# GSK3β-Targeted Library

*Medicinal and Computational Chemistry Dept., ChemDiv, Inc., 6605 Nancy Ridge Drive, San Diego, CA 92121 USA, Service: +1 877 ChemDiv, Tel: +1 858-794-4860, Fax: +1 858-794-4931, Email: ChemDiv@chemdiv.com*

## INTRODUCTION

GSK3 was discovered nearly three decades ago in rabbit skeletal muscle as a protein kinase that phosphorylates and inactivates glycogen synthase, the final enzyme of glycogen biosynthesis. GSK3 is a multifunctional Ser/Thr kinase with diverse roles in various human diseases, including diabetes, inflammation, neurological disorders and various neoplastic diseases. To date, two members of the mammalian GSK3 family (α and β) are known. They are ubiquitously expressed and highly conserved and are members of the CMGC family of protein kinases. Many of the substrates of GSK3 need a "priming phosphate" (which is a Ser/Thr residue) located four amino acids (aa) C-terminally from the site of phosphorylation. GSK3 is constitutively active in nonstimulated cells, where the majority of its substrates undergo inactivation/proteolysis by phosphorylation, and undergoes a rapid and transient inhibition in response to a number of external signals. Physiological regulation of GSK3 activity by various upstream kinases in different physiological and pathological condition is established [1].

Thus, in resting cells, constitutive activity of GSK-3β phosphorylates/inactivates a multitude of substrates (e.g., transcription factor [β-catenin], translation initiation factor [e.g., eIF2B], and structural proteins [e.g., tau]). Conversely, receptor tyrosine kinases (e.g., insulin receptor), G protein–coupled receptors, Wnt receptors, and hyperglycemia cause Ser9-phosphorylation/inactivation of GSK-3β, relieving the suppression of the aforementioned substrates by GSK-3β [2]. The biological importance of GSK-3β is reinforced by a number of observations. In 1998, Pap and Cooper [3] showed that overexpression of GSK-3β induced apoptosis in rat pheochromocytoma PC12 cells. In 2000, Hoeflich et al. [4] showed that disruption of GSK-3β gene in mice caused embryonic lethality.

The therapeutic potential of GSK3beta inhibitors has become an important area of investigation. However, GSK3beta also participates in neoplastic transformation and tumor development. The role of GSK3beta in tumorigenesis and cancer progression remains controversial; it may function as a "tumor suppressor" for certain types of tumors, but promotes growth and development for some others. GSK3beta also mediates drug sensitivity/resistance in cancer chemotherapy. Therefore, although GSK3beta is an attractive therapeutic target for a number of human diseases, its potential impact on tumorigenesis and cancer chemotherapy needs to be carefully evaluated [5].

## 1. Insulin as endogenous regulator of GSK-3β

It has been clearly demonstrated that at the developing and adult neuronal cell levels, insulin promotes axon myelination, formation of synaptic network, synaptic plasticity, and prevents Alzheimer's diseaserelated tau hyperphosphorylation, which are mediated largely via inhibiting GSK-3β [6]. Extracellular stimuli (e.g., insulin) catalyze inhibitory Ser9-phosphorylation of GSK-3β, turning on signaling and causing other biological consequences otherwise constitutively suppressed by GSK-3β. Regulated and dysregulated activities of GSK-3β are pivotal to health, disease, and therapeutics (e.g., insulin resistance, neurodegeneration, tumorigenesis, inflammation); however, the underlying mechanisms of multifunctional GSK-3β remain elusive. In cultured bovine adrenal chromaffin cells, Nemoto et al. (19) showed that insulin (100 nM) caused a rapid (<5 min) and sustained (up to 24 h) increase of Ser9-phosphorylation of GSK-3β up to 104%, which was followed by decreases of insulin receptor substrate-1 (IRS-1) and IRS-2 protein levels up to 72%. Treatment with LiCl (1 – 20 mM for ≥12 h) increased Ser9-phosphorylated GSK-3β and β-catenin levels up to 59%, while decreasing cell surface 125I-insulin binding capacity, cellular levels of insulin receptor, IRS-1, IRS-2, and Akt1 up to 62% in a time- and concentration-dependent manner. SB216763 (0.1 – 30 μM for ≥12 h), a selective inhibitor of GSK-3, increased β-catenin level, while decreasing insulin receptor signaling molecule levels, as did LiCl (19 – 21). LiCl-induced IRS-1 reduction was abolished, while LiCl-induced IRS-2 reduction was partially blocked by β-lactone or lactacystin, a proteasome inhibitor; LiCl decreased mRNA levels of insulin receptor, IRS-2, and Akt1. Changes of these molecules induced by insulin, LiCl, or SB216763 were all restored to the control levels after washout of either test compound–treated cells at 12 h. Thus, constitutive activity of GSK-3β controls proteasomal degradation of IRS-1 and IRS-2 and mRNA levels of insulin receptor, IRS-2, and Akt, thereby maintaining steady-state levels of these proteins. In cultured bovine adrenal chromaffin cells, Sugano et al. [7] showed that nicotinic receptor stimulation by nicotine (1 – 300 μM for 12 – 48 h) caused time- and concentration-dependent increases of IRS-1 and IRS-2 mRNA and protein levels up to 125%, which was mediated via sequential activation of PKC-α and ERK; in these cells, insulin (100 nM for 10 min)-induced Ser9-phosphorylation of GSK-3β was enhanced. In dorsal root ganglion neurons, PC12 cells, and NG108-15 cells, NGF- or IGF-I–induced Ser9-phosphorylation of GSK-3β or GSK-3 inhibitors (e.g., lithium, SB216763, and valproic acid) promoted axon–dendrite neuronal polarity, increasing axon growth cone size [8].

In cultured bovine adrenal chromaffin cells, insulin (100 nM for ≥12 h) or IGF-I increased the cell surface number of Nav1.7 up to 49%, as evidenced by cell surface [3H]saxitoxin binding assay. By using phosphoinositide 3-kinase (PI3K) inhibitors, LiCl, SB216763, or valproic acid, it was shown that upregulation of Nav1.7 was mediated by PI3K-induced GSK-3β inhibition and involved increased levels of Nav1.7 mRNA and auxiliary β1-subunit mRNA [9]. Up-regulation of Nav1.7 augmented veratridine-induced 22Na+ influx via Nav1.7, 45Ca2+ influx via voltagedependent calcium channel, and exocytic secretion of catecholamine.

Evidence has accumulated that multiple signaling pathways (e.g., insulin) converge on GSK-3β, thus GSK-3β being pivotal to health, disease, and therapeutics. Therefore, both (1) regulation of insulin receptor signaling molecules by GSK-3β and (2) modification of GSK-3β signaling strength by various stimuli are the current issues of great interest.

**2. GSK-3β in Wnt/Frizzled/LRP signaling system.**

Recent advances in stem cell biology have provided new insights that may lead to the development of regeneration therapy in the central nervous system to replenish lost neurons and to reconstitute neural circuits. The strategies for regeneration can be classified into two approaches: i) activation of endogenous neural stem cells and ii) transplantation of donor cells to replace lost cells. GSK-3β is a promising drug target for neural regeneration via Wnt signaling that has been shown to control proliferation [[10]]. It also has been found that the Wnt signaling pathway is involved in retinal regeneration and that exogenous application of Wnt3a or Wnt2b increases the number of BrdU-labeled, proliferative cells in the injured retina, thereby increasing the number of newly generated photoreceptors [[11]]. Interestingly, low molecular weight inhibitors of GSK-3β mimic these effects and similarly promote the proliferation of Müller glia–derived progenitors. In the canonical pathway, binding of Wnt ligand to its receptor Frizzled and co-receptor LRP5/6 inhibits the activity of the destruction complex that degrades β-catenin via an ubiquitin-proteasome, leading to the accumulation of β-catenin in the cytoplasm (Fig. 1). In more detail, after the Wnt proteins bind to the receptor complex, cytoplasmic disheveled (Dvl), a protein downstream of the receptor complex, is phosphorylated and inhibits GSK-3β by causing their retention at the scaffolding protein axin, resulting in the accumulation of nonphosphorylated β-catenin in the cytoplasm. Nonphosphorylated β-catenin avoids degradation and translocates into the nucleus, where it binds to lymphoid enhancer–binding factor/T-cell–specific transcription factor (LEF/TCF) transcription factors and then activates the transcription of target genes such as cyclin D1.

Accumulating evidence points to a role for Wnt/β-catenin signaling in regulating various types of stem cells. In the adult hippocampus, Wnt3 derived from astrocytes promotes neuronal differentiation and enhances adult neurogenesis [[12]]. Activation of β-catenin signaling by GSK-3β inhibitors increases the proliferation of neural stem cells in the SVZ, thereby promoting neurogenesis in the olfactory bulb.

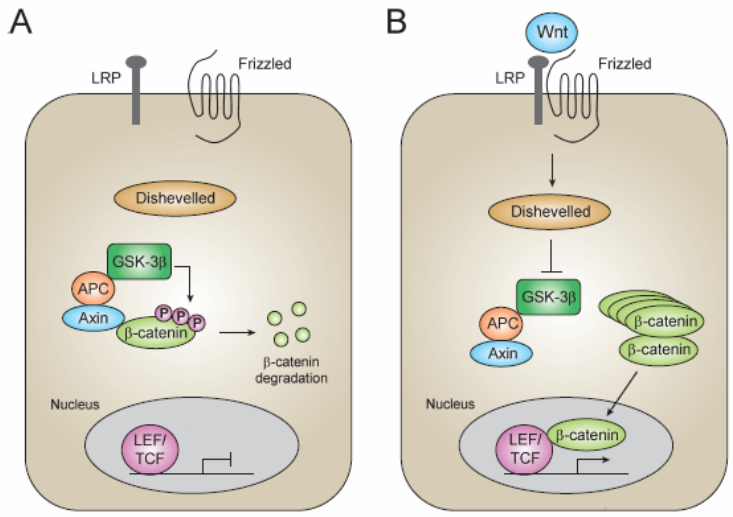



**Fig. 1**. Canonical Wnt signaling pathway. **A**: In the absence of Wnts, β-catenin forms a complex with Axin, APC, and GSK-3β; and it is degraded by the ubiquitin-proteasome system. **B**: When Wnts bind their receptors, such as Frizzled and LRP, GSK-3β is inactivated via Dishevelled. Cytoplasmic β-catenin is stabilized and the accumulated β-catenin is translocated into the nucleus, resulting in the activation of the transcription factor, LEF /TCF. LRP, low-density lipoprotein receptor–related protein; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; LEF/TCF, lymphoid enhancer–binding factor/T-cell factor.

### 2.1. Role of GSK-3β in cancer proliferation and progression

Cell signaling cascades activated by Wnt proteins (i.e., the Wnt signaling pathways) are well conserved through evolutionary processes across a variety of species. As well as regulating cellular processes such as proliferation, differentiation, motility, and survival / apoptosis, the Wnt signaling pathways play key roles in embryonic development and maintenance of homeostasis in mature tissues [13]. Of four known Wnt signaling pathways, [the Wnt/β-catenin (canonical) pathway, the planar cell polarity (PCP) pathway, the Wnt/Ca2+ pathway, the protein kinase A pathway], the Wnt/β-catenin signaling pathway is best characterized [14]. The activity of the Wnt/β-catenin signaling pathway is dependent on the amount of β-catenin in the cytoplasm. As described above, normally, the cytoplasmic β-catenin level is kept low through continuous ubiquitin-proteasome system–mediated degradation, which is regulated by a multiprotein complex containing axin, adenomatous polyposis coli (APC), and glycogen synthase kinase-3β (GSK-3β). The activity of GSK-3β is decreased by the phosphorylation of Ser9 and several studies have shown that Ser9 in GSK-3β is phosphorylated by Akt, a serine/threonine kinase that is activated by phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1), a protein kinase downstream of the mitogen-activated protein kinase (MAPK) cascade, and p70 ribosomal S6 kinase-1 [15].

Since several oncogenes are included amongst the target genes, constitutive activation of the Wnt/β-catenin signaling pathway can lead to cancer [16]. One oncogene, the cyclin D1 gene CCND1, is a well-known Wnt/β-catenin target gene. The level of the cyclin D1 protein is regulated by an ubiquitin-dependent mechanism throughout the progression of the cell cycle. Cyclin D1 is transported from the nucleus to the cytoplasm where it is degraded by the 26S proteasome. Although GSK-3β is a cytosolic protein, it is translocated into the nucleus when activated and phosphorylates cyclin D1 on Thr286, thereby stimulating cyclin D1 turnover in response to mitogenic signals. Phosphorylation of cyclin D1 on Thr286 by GSK-3β facilitates its association with CRM1, which is a nuclear protein that mediates the nuclear export of proteins, resulting in the exclusion of cyclin D1 from the nucleus to

initiate its proteasomal degradation [17]. As described above, cyclin D1 gene expression is activated by Wnt/β-catenin signaling, in which GSK-3β plays a critical role in its regulation, and cyclin D1 protein degradation is regulated by GSK-3β. Thus activation of GSK-3β is expected to lead to a reduction in the level of cyclin D1 mRNA at the transcriptional level and the protein at the degradation level. While many diseases, including diabetes mellitus and Alzheimer's disease, can be ameliorated by the use of GSK-3β inhibitors, cancers, especially cancers in which cyclin D1 is overexpressed, are likely to be more susceptible to pharmacological activation of GSK-3β.

Differentiation-inducing factors (DIFs) were identified in *Dictyostelium discoideum* as the morphogens required for stalk cell differentiation [18]. In the DIF family, DIF-1 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone] was the first to be identified and DIF-3, the monochlorinated analog of DIF-1, is a natural metabolite of DIF-1 in Dictyostelium [19]. However, the actions of DIFs are not limited to Dictyostelium. They also have strong effects on mammalian cells. DIF-1 and /or DIF-3 strongly inhibit proliferation and induce differentiation in several leukemia cells, including the murine erythroleukemia cell line B8, human leukemia cell line K562, and human myeloid leukemia cell line HL-60 [20]. DIF-3 has been reported to have the most potent antiproliferative effect on mammalian leukemia cells among the DIF analog examined to date. However, the target molecule (receptor) of DIFs is unknown and it is not clear even in Dictyostelium how DIFs induce antiproliferative effects and cell differentiation. Although the precise mechanisms underlying their antiproliferative effects are not yet known, it has recently been found that DIFs (DIF-1 and DIF-3) inhibited mammalian cell proliferation by suppressing the expression of cyclin D1 mRNA and protein through the activation of GSK-3β [21]. DIFs dephosphorylated Ser9 of GSK-3β by an unknown mechanism and thus activated this kinase. Activated GSK-3β by DIFs induced β-catenin degradation and suppressed β-catenin /TCF–dependent transcription activity, indicating that DIFs inhibit the Wnt /β-catenin signaling pathway (see Fig. 1). It also has been found that DIFs reduced the activity of a reporter gene driven by the human cyclin D1 promoter (+134/−961 bp) via a TCF binding site (−75/−81 bp) [22]. This result suggests that DIFs inhibited cyclin D1 mRNA expression via the inhibition of β-catenin /TCF–dependent transcription activity. On the other hand, the activated GSK-3β translocates to the nucleus and phosphorylates cyclin D1 on Thr286 to trigger the degradation of cyclin D1 by an ubiquitin-dependent mechanism. Correlated with the above observations, DIFs induced G0/G1 cell cycle arrest, which was rescued by the overexpression of cyclin D1, suggesting that DIFs were likely to induce cell cycle arrest by reducing the expression of cyclin D1.

Cyclin D1 degradation is facilitated by the phosphorylation of specific threonine residues, not only 286 but also 288, according to previous reports [23]. Zou et al. [24] reported that dual-specificity tyrosinephosphorylation–regulated kinase 1B (DYRK1B), a member of the DYRK family, phosphorylates cyclin D1 on Thr288, also resulting in cyclin D1 degradation. Therefore, the effect of DIF-3 on DYRK1B was examined and it was found that not only GSK-3β but also DYRK1B was involved in the phosphorylation of cyclin D1 to trigger its degradation. This may have an important implication in DIFs-induced cyclin D1 degradation because DIFs induce rapid and strong degradation of cyclin D1 (within 1 h). Clarified DIFs action is summarized in Fig. 2. Thus, GSK-3β plays a critical role in the regulation of the amount of cyclin D1, as this kinase is involved in both cyclin D1 mRNA transcription and ubiquitin-dependent proteolysis. DIFs act as an inhibitor of the Wnt/β-catenin signaling pathway via the activation of GSK-3β, whereas the target molecule is not clarified

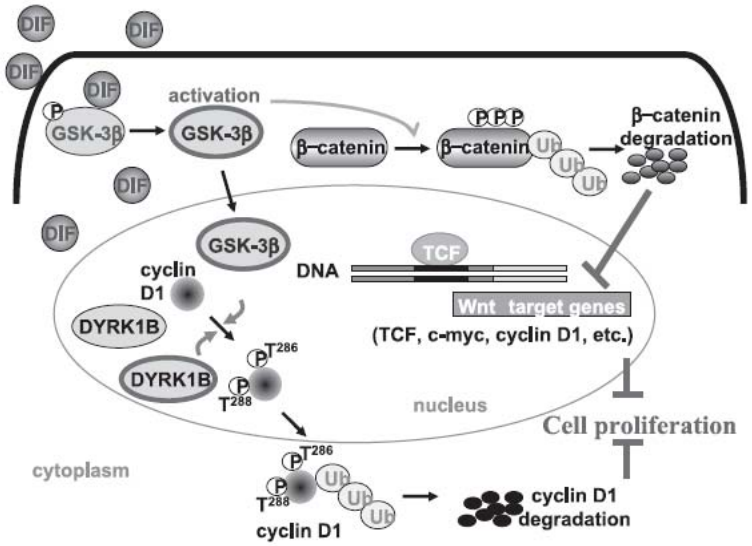



**Fig. 2**. DIFs action and the Wnt/β-catenin signaling pathway. DIFs enter into the cell and dephosphorylate GSK-3β at Ser9 by unknown mechanisms, resulting in the activation of this kinase. Activated-GSK-3β translocates into nucleus and phosphorylates Thr286 of cyclin D1. DIFs also activated DYRK1B, which is present in nucleus, by an unknown mechanism, and activated DYRK1B phosphorylates Thr288 of cyclin D1. Phosphorylated cyclin D1 is exported from the nucleus, resulting in its degradation by the 26S proteasome system after ubiquitination. Activated-GSK-3β also phosphorylates β-catenin in the cytoplasm. Phosphorylated β-catenin is degraded, resulting in the inhibition of transcription of the target genes, such as cyclin D1 and c-myc. GSK-3β, glycogen synthase kinase-3β; DYRK1B, dual-specificity tyrosine phosphorylation-regulated kinase 1B; Ub, ubiquitin.

### 3. GSK-3β in cardiovascular diseases

Roles of GSK-3β in cardiac glycogen metabolism and its function in counteracting ventricular hypertrophy have been well characterized [25]. Recently, accumulating evidence suggests that GSK-3β is also critically involved in the fate of cells subjected to extracellular stress, including ischemia/reperfusion [26]. GSK-3β has recently received attention as a possible regulator of mitochondrial permeability transition pore (mPTP) opening since this kinase is a common target of multiple signal pathways that lead to myocardial protection from infarction. In fact, myocardial infarct size is reduced by ischemic preconditioning and by treatment with δ-opioid, insulin, insulin-like growth factor, erythropoietin, or isoflurane; and all of these interventions induce Ser9-phosphorylation of GSK-3β. However, different protein kinases (i.e., protein kinase C [PKC], Akt, ERK1/ 2) are responsible, depending on the intervention, for direct phosphorylation of GSK-3β in the cardioprotection. Evidence for a regulatory role of GSK-3β in mPTP opening was first reported by Juhaszova et al. [27]. They determined the threshold for opening of the mPTP by monitoring mitochondrial membrane potential in isolated cardiomyocytes and used ROS generated by laser irradiation as a trigger of mPTP opening. The threshold for mPTP opening was significantly elevated by inhibitory phosphorylation of GSK-3β at Ser9, transfection of a dominant negative mutant of GSK-3β, or reduction of GSK-3β expression by siRNA. Recently Gomez et al. [28] assessed the involvement of GSK-3β in inhibition of mPTP opening by ischemic postconditioning. They isolated mitochondria from the myocardium after ischemia/reperfusion and determined the threshold for mPTP opening as the amount of loading $Ca^{2+}$ required to induce irreversible $Ca^{2+}$ release from the mitochondria. Postconditioning significantly elevated the threshold of mPTP opening in cardiac mitochondria from wild-type mice, but such a protective effect was not detected in mitochondria from transgenic mice expressing GSKS9A, which cannot be inactivated by phosphorylation at Ser9.

Taken together, these findings support the notion that phospho-GSK-3β regulates the threshold for mPTP opening in response to ROS and/or $Ca^{2+}$ overloading.

The intracellular localization of GSK-3β and interaction of GSK-3β with mPTP before and after ischemia/reperfusion have been examined in isolated perfused rat hearts [29] to get an insight into the molecular mechanism by which phospho-GSK-3β regulates mPTP opening. Thus, GSK-3β was predominantly found in the cytosolic fraction under baseline conditions, but ischemia/reperfusion significantly increased GSK-3β level in the mitochondrial fraction. Reperfusion also increased phospho-GSK-3β level in all cell fractions. GSK-3β was co-immunoprecipitated with ANT and with VDAC before ischemia, but levels of these complexes were increased after reperfusion by approximately 50%, indicating that GSK-3β translocated after reperfusion binds to mPTP.

Based on the finding of reperfusion-induced GSK-3β interaction with ANT and VDAC, it has been hypothesized that elevation of the threshold for mPTP opening by phosphorylation of GSK-3β is achieved by direct interaction of phospho-GSK-3β with mPTP subunit proteins [30]. To test this hypothesis, GSK-3β phosphorylation at Ser9 was induced by ischemic preconditioning and erythropoietin-receptor activation in rat hearts. Phospho-GSK-3β in the reperfused myocardium was increased by preconditioning and erythropoietin-receptor activation in a PKC- and Akt-dependent manner, and the increased phospho-GSK-3β was co-immunoprecipitated with ANT but not with VDAC. Furthermore, the level of CyP-D co-immunoprecipitated with ANT was significantly reduced to 40% in association with a 50% increase in phospho-GSK-3β ANT binding. These results suggest that interaction of phospho-GSK-3β with ANT inhibits CyP-D–ANT interaction, resulting in prevention of mPTP opening. In addition to ANT, p53 may be involved in suppression of mPTP opening by phosphorylation of GSK-3β. p53 is one of more than 20 substrates of GSK-3β, and its phosphorylation enhances functional activity and translocation of p53 to the nucleus and mitochondria [31]. A recent study by Venkatapuram et al. [32] showed that an inhibitor of p53, pifithrin-α, lowered the threshold of isoflurane-induced limitation of infarct size in rabbit hearts. Interestingly, this beneficial effect of a p53 inhibitor was abolished by an activator of mPTP, atractyloside. Thus, inhibition of GSK-3β–mediated p53 phosphorylation may also contribute to suppression of myocardial necrosis by GSK-3β phosphorylation.

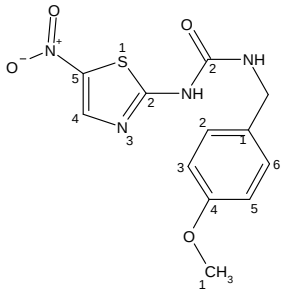
There are redundant signal pathways that induce phosphorylation of GSK-3β in cardiomyocytes [33], and more than one pathway is frequently activated by cardioprotective receptor agonists and ischemic preconditioning [34]. However, some of the cytoprotective signal pathways suffer from impairment by concurrent diseases associated with coronary artery diseases (such as diabetes mellitus, hypercholesterolemia, and post-infarct ventricular remodeling). It has been found that post-infarct remodeling impairs activation of PKC-ε after ischemic preconditioning and Jak2–PI3K–Akt signaling by erythropoietin-receptor activation [35]. Erythropoietin-receptor activation also failed to activate the Jak2–PI3K–Akt pathway in the myocardium of an animal model of type 2 diabetes [36]. Thus, from the viewpoint of clinical application, a strategy to directly inhibit GSK-3β would be more preferable to indirect GSK-3β inhibition by use of its up-stream signaling pathways.

Phospho-Ser9-GSK-3β plays a critical role in intracellular signal–mediated interventions that protect cardiomyocytes from ischemia/reperfusion-induced necrosis. The mechanism of GSK-3β phosphorylation differs depending on the receptor activated by the intervention, but inactivation of GSK-3β by phosphorylation at Ser9 elevates the threshold for mPTP opening, which reduces myocyte necrosis. Although the mechanism by which phospho-Ser9-GSK-3β elevates the threshold for mPTP opening is unclear, suppression of ANT–CyP-D interaction by binding of phospho-Ser9-GSK-3β to ANT and reduction in GSK-3β–mediated phosphorylation of p53 may be

involved. The level of phospho-Ser9-GSK-3β at the time of reperfusion is a determinant of infarct size, and GSK-3β inhibitors are promising as agents for myocardial salvage in patients with AMI.

Summarizing these observations, it has become clear that there is a clinical need for novel therapy to protect cardiomyocytes from ischemia/reperfusion-induced necrosis. An advantage of a GSK-3β inhibitor for clinical use is its efficiency even when administered immediately before reperfusion. Another favorable profile of GSK-3β inhibitors is that their protective effect would not be impaired by common co-morbidities in AMI patients that modify signaling to cytoprotective kinases such as PKC-ε and Akt. Although chronic inhibition of GSK-3β raises concern about risks of cancer development and ventricular hypertrophy [37], such risks would be negligible for single injection of a GSK-3β inhibitor before reperfusion therapy in AMI patients.

It has been found that GSK3β selectively interacts with 5-hydroxytryptamine-1B receptors (5-HT1BR) that have important functions in serotonin neurotransmission and behavior [38]. Using molecular, biochemical, pharmacological, and behavioral approaches, authors tested 5-HT1BR's interaction with G(i)α(2) and β-arrestin2 and 5-HT1BR-regulated signaling in cells, serotonin release in mouse cerebral cortical slices, and behaviors in wild-type and β-arrestin2 knockout mice. Molecular ablation of GSK3β and GSK3 inhibitors abolished serotonin-induced change of 5-HT1BR coupling to G(i)α(2) and associated signaling but had no effect on serotonin-induced recruitment of β-arrestin2 to 5-HT1BR. This effect is specific for 5-HT1BR because GSK3 inhibitors did not change the interaction between serotonin 1A receptors and G(i)α(2). Two GSK3 inhibitors, N-(4-methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418) and 3-(5-bromo-1-methyl-1H-indol-3-yl)-4-(benzofuran-3-yl)pyrrole-2,5-dione (BIP-135), efficiently abolished the inhibitory effect of the 5-HT1BR agonist anpirtoline on serotonin release in mouse cerebral cortical slices.



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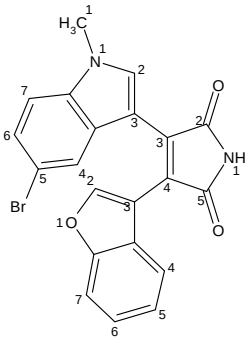

BIP-135

GSK3 inhibitors also facilitated the 5-HT1BR agonist anpirtoline-induced behavioral effect in the tail suspension test but spared anpirtoline-induced locomotor activity. These results suggest that GSK3β is a functional selective modulator of 5-HT1BR-regulated signaling, and GSK3 inhibitors fine-tune the physiological and behavioral actions of 5-HT1BR. Future studies may elucidate the significant roles of GSK3 in serotonin neurotransmission and implications of GSK3 inhibitors as functional selective modulators of 5-HT1BR.

**4. Small molecule GSK-3β inhibitors**

To date, more than 750 small-molecule compounds have been identified as potent GSK-3β inhibitors (Fig. 3). As shown in figure 3, compounds belong to a wide range of chemotypes with different features. Among these compounds at least 7 agents are currently evaluated in advanced clinical trials (Table 1). Hundred papers dedicated to the synthesis of novel GSK-3β inhibitors have been published to date. Several recent examples are listed below.

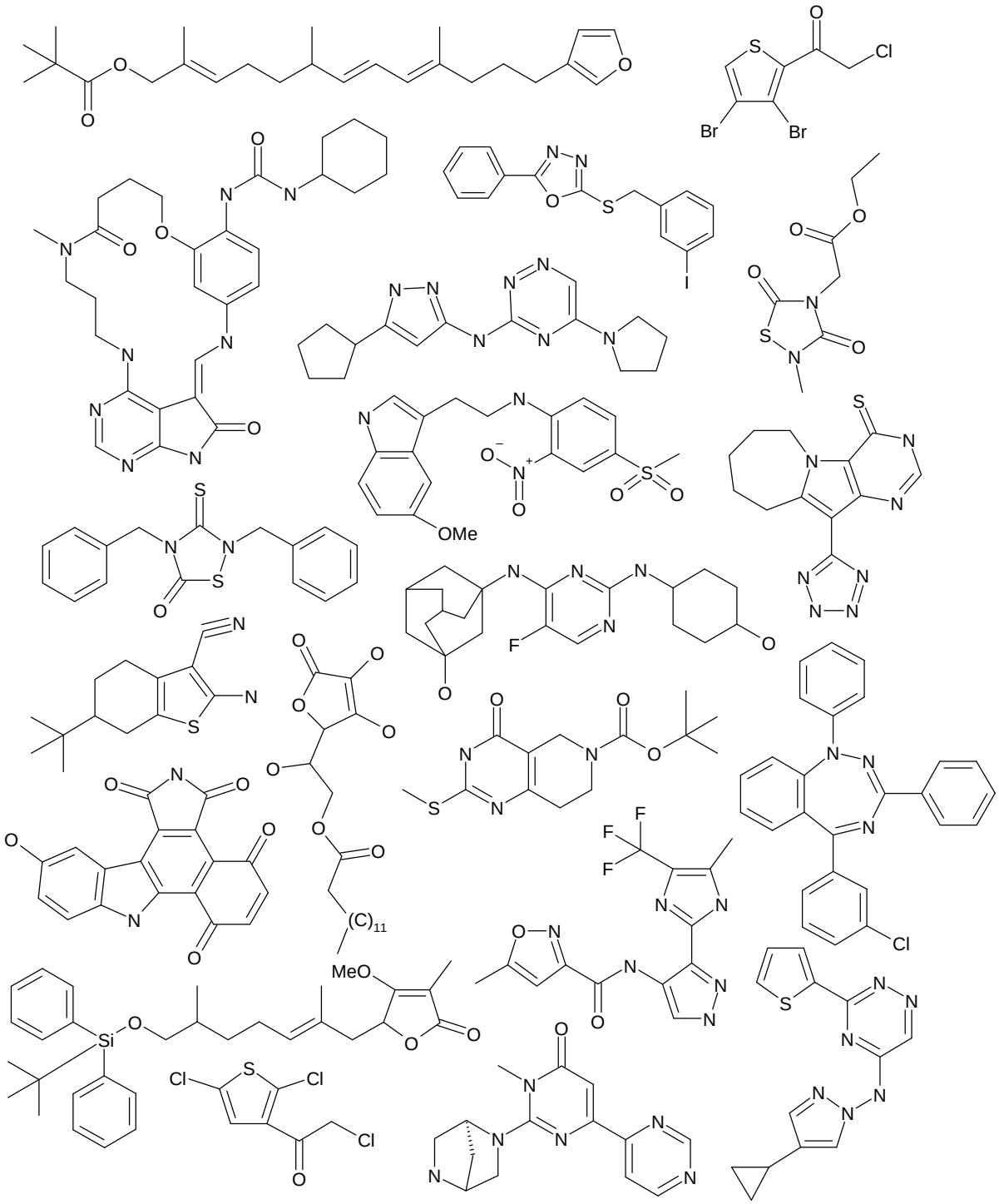

**Fig. 3**. Representative examples of GSK-3β inhibitors investigated in biological trials

**Table 1**. Examples of GSK-3β inhibitors currently evaluated in different clinical trials

| Compound | Name | Originator | Mechanism | Therapeutic group |
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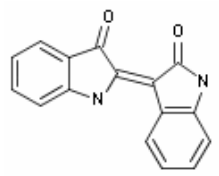
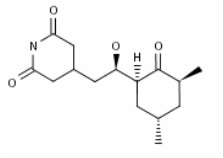
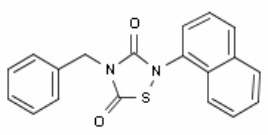
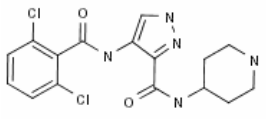
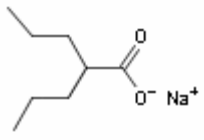
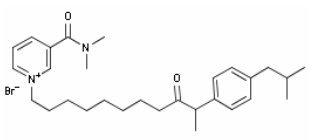
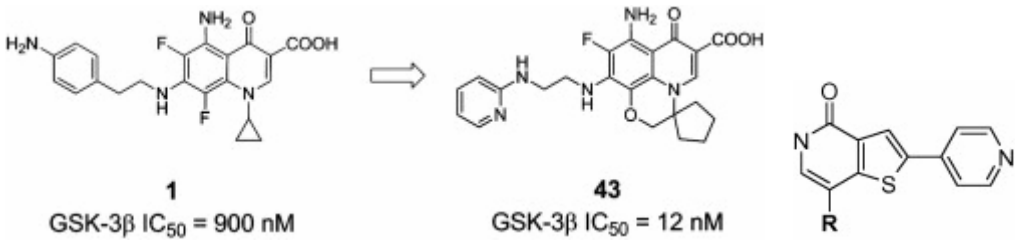
|  Clinical | Indirubin | Chinese Academy of Sciences (Originator) Institute of Materia Medica, Beijing (Originator) | CDK1 Inhibitors CDK2 Inhibitors CDK4 Inhibitors CDK5 Inhibitors Glycogen Synthase Kinase 3 beta (GSK- | Myeloid Leukemia Therapy |

| | | | | |
|---|---|---|---|---|
| | | | 3beta; tau Protein Kinase I) Inhibitors | |
|  Launched | Cycloheximide | Pfizer | Glycogen Synthase Kinase 3 beta (GSK-3beta; tau Protein Kinase I) Inhibitors | Antifungal Agents Oncolytic Drugs |
|  Phase II | Tideglusib | Noscira | Glycogen Synthase Kinase 3 beta (GSK-3beta; tau Protein Kinase I) Inhibitors | Alzheimer's Dementia, Treatment of Cerebrovascular Diseases, Treatment of Neurologic Drugs (Miscellaneous) |
|  Phase I/II | | Astex Pharmaceuticals | CDK1 Inhibitors CDK2 Inhibitors Glycogen Synthase Kinase 3 beta (GSK-3beta; tau Protein Kinase I) Inhibitors | Antifungal Agents Oncolytic Drugs |
| Phase II | LY-2090314 | Lilly | Glycogen Synthase Kinase 3 beta (GSK-3beta; tau Protein Kinase I) Inhibitors | Leukemia Therapy Oncolytic Drugs |
|  Launched - 1981 | Valproic acid sodium salt | Abbott | GABAergic Transmission Enhancers Glycogen Synthase Kinase 3 (GSK-3) Inhibitors | Epilepsy Mania Bipolar disorder |
|  Phase I | IBU-PO | Israel Institute for Biological Research | Glycogen Synthase Kinase 3 (GSK-3) Inhibitors beta-Amyloid (Abeta) Protein Neurotoxicity Inhibitors | Alzheimer's Dementia |

Cociorva et al [39] have recently reported the synthesis, GSK-3β inhibitory activity, and anti-microbial activity of bicyclic and tricyclic derivatives of the 5,7-diamino-6-fluoro-4-quinolone-3-carboxylic acid scaffold (Fig.

4A). Kinase selectivity profiling indicated that members of this class were potent and highly selective GSK-3 inhibitors. Gentile and colleagues [40] have discovered a novel series of 2-(4-pyridyl)thienopyridinone GSK-3β inhibitors (Fig. 4B). X-ray crystallography reveals its binding mode and enables rationalization of the SAR. The initial optimization of the template for improved cellular activity and predicted CNS penetration is also presented.




(A) (B)

**Fig. 4**. 4-Quinolone-3-carboxylic acids (A) and 2-(4-pyridyl)thienopyridinones as potent GSK-3β inhibitors

**5. Computational approaches for the discovery of novel GSK-3β inhibitors**

Many various computational approaches have been effectively applied for the design of novel small-molecule GSK-3β inhibitors including 2D-structural similarity, bioisosteric/isosteric morphing procedures, artificial neural-net algorithms (ANN), 3D-pharmacophore modeling and searching as well as 3D-molecular docking. Several representative examples are highlight below.

Caballero et al [41] have been performed the docking of 3-amino-1H-indazoles complexed with GSK3β to gain insight into the structural requirements and preferred conformations of these inhibitors. The study was conducted on a selected set of 57 compounds with variation in structure and activity. Authors have found that the most active compounds established three hydrogen bonds with the residues of the hinge region of GSK3β, but some of the less active compounds have other binding modes. In addition, models able to predict GSK3β inhibitory activities ($IC_{50}$) of the studied compounds obtained by 3D-QSAR methods CoMFA and CoMSIA. Ligand-based and receptor-guided alignment methods have been utilized. Adequate R(2) and Q(2) values have been obtained by each method, although some striking differences existed between the obtained contour maps. Each of the predictive models exhibited a similar ability to predict the activity of a test set. The application of docking and quantitative structure-activity relationship together allowed conclusions to be drawn for the choice of suitable GSK3β inhibitors.

Structure-based virtual screening of GSK3β inhibitors including the analysis of scoring functions applied to large true actives and decoy sets, has been described in [42]. Thus, comparative assessment of nine different scoring functions (OpenEye and Tripos implementation) applied to structure-based virtual screening based on rigid docking of the pregenerated conformations library of GSK-3β inhibitors has been carried out. The functions studied belong to the following types: Gaussian (Chemgauss3, Shapegauss), empirical (Chemscore, OEChemscore, Piecewise Linear Potential, Screenscore), force field-based (D_score and G_score), and potential of mean force (PMF_score). Overall enrichment of the large true inhibitors set against the set of true non-inhibitors, Directory of Useful Decoys (DUD), cyclin-dependent kinase 2 subset, and NCI Diversity Set was evaluated by means of ROC (receiver operating characteristic) method. According to this analysis, scoring function Chemscore leads to the best enrichment of the inhibitors whereas the best early enrichment of the actives may be obtained with the help of Chemgauss3 function as estimated by BEDROC (Boltzmann-enhanced discrimination of ROC) metrics.

Fang et al [43] have suggested a new lead identification protocol that predicts new GSK-3β ATP competitive inhibitors with topologically diverse scaffolds (Fig. 5). First, three-dimensional quantitative structure-activity

relationship (3D QSAR) models were built and validated. These models are based upon known GSK-3β inhibitors, benzofuran-3-yl-(indol-3-yl) maleimides, by means of comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA). Second, 28826 maleimide derivatives were selected from the PubChem database. After filtration via Lipinski's rules, 10429 maleimide derivatives were left. Third, the FlexX-dock program was employed to virtually screen the 10429 compounds against GSK-3β. This resulted in 617 virtual hits. Fourth, the 3D QSAR models predicted that from the 617 virtual hits, 93 compounds would have GSK-3β inhibition values of less than 15 nM. Finally, from the 93 predicted active hits, 23 compounds were confirmed as GSK-3β inhibitors from literatures; their GSK-3β inhibition ranged from 1.3 to 480 nM. Therefore, the hits rate of our virtual screening protocol is greater than 25%. The protocol combines ligand- and structure-based approaches and therefore validates both approaches and is capable of identifying new hits with topologically diverse scaffolds.

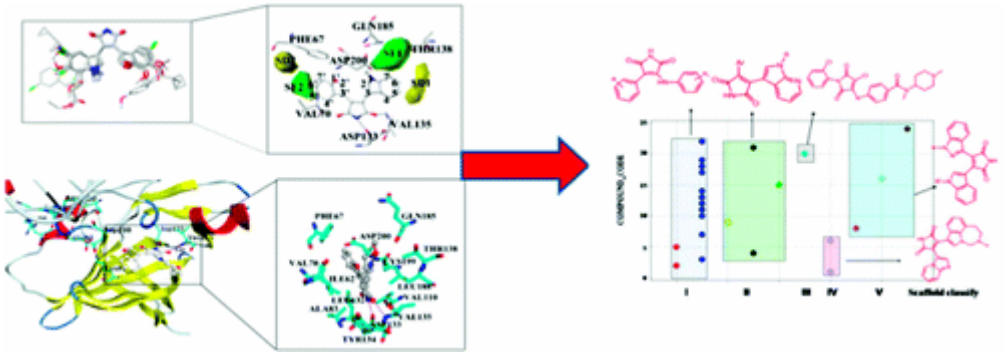


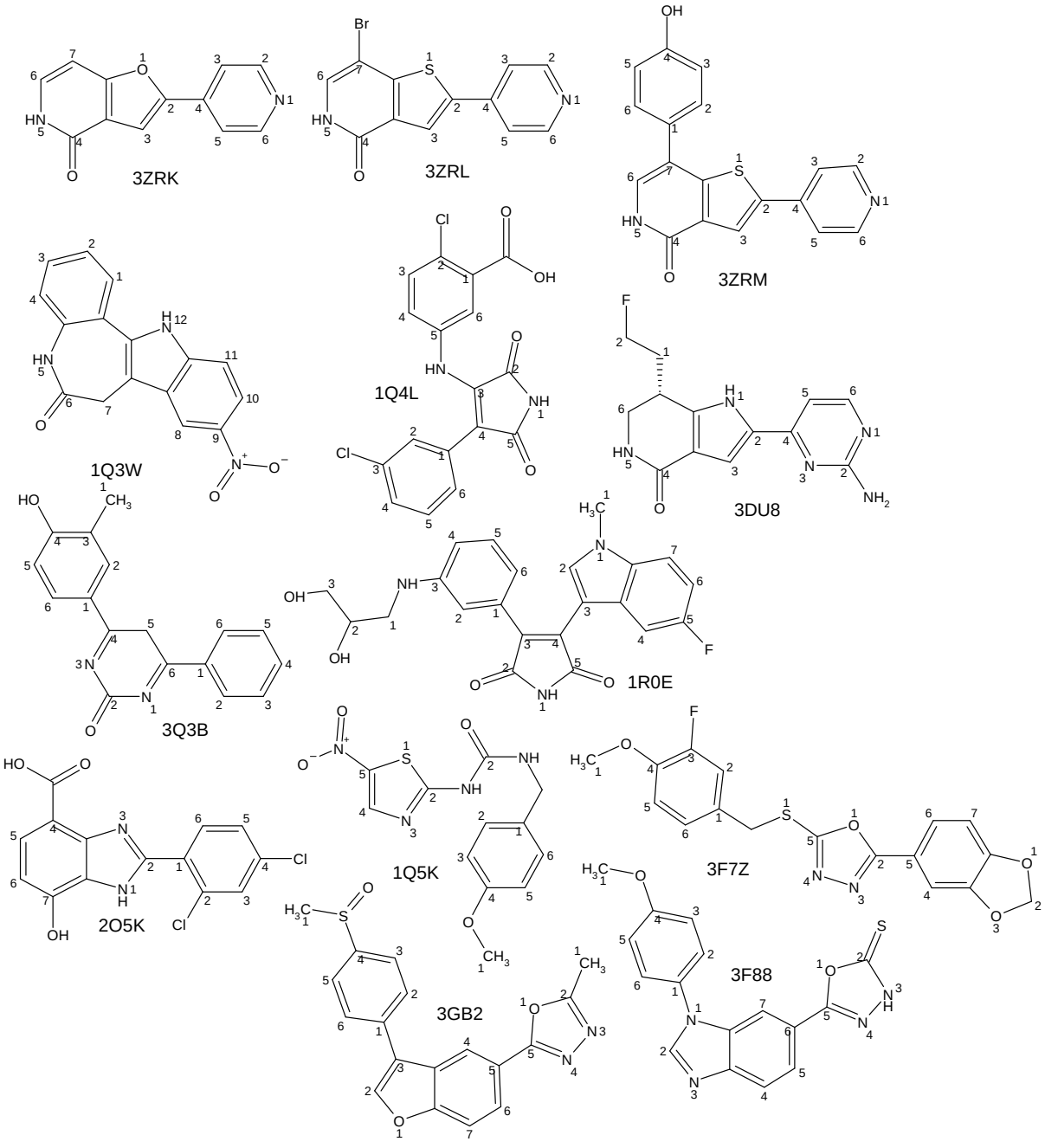
**Fig. 5**. Schematic view of novel lead-identification protocol suggested for GSK-3β inhibitors by Fang et al [44]

Licht-Murava and colleagues [45] have explored substrate recognition and competitive inhibition of GSK3β using molecular and computational tools. In previous work, authors described Gln89 and Asn95 within GSK-3β as important substrates binding sites. In the cited work, they show that the cavity bordered by loop 89-QDKRFKN-95, located in the vicinity of the GSK-3β catalytic core, is a promiscuous substrate binding subsite. Mutations within this segment highlighted Phe93 as an additional essential contact residue for substrates' recognition. However, unlike Gln89 and Asn95, Phe93 was also important for the binding of our previously described substrate competitive inhibitor, L803 [KEAPPAPPQS(p)P], and its cell-permeable variant L803-mts. The effects of the substitution of charged or polar residues within L803 further suggested that binding to GSK-3β is governed by hydrophobic interactions. The described computational model of GSK-3β bound to L803 was in agreement with the experimental data. It revealed L803 binding with a hydrophobic surface patch and identified interactions between Pro8 (L803) and Phe93 (GSK-3β). Computational modeling of new L803 variants predicted that inhibition would be strengthened by adding contacts with Phe93 or by increasing the hydrophobic content of the peptide. Indeed, the newly designed L803 variants showed improved inhibition. The study identified different and overlapping elements in GSK-3β substrate and inhibitor recognition and provides a novel example for model-based rational design of substrate competitive inhibitors for GSK-3.

An original application of two MIFs-based tools (Volsurf+ and Pentacle) to binary QSAR, the case of a palinurin-related data set of non-ATP competitive GSK-3β inhibitors, has recently been evaluated by Ermondi et al [46]. VolSurf+ and GRIND descriptors extract the information present in MIFs calculated by GRID: the first are simpler to interpret and generally applied to ADME-Tox topics, whereas the latter are more sophisticated and thus more suited for pharmacodynamics events. The paper describes the approach which compares binary QSAR models

obtained with VolSurf+ descriptors and GRIND for a data set of non-ATP competitive GSK-3β inhibitors chemically related to palinurin for which the biological activity is expressed in binary format. Results suggest not only that the simpler Volsurf+ descriptors are good enough to predict and chemically interpret the investigated phenomenon but also a bioactive conformation of palinurin which may guide future design of ATP non-competitive GSK-3 inhibitors.

A large number of described 3D-studies, including 3D-pharmacophore modeling and 3D-molecular docking, are based on available X-Ray data obtained for several small-molecule GSK-3β inhibitors (Fig. 6).



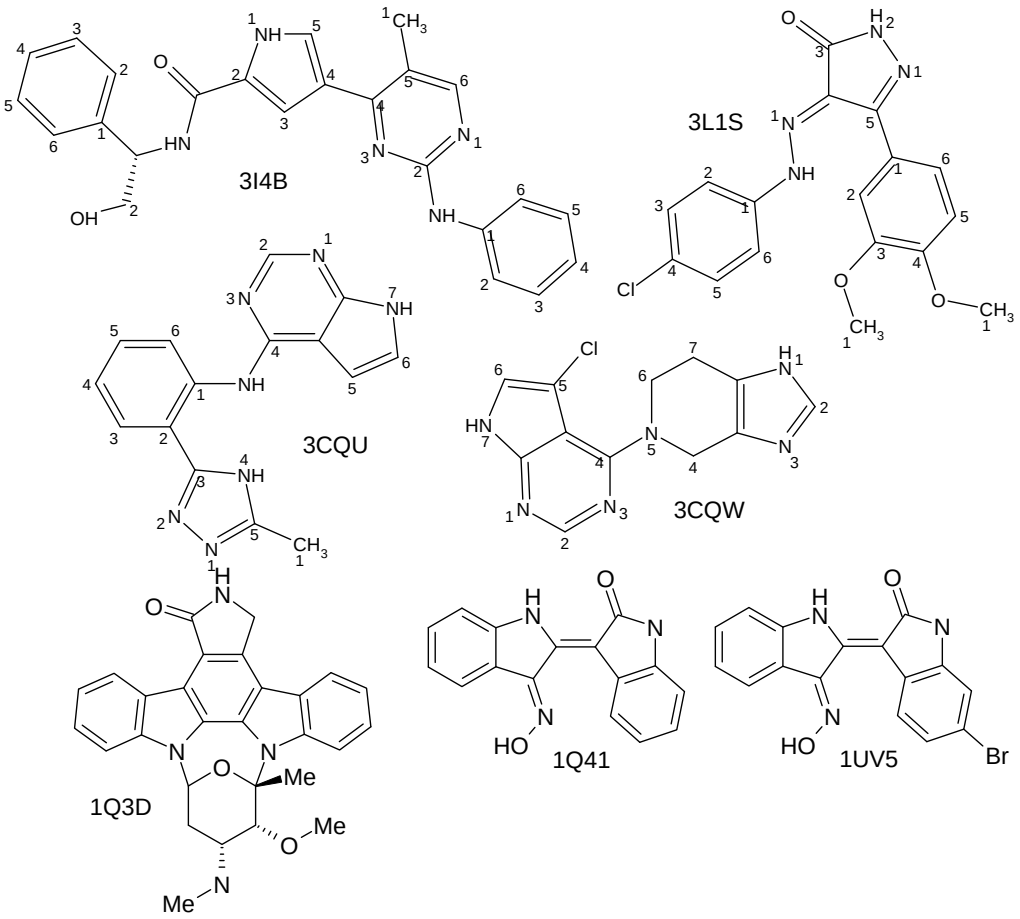



**Fig. 6**. Small-molecule GSK-3β inhibitors supported by crystallographic data.

**6. Concept and Applications**

GSK3β-targeted library design at CDL involves:

• *A combined profiling methodology that provides a consensus score and decision based on various advanced computational tools and theoretical knowledge*:

(A) Front-line approaches

1. Bioisosteric/isosteric morphing procedure
2. 2D-structural similarity approach
3. "Targeted diversity" concept applied for the library design

(B) Second-line approaches

1. 3D-molecular docking
2. 3D-pharmacophore modeling/searching
3. Methods of chemogenomics
4. Computational-based `*in silico*` ADME/Tox assessment for novel compounds includes prediction of human CYP P450-mediated metabolism and toxicity as well as many pharmacokinetic parameters, such as Human Intestinal Absorption (HIA), Plasma Protein binding (PPB), Plasma half-life time ($T_{1/2}$), Volume of distribution in human plasma ($V_d$), etc.

The fundamentals for these applications are described in a wide series of our publications on the design of exploratory small molecule chemistry for bioscreening [for related data visit ChemDiv. Inc. online source: www.chemdiv.com].

• *Synthesis, biological evaluation and SAR study for the selected structures*:

1. High-throughput synthesis with multiple parallel library validation. Synthetic protocols, building blocks and chemical strategies are available.

2. Library activity validation via bioscreening with related SAR study.

**Front-line approaches**

*Bioisosteric/isosteric morphing procedure*

Bioisosteric transformation is one of the tools that allow to balance different lead-like papameters including specificity, physicochemical and PKPD properties in the SAR studies. In addition, bioisosteric morphing provides insight into patentability of lead candidates. Numerous GSK-3β inhibitors have been designed using this technique. We have applied this approach to design of our GSK3β-targeted library. Typical examples of bioisosteric modifications for this class of molecules are shown in Fig. 7.

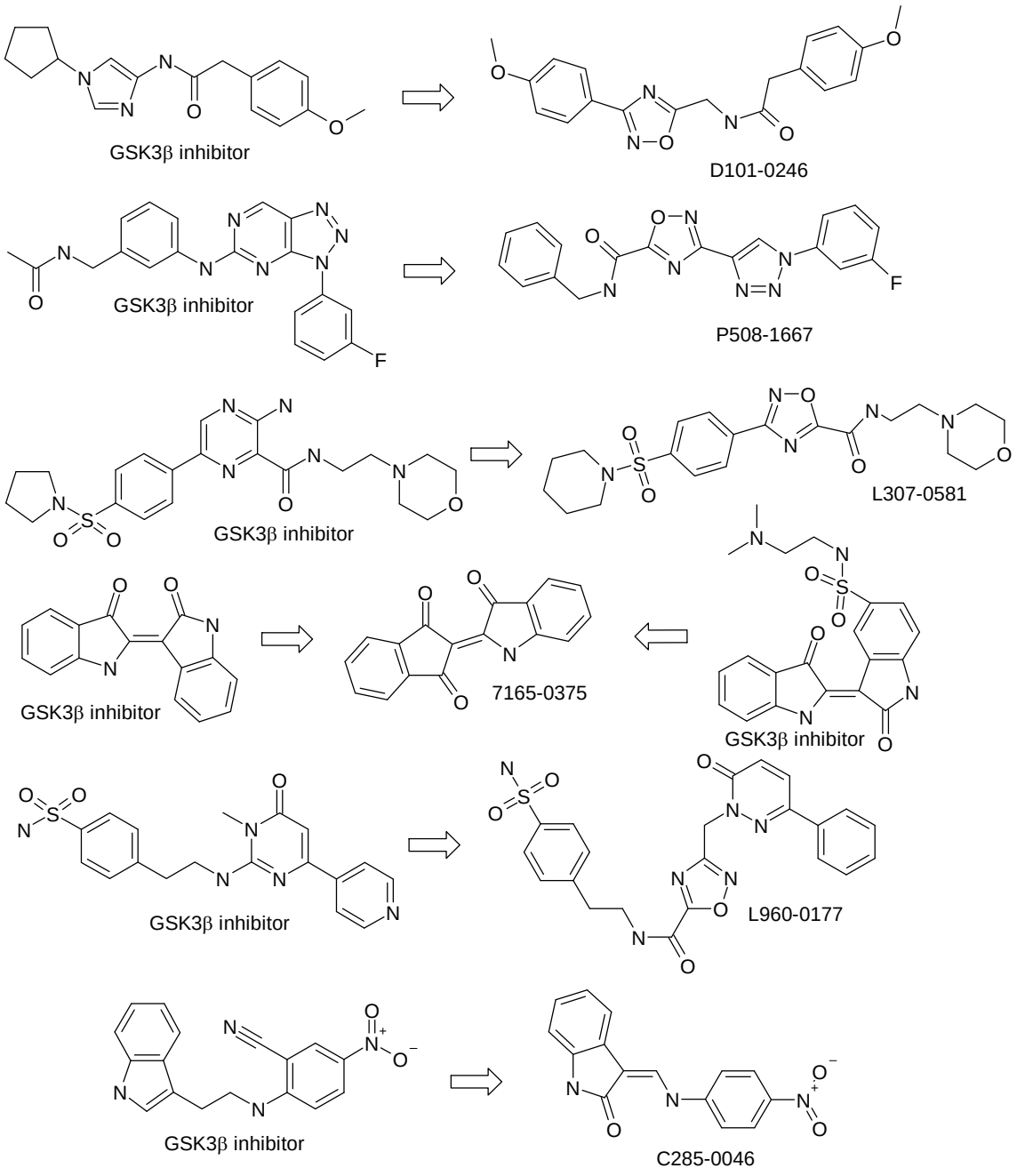
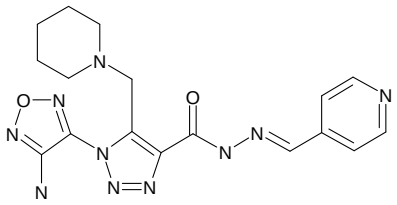
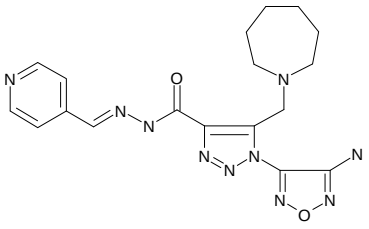
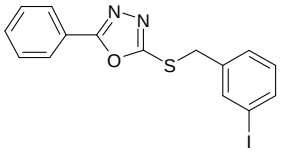
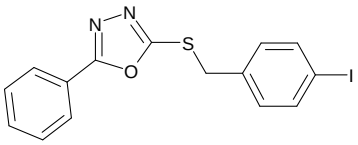
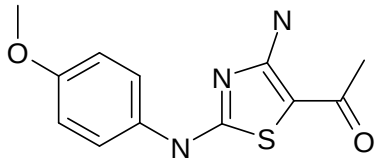
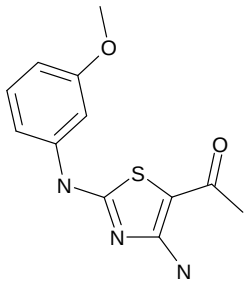
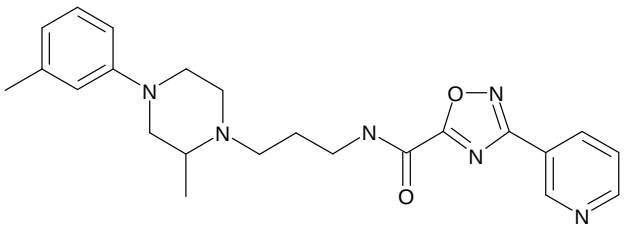
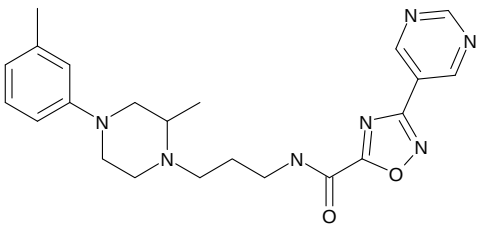
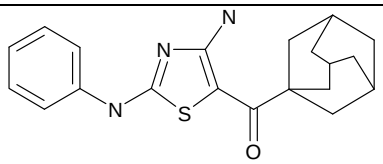
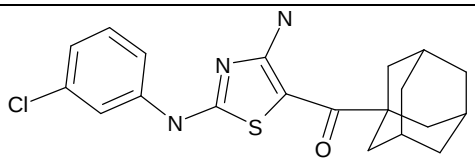



**Figure 7**. Bioisosteric transformations within GSK3β-targeted library

*2D-structural similarity approach*

We also have applied the typical 2D-similarity approach to select the most similar ChemDiv compounds to known GSK3β inhibitors (Table 2). As shown in the table below, selected ChemDiv compounds have high structural similarity toward reported GSK3β inhibitors and represent promising hit-candidates against GSK3β. Heterocyclic analogues of core moieties as well as similar peripheral fragments with regard to lipophilicity and key binding points are shown.

**Table 2**. Representative examples of most similar compounds from the targeted library to reported GSK3β inhibitors

| GSK3β inhibitor | 2D-similarity coefficient* | Compound from GSK3β-targeted library |
|---|---|---|
|  | 0.98 |  |
|  | 0.96 |  |
|  | 0.94 |  |
|  | 0.84 |  |
|  | 0.83 |  |

| | | |
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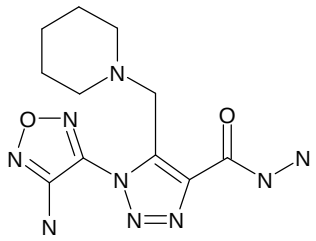
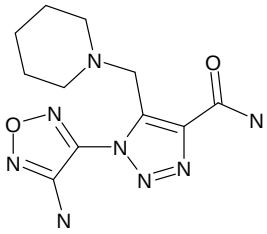
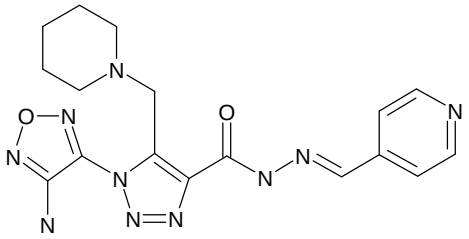
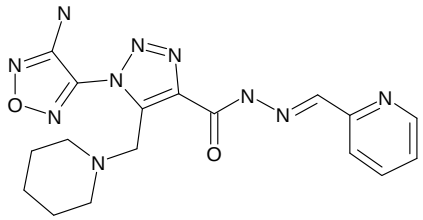
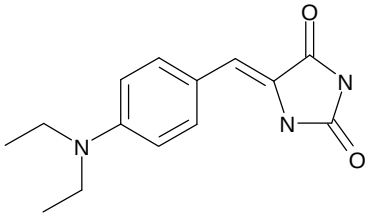
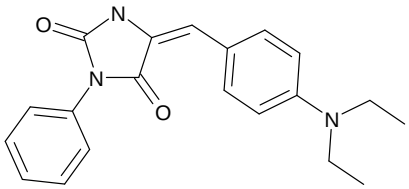
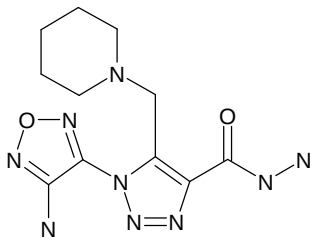
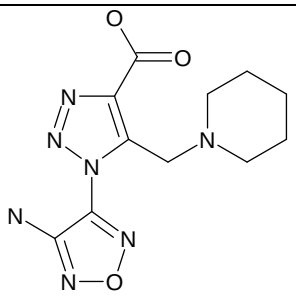
|  | 0.81 |  |
|  | 0.80 |  |
|  | 0.73 |  |
|  | 0.7 |  |

* - Tanimoto similarity measure

In addition, we also have applied the main concept of "*Targeted Diversity*" for the GSK3β-library design. Thus, we have selected a set of compounds from the common kinase library based on the fundamental principles of the underlying concept. As a result, approx. 20% of compounds within the library have been recruited following this approach.

**(B) Second-line approaches**

*3D-molecular docking*

We also have developed 3D-molecular docking model based on the obtained crystallographic data (3F7Z) for known GSK3β inhibitor (2-(1,3-benzodioxol-5-yl)-5-[(3-fluoro-4-methoxybenzyl)thio]-1,3,4-oxadiazole) (see Fig. 8). This model has been used for the assessment of most represented chemotypes within the target library. Representative examples of ChemDiv molecules with related docking score are depicted in Figs. 8 and 9. As shown in Figure 9, compounds from the target library have a similar binding mode as compared to the template molecule. Representative examples of compounds from GSK3β-targeted library are shown in Fig. 10.

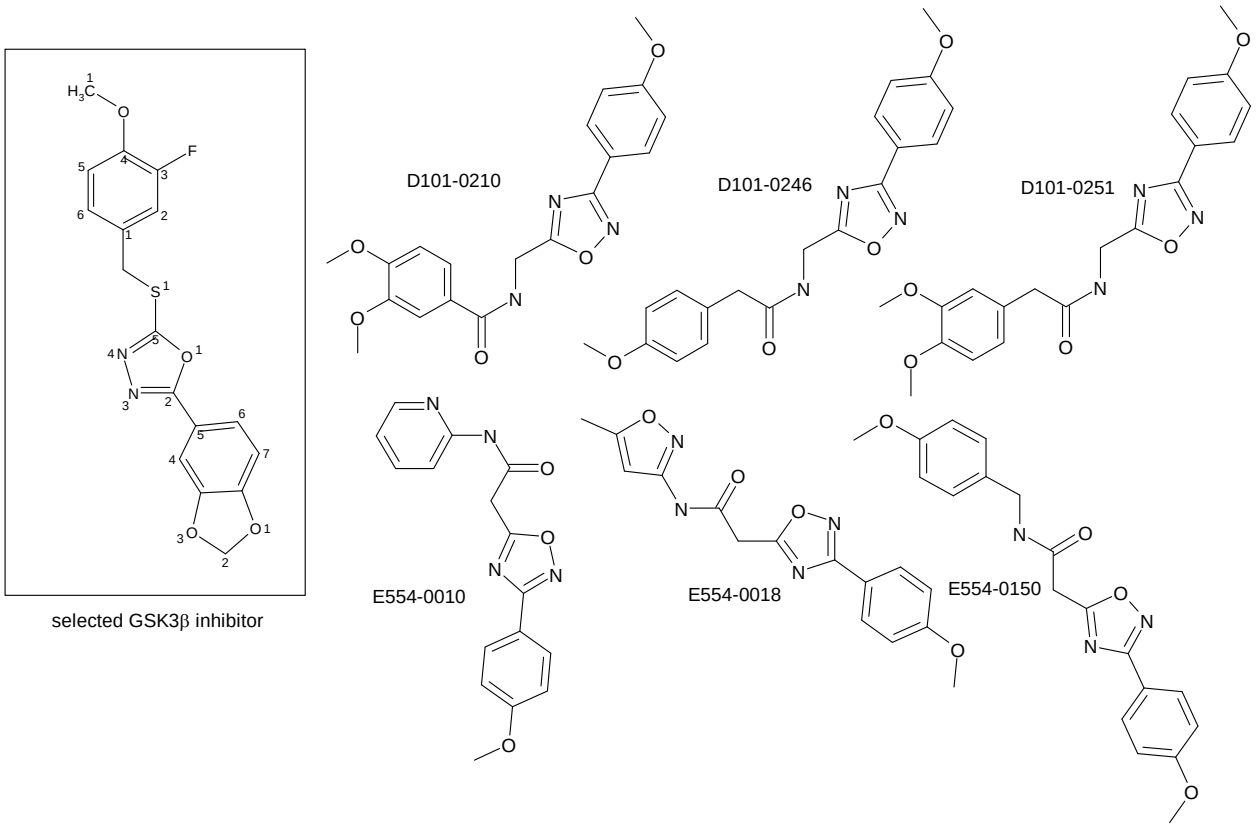
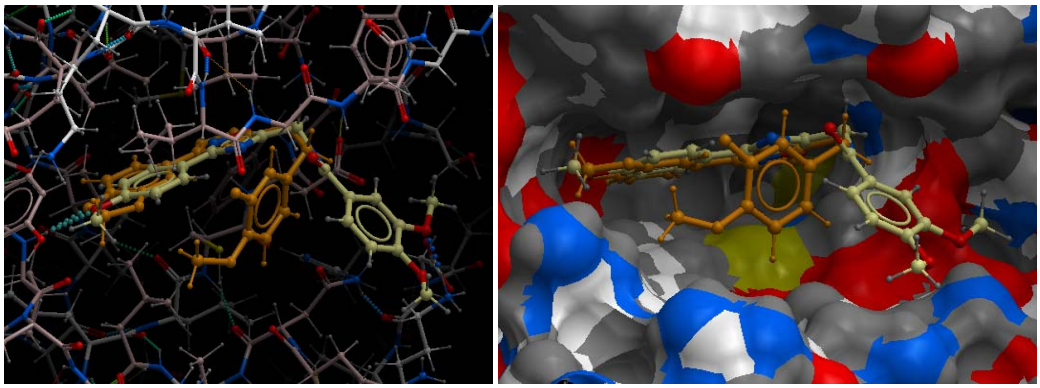
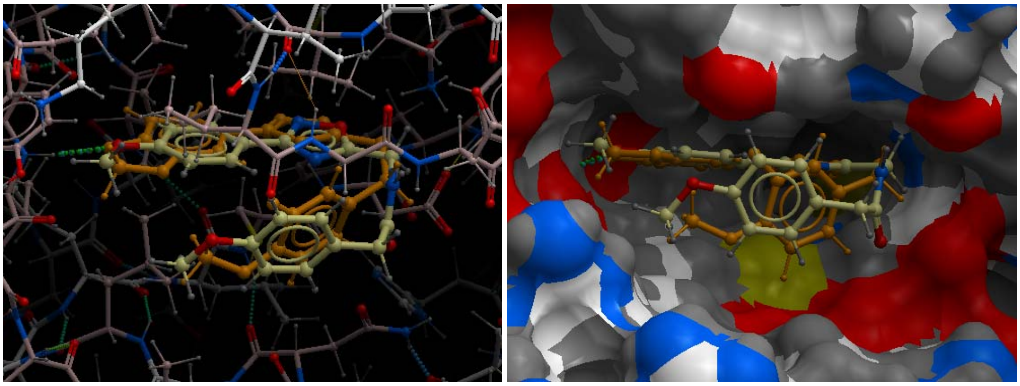


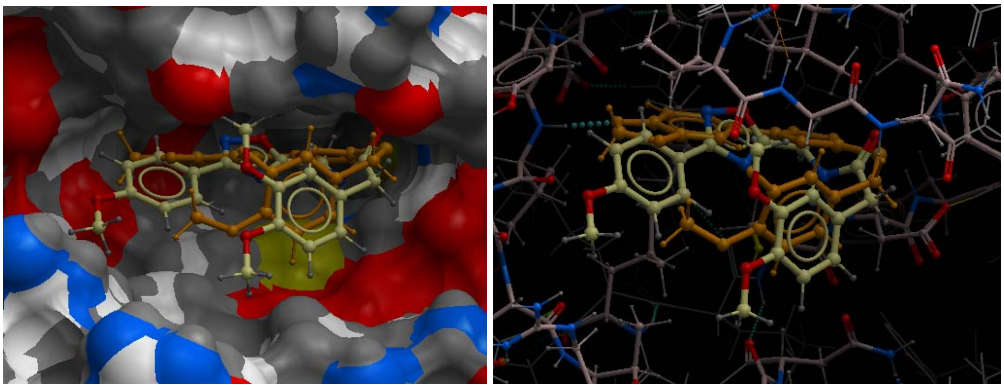

**Fig. 8**. The template GSK3β inhibitor and examples of docked compounds from the library.



**D101-0210 (MEDIUM SCORE)**



**D101-0246 (LOW/MED SCORE)**



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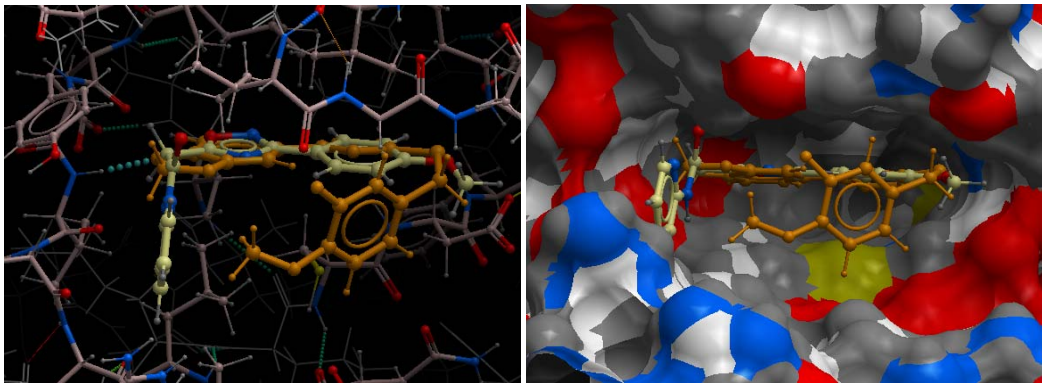

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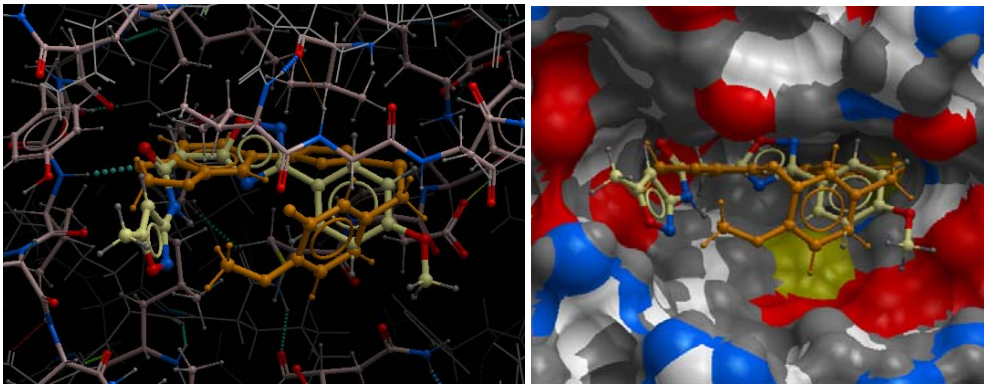

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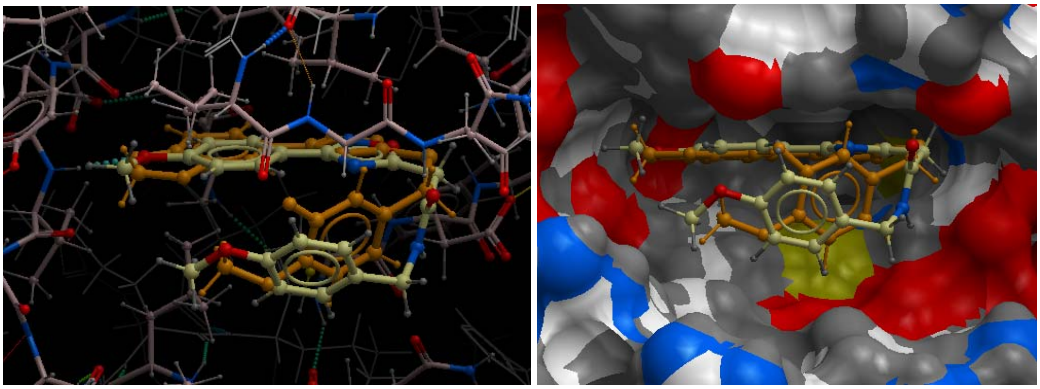

**E554-0150 (LOW/MED SCORE)**

**Fig. 9**. Examples of compounds from the most representative class in the target library with the calculated docking score

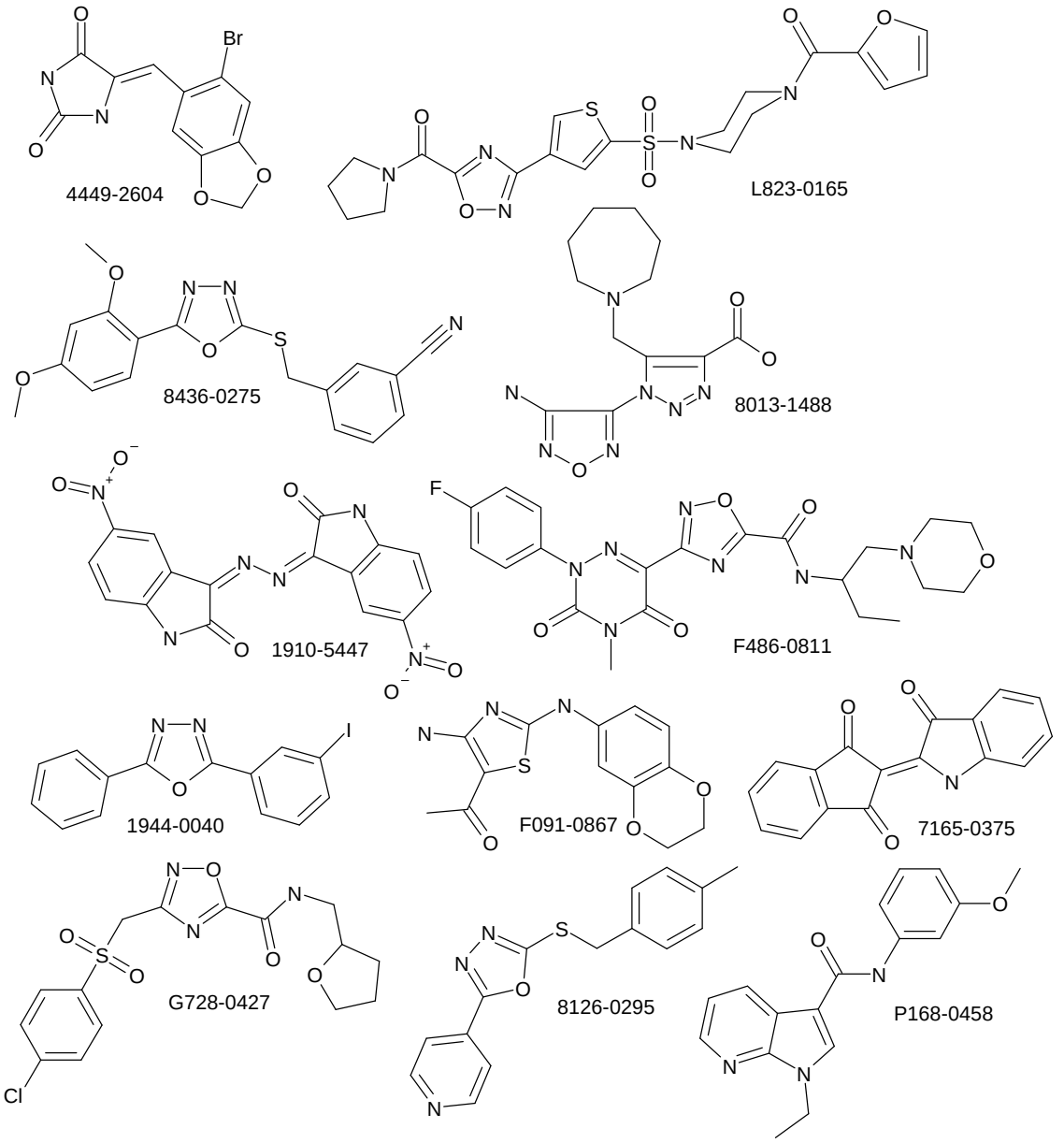


**Fig. 10**. Representative examples of compounds from GSK3β-targeted library

## Conclusion

Here we provide an efficient approach for the design of novel GSK3β-active compounds. Based on the accumulated knowledgebase, concept of target diversity as well as unique structure- and target-based computational models we have designed GSK3β-targeted library of more than 4K small molecule compounds. As a result, the library is renewed each year, proprietary compounds comprising 50-75% of the entire set. Clients are invited to participate in the template selection process prior to launch of our synthetic effort.

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