke, bipolar disorders, chronic inflammatory processes, cancer and Type II diabetes. The full potential of GSK-3 inhibitors is yet to be realised and the number of drug candidates being developed by both academic centres and pharmaceutical companies has increased exponentially in the last three years. This review discloses recent discoveries on peptides and small molecules targeting GSK-3. Antisense therapy for the modulation of GSK-3 expression is also discussed. Focusing attention on this exciting target could thus reap considerable clinical and economic rewards.

Keywords: Alzheimer’s disease, cancer, glycogen synthase kinase-3 (GSK-3) inhibitors, inflammation, Type II diabetes


1. Introduction

Glycogen synthase kinase-3 (GSK-3) was cloned in 1990 and is a key regulator of glycogen synthase, one of the principal modulators of glycogen metabolism and hence glucose levels. GSK-3, a serine/threonine kinase for which two isoforms, GSK-3α and GSK-3β, have been identified, phosphorylates and thereby regulates the functions of many metabolic, signalling and structural proteins [1,201]. These two isoforms share 97% sequence similarity within their kinase catalytic domains but differ significantly from one another outside this region, with GSK-3-α possessing an extended N-terminal glycine-rich tail. The two isoforms are encoded by two different mRNAs that are variably expressed in different tissues [2]. Whilst GSK-3-α is found in the lung, ovary, kidney and testis, GSK-3-β is highly expressed in the lung, kidney and brain. Recently, another isoform of GSK-3-β, with a 13 amino acid insert in the catalytic domain, was discovered [3]. The alternative transcripts were found in the brains of mice, rats and humans, with highly conserved sequences.

Notable among the signalling proteins regulated by GSK-3 are many transcription factors, including activator protein-1, cyclic AMP response element binding protein (CREB), the nuclear factor (NF) of activated T cells, heat shock factor-1, β-catenin and NFκ−β [4]. The use of transgenic mice has, however, suggested a much wider range for this new therapeutic target. Over expression of GSK-3-β in the brain of adult mice was found to produce neurodegeneration exhibiting many of the characteristics of Alzheimer’s disease, including tau hyperphosphorylation [5]. Indeed, GSK-3 has been linked to all the primary abnormalities associated with Alzheimer’s disease [6]. In addition, inhibition of GSK-3 was shown to attenuate apoptotic signals, implicating its importance in more acute conditions. Consequently, GSK-3 inhibitors are in development for Alzheimer’s disease [7] and protection against cell death. Finally, the effects of two mood-stabilising drugs in common
use, lithium and valproic acid (Depakene™, Abbott Laboratories) appear to be mediated, at least in part, through the inhibition of GSK-3 and hence specific inhibitors of this enzyme may represent improved treatments for this bipolar disorder [8].

Recently, GSK-3 has emerged, in the medicinal chemistry research field, as one of the most attractive therapeutic targets for the development of selective inhibitors as new promising drugs for numerous serious pathologies including Type II diabetes, Alzheimer’s disease, stroke, manic depression, chronic inflammatory processes and cancer [9]. The potential of GSK-3 inhibitors is currently being evaluated and the number of therapeutic candidates in development is still limited [10]. Focusing attention on this exciting target could ultimately reap considerable clinical and economic rewards.

This article reviews the patent literature reported in the last four years, focusing on the discovery of new GSK-3 inhibitors and their primary screening methods.

2. Peptidic glycogen synthase kinase-3 inhibitors

Targeting GSK-3 has therapeutic potential for many different pathologies, and the search for GSK-3 inhibitors is a very active field of research for both academic centres and pharmaceutical companies.

The University of Dundee claimed one of the first screening methods developed for discovering agents capable of affecting the activity of kinases GSK-3 and protein kinase B (PKB) [101]. Polypeptides that are useful in modulating the activity of GSK-3, as well as methods for identifying compounds that are capable of inhibiting the activity of GSK-3 towards phosphate-dependent substrates to a greater extent than towards non-phosphate-dependent substrates, are also claimed [102].

Some peptides have recently been reported as GSK-3 inhibitors. These include GBP, a maternal XGSK-3-binding protein that is homologous to a T cell proto-oncogene [11], and p24, a heat resistant GSK-3 binding protein [12].

Ramot University has claimed small, synthetic peptide inhibitors such as compound 1. The templates used as models in the synthetic strategy were two known substrates of GSK-3, CREB and heat shock factor-1 (HSF-1) [103]. In all cases, a phosphorylated serine is included in the sequence for the specific substrate GSK-3 recognition. Inhibition of GSK-3 by synthetic peptide inhibitors was determined using in vitro kinase assays with a peptide substrate and determination of 32P incorporation into the substrate. Lineweaver-Burk plots revealed that all the synthetic peptides assayed were ATP-competitive inhibitors with Kᵢ values ranging from 30 to 80 µM.

3. Small molecules targeting glycogen synthase kinase-3

3.1 Purine and pyrimidine derivatives

Purine derivatives, developed by Chiron, were probably the first synthetic molecules specifically reported as GSK-3 inhibitors [104]. The most active compound tested (compound 2) showed 63% inhibition of GSK-3 activity at 1 µM. Recently, Chiron has focused its efforts on pirazine and pyrimidine derivatives such as compounds 3 – 5, which have shown an IC₅₀ value of 1 mM in a cell-free assay for GSK-3 inhibitory activity [105-107]. Transient transfection of COS cells with a GSK-3 expression plasmid and tau expression are used in the screening method in which an enzyme-linked immuno-sorbent assay (ELISA) reader is used for the detection of phosphorylated tau in cell lysates. Recently, Chiron claimed truncated GSK-3 polypeptides capable of crystallisation [108] as well as a 3D structure of a human GSK-3 construct [109] for use in the identification and optimisation of GSK-3 inhibitors. It is
Figure 2. Purine, pyrimidine and pyrimidone derivatives as glycogen synthase kinase-3 inhibitors.
worth mentioning that crystalline 3D structures of GSK-3 have been reported by three independent groups (Protein Data Bank (PDB) entries: 1H8F and 1109) [13-15].

Compounds 3–5 are being developed as possible drugs for the treatment of diabetes [110]. Under normal conditions, insulin is able to inhibit GSK-3 and so during insulin resistant states GSK-3 activity is increased. Enhanced GSK-3 activity has been reported in both adipose and skeletal muscle tissue taken from diabetic subjects and this is generally accepted to play a major role in the hyperglycaemic effects of insulin resistance. Thus, the development of GSK-3 inhibitors has received attention in the attempt to control the serious and growing clinical problem of diabetes. It is therefore important to note that derivative 3, when administered at a dose of 30 mg/kg to obese mice, shows high bioavailability and tissue penetration and exhibits a reduction in the areas under the blood glucose curve comparable to troglitazone (Rezulin™, Warner-Lambert Company) [111]. Additionally, the antidiabetic efficacy of compounds 3–5 were assessed in a glucose tolerance assay in mice.

Sanofi-Synthelabo, with Mitsubishi-Tokyo Pharmaceuticals as the second applicant, claimed three compound families with GSK-3β inhibitory properties: aryl and heteroaryl alkylamino pyrimidones (6–10) [112-116], nitrogen-heterocycle pyrimidones (11–13) [117-119] and fused heterocyclic pyrimidones (14–6) [120-122]. The chemical structures of some of the compounds in these families are depicted in Figure 2. The ability of the compounds to inhibit GSK-3β activity is determined in vitro following two protocols using different concentrations of prephosphorylated glycogen synthase peptide (GS-1), and [32P]-ATP. The enzyme, peptide, [32P]-ATP and test compounds are incubated together. Incorporation of [32P] radioactivity is determined by liquid scintillation spectrometry, and the IC50 values of the compounds are in the range 2 nM – 10 μM.

3.2 Azoles and related compounds

During the last two years, many amine pyrazole derivatives, including the enzyme GSK-3β, have been developed as protein kinase (PK) inhibitors by Vertex Pharmaceuticals. Amine pyrazole derivatives with benzopyridine (17) [123], benzopyrimidine (18) [124] and pyridopyrimidine (19) substituents [125], have the ability to inhibit GSK-3, Aurora-2, extracellular signal related kinase-2 (ERK-2) and Src kinases in vitro with K_i values of <0.1, <0.1, 1.0 mM and <100 nM, respectively. Aurora-2 is a serine/threonine protein kinase implicated in the cell cycle. ERK-2 phosphorylates many regulatory proteins and transcription factors and is involved in cancer and other pathologies. Scr kinase is a mitogen activated protein kinase (MAPK) kinase implicated in hypercalcaemia and osteoporosis.

Substitution of the pyrazole heterocycle by a triazol moiety results in the same inhibition data (compound 20) [126]. Triazine (21) [127], pyrimidine (22) [128] and pyridazine (23) derivatives [129], have also been assessed versus cyclin dependent kinase-2 (CDK-2) and AKT (protein kinase B) but no resulting inhibition data are presented.

Vertex has also developed 5-pyrazol or 5-pyrrol pyrazoles that inhibit JNK (c-Jun N-terminal kinase), Aurora-2, GSK-3, kinase domain receptor (KDR), AKT and related PKs [130,131]. These compounds are stated to be particularly useful for inhibiting ERK. The ERK2 inhibitory activity of 177 compounds was measured. The specified compound (24) has a K_i value of < 1 μM versus ERK2, a K_i value of < 1 mM in a JNK inhibition assay and an IC50 value of <5 μM in an Aurora-2 inhibition assay.

Pfizer has developed various pyrazoles as PK inhibitors [132,133]. They are implicated in the treatment of abnormal cell growth and neurodegenerative disorders, by altering dopamine-mediated neurotransmission. The compounds (25 and 26) are claimed to have demonstrated IC50 values of <50 μM when assayed for CDK5 and GSK-3β inhibition.

Pyrazol amide compounds with in vitro inhibitory effects against GSK-3β are described by Welfide [134]. The specified compound (27) was the most potent inhibitor tested, showing an IC50 value of 0.12 μmol/l out of a range extending to 9.5 μmol/l.

Pyrazolopyridines and pyrazolopyridazines are claimed by SmithKline Beecham as antidiabetic agents (compound 28) [135]. The compounds are stated to be particularly potent and selective inhibitors of the protein kinase GSK-3. The activity of the compounds against GSK-3 was tested in vitro by examining the ability of the enzyme to phosphorylate a biotinylated peptide GS-1. The peptide was then captured onto Streptavidin-coated Scintillation Proximity assay (SPA) beads, the mixture was centrifuged and the activity determined using scintillation counter. It is stated that the most potent compounds gave IC50 values in the range 1–500 nM, but no specific biological data are presented.

Novo Nordisk evaluate the insulin releasing effect of 2,4-diaminothiazole derivatives, which inhibit GSK-3, using pancreatic islets and single B cells [136]. Compound 29 shows an IC50 value < 5 μM for the inhibition of GSK-3. Furazanyl-triazole derivatives are stated as GSK-3 inhibitors [137] with IC50 values of <10 μM for the specified compound (30).

3.3 Maleimide derivatives

One of the most commonly used families of GSK-3 inhibitors are those with structures containing a maleimide core. Recent studies have demonstrated that the bisindolylmaleimide derivatives of staurosporine, GF 109203x (compound 31) and Ro 31-8220 (compound 32), widely used as specific inhibitors of protein kinase C (PKC), are potent inhibitors of GSK-3, showing IC50 values for this enzyme in the nanomolar range [138]. The proposed mechanism of action is reversibly competitive with ATP at the nucleotide-binding site of GSK-3β, such as has been suggested for PKC.

Based on high-throughput screening techniques, SmithKline Beecham identified a number of maleimide derivatives as potent inhibitors against rabbit GSK-3α, focusing their efforts on the structures related to anilinomaleimide derivatives, such as the
Figure 3. Pyrazoles and other azole derivates as GSK-3 inhibitors.

Structures 17–19 and 21–23: GSK-3 \( K_i < 0.1 \) µM; Aurora2 \( K_i < 0.1 \) µM; ERK2 \( K_i < 1.0 \) µM; Src \( K_i < 100 \) nM

Structure 20: GSK-3 \( K_i < 0.1 \) µM; Aurora2 \( K_i = 0.1 \) µM; ERK2 \( K_i < 1.0 \) µM; Src \( K_i < 100 \) nM

CDK: Cyclin dependent kinase; ERK: Extracellular signal-regulated kinase; GSK: Glycogen synthase kinase.
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The synthetic route is carried out using an automated synthetic approach in the final step of the process [17]. Enzymatic assays against recombinant human GSK-3α have identified the arylindolemaleimide family as potent GSK-3α inhibitors, showing equal potency for GSK-3β [135].

The GSK-3 inhibitory activity of the specified compound (34) was determined in two in vitro assays, which used a prephosphorylated 26-mer peptide and a biotinylated 27-mer peptide as phosphorylation substrates. The sequence of both peptides are derived from the phosphorylation site of glycogen synthase. The IC50 values against both rabbit and human GSK-3 are 3.3 and 8 nM, respectively. In common with most of the other protein kinase inhibitors, these compounds inhibited GSK-3α in an ATP competitive manner. However, measurement of selectivity, which was evaluated against a panel of >25 kinases, showed inhibition values for the majority of these kinases <50% at 10 μM. As previously mentioned, GSK-3 phosphorylates and modulates the activity of a number of key regulatory proteins in the cell. Pharmacological studies on the maleimide derivatives, SB-216763 (compound 35, IC50 = 34 nM) and SB-415286 (compound 36, IC50 = 78 nM), have shown that these compounds represent a valuable tool in elucidating the role of GSK-3β in cell signalling pathways. These compounds stimulate glycogen synthesis in human liver cells and induce expression of a β-catenine lymphoid enhanced factor/T-cell factor (LEF/TCF) regulated reporter gene in human embryonic kidney (HEK293) cells. Therefore, these compounds are capable of eliciting responses that have previously been attributed to the inhibition of GSK-3 activity [18]. Additionally, these compounds are able to repress transcription of the rate determining enzymes in the gluconeogenic pathway, showing their potential for the treatment of diabetes. It is worth mentioning that derivative 37 was developed specifically for the treatment of diabetes [136]. However, further research is still needed to determine whether the inhibition of other cellular processes, in which GSK-3 is implicated, will lead to unacceptable side effects. Together with the previous

![Figure 4. Maleimide core containing structures.](image-url)
and GSK-3 [21]. Hymenialdisine competes with ATP for binding to all these kinases. The activity of this compound in inhibiting protein kinases was determined in vitro in the presence of 15 µM ATP. The IC_{50} values are 22, 70, 700, 10 and 35 nM against CDK-1, CDK-2, CDK-5, GSK-3 and CK-1, respectively [138]. Hymenaldisine also blocks the in vivo phosphorylation of the microtubule-binding protein tau at sites that are hyperphosphorylated by GSK-3β and CDK5/p35 in Alzheimer's disease. Additionally, it is known that GSK-3 is required for nuclear functioning of NFκB and this implies that small molecule inhibitors of GSK-3 should be potent anti-inflammatory agents. The Ontario Cancer Institute stated that, by inhibiting GSK-3, the inflammatory immune responses mediated by NFκB are ameliorated [139]. It is worth mentioning at this point, that dibromohymenaldisine was initially isolated on the basis of its anti-inflammatory properties and was later shown to inhibit GSK-3, which indicates that these compounds may have potential application in the treatment of inflammatory processes [22].

Paulones constitute a new family of benzazepinones with promising antitumour activity. They have been described as potent, ATP-competitive, CDK regulating inhibitors of the cell cycle [23]. More recently, paulones have been proven to be potent CDK5/p25 and GSK-3β ATP-competitive inhibitors with IC_{50} values between 20 - 200 nM and 4 - 80 nM, respectively [140]. Compound 40 inhibits the phosphorylation of tau in vivo at sites which are typically phosphorylated by GSK-3 in Alzheimer's disease, and also inhibits the CDK5/p25-dependent phosphorylation of the DA- and cAMP-regulated phosphoprotein (Mr 45,000) (DARPP-32) in mouse striatum slices in vitro.

Indirubins are the main component of traditional Chinese medicines used to treat chronic diseases such as leukaemias, and have been demonstrated to be powerful inhibitors (IC_{50} = 5 - 50 nM) of GSK-3β, while other indigoids are inactive [24]. Testing of a series of indoles and bis-indoles against GSK-3β, CDK1/cyclin B and CDK5/p25 showed that only indirubins inhibit these kinases [141]. GSK-3β is one of the evolutionarily closest enzymes to the CDK family. It is confirmed that indirubins inhibit GSK-3β as they do CDKs, by competing with ATP for binding to the catalytic site. Compound 41 has an IC_{50} value of 100 nM, for both GSK-3 and CDK5 in in vitro assays. The GSK-3 enzyme used in these assays was purified from rabbit muscle or expressed on Sf 9 insect cells. CDK5/p25 was formulated using an equal mixture of CDK5 and p25 from mammalian recombinants, expressed on Escherichia coli.

**3.4 Miscellaneous heterocyclic compounds**

The marine sponge constituent hymenaldisine (compound 39) is a potent inhibitor of CDK's, casein kinase 1 (CK-1) and GSK-3 [21]. Hymenaldisine competes with ATP for binding to all these kinases. The activity of this compound in inhibiting protein kinases was determined in vitro in the presence of 15 µM ATP. The IC_{50} values are 22, 70, 700, 10 and 35 nM against CDK-1, CDK-2, CDK-5, GSK-3 and CK-1, respectively [138]. Hymenaldisine also blocks the in vivo phosphorylation of the microtubule-binding protein tau at sites that are hyperphosphorylated by GSK-3β and CDK5/p35 in Alzheimer's disease. Additionally, it is known that GSK-3 is required for nuclear functioning of NFκB and this implies that small molecule inhibitors of GSK-3 should be potent anti-inflammatory agents. The Ontario Cancer Institute stated that, by inhibiting GSK-3, the inflammatory immune responses mediated by NFκB are ameliorated [139]. It is worth mentioning at this point, that dibromohymenaldisine was initially isolated on the basis of its anti-inflammatory properties and was later shown to inhibit GSK-3, which indicates that these compounds may have potential application in the treatment of inflammatory processes [22].

**3.5 ATP-non-competitive inhibitors**

Small thiazoloinodinone (TDZD) derivatives are the first ATP-non-competitive GSK-3 inhibitors reported to date [25]. The kinase inhibitory activity of these compounds, developed as secondary products in the preparation of potassium channel openers [26], muscarinic agonists [27] or acetylcholinesterase inhibitors, further data have revealed the role of these compounds in the prevention of neuronal death [19]. Derivatives 35 and 36 have been shown to protect primary neurons from death induced by reduced phosphatidylinositol 3-kinase (PI-3) pathway activity. The inhibition of neuronal death correlated with inhibition of GSK-3 activity and modulation of GSK-3 substrates and β-catenin, suggesting the therapeutic value of these compounds in the treatment of neurodegenerative disorders, such as Alzheimer's disease, or for the prevention of neuronal apoptosis after stroke.

Recently, Hoffmann La-Roche claimed that inhibition of GSK-3 by arylindolyl-maleimide derivatives, such as compound 38, reduces the level of CD4+ T helper 2 cells (Th2), which produce cytokines such as IL-4, IL-5, IL-13 and promote IgE production and eosinophil differentiation [137]. This is an important discovery, due to the involvement of Th2 specific cytokines in the pathogenesis of disorders such as allergies and asthma [20].

**Figure 5. Miscellaneous glycogen synthase kinase-3 ATP-competitive inhibitors.**

CDK: Cyclin dependent kinase; CK: Casein kinase; GSK: Glycogen synthase kinase.
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Figure 6. Non-ATP competitive GSK-3 inhibitor.

4. Antisense therapy

Antisense technology is emerging as a novel drug discovery method. Antisense drugs are complementary strands of small segments of mRNA. They are created by linking nucleotides together in short chains designed to bind to a specific sequence of nucleotides in the mRNA target, thereby inhibiting production of the protein encoded by the target mRNA. Isis Pharmaceuticals employs oligomeric antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding GSK-3α and which modulate the expression of GSK-3α [143]. Moreover, antisense oligonucleotides exert protection against neuronal death [30].

5. Expert opinion

GSK-3 is a fascinating enzyme with an astoundingly diverse range of actions in intracellular signalling pathways. It plays a crucial role in several human diseases and therefore has great potential for therapeutic intervention. Knowledge of the role of GSK-3 inhibitors in many different cellular processes has increased over the last five years, and as new pharmacological and genetic tools become available, this knowledge is likely to develop further. GSK-3 inhibitors have arisen as promising drugs for the pharmacotherapy of several acute pathologies, such as cancer, diabetes, stroke, mood disorders, inflammation and Alzheimer’s disease, among others.

Patenting activity relating to the discovery of GSK-3 inhibitors has increased exponentially in the last four years. A number of diverse drug-like molecules (small molecules capable of crossing biological barriers such as the blood–brain barrier and gastrointestinal tract) have emerged [104-142]. However, agents for clinical use must be able to specifically target the appropriate enzymatic process. Nonspecific protein kinase inhibition by ATP site-directed inhibitors might have widespread undesirable effects. This is the case in the majority of GSK-3 inhibitors discovered to date. All show activity on many other kinases, thus diminishing their drug development possibilities. Only ATP-non-competitive GSK-3 selective inhibitors represent an efficient pathway for providing promising drugs for therapeutic intervention. TDZD compounds [141] have been described as the first drug-like candidates with this biochemical behaviour.

The relevance of GSK-3 as a therapeutic target may become testable with the availability of specifically designed transgenic animal models. The body of evidence pointing toward the seminal role of GSK-3 in neurodegeneration is substantial, and much research has been carried out in the area. Several transgenic animal models, using species such as lampreys, mice and rats, provide in vivo models in which therapies for neurodegenerative disease can be assessed [31]. To date, three transgenic mice that overexpress GSK-3 in the nervous system have been reported [32]. Conversely, GSK-3 knockout mice die during embryonic life, pointing to the crucial role of this kinase in development. Implication of GSK-3 in neurodegeneration has also been firmly established from these in vivo genetic approaches. The conditional transgenic model is useful in testing the neuroprotective effect of the forthcoming GSK-3 specific inhibitors [144].

Through intense research, understanding of the molecular mechanisms underlying GSK-3 signalling pathways has grown rapidly [33]. Technology has been developed that enables the search for and discovery of new potent and selective inhibitors of GSK-3 [145]. Moreover, the crystal structure of GSK-3 provides a powerful tool to improve the creativity of the medicinal chemist for the rational design of specific inhibitors that target selective aminoacids within the enzyme. In summary, basic and applied research has provided a technological platform for the generation of selective GSK-3 inhibitors to interfere with and delay the progression of neurodegeneration, and other important pathologies, for which specific treatment is expected to be developed in the near future.
Bibliography

Papers of special note have been highlighted as either of interest (+) or of considerable interest (+++) to readers.


++ Presents a complete description of the biological functions of GSK-3.


+ Describes the in vivo experiments that validate GSK-3β as a target for neurodegenerative disorders.


• Reviews the scientific literature surrounding small molecule GSK-3 inhibitors.

• Highlights the potential of GSK-3β as a new therapeutic target as well as the properties desirable for its inhibitors.

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Expert Opin. Ther. Patents (2002) 12:10
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**Patents**


**Website**

201. [http://www.stke.org/cgi/content/full/OC_sigtrans;2001/100/re12](http://www.stke.org/cgi/content/full/OC_sigtrans;2001/100/re12)


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