

Glycogen Synthase Kinase 3 as an Anticancer Drug Target: Novel Experimental Findings and Trends in the Design of Inhibitors

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Abstract: Glycogen synthase kinase 3 (GSK-3) is a ubiquitous serine/threonine protein kinase participating in numerous pathways. Very important among them are cancer-related pathways, such as Wnt pathway and nuclear factor κ B pathway. The recent findings concerning possible applicability of GSK-3 inhibitors as anticancer agents are summarized in this review, and controversies in the data are highlighted. The most actual concepts of GSK-3 inhibitor design are also thoroughly discussed.

Keywords: Glycogen synthase kinase 3, molecular design, cancer treatment, kinase inhibitors, Wnt pathway.

INTRODUCTION

Glycogen synthase kinase 3 (GSK-3) is a constitutively active serine/threonine protein kinase first identified in 1980 during studies of glycogen biosynthesis [1]. Further studies have shown potential therapeutic role of the inhibitors of this kinase in various diseases such as Alzheimer's disease (AD) [2], non-insulin dependent diabetes mellitus [3,4], inflammation [5,6], and cardiac hypertrophy [7]. They can also be used as cognition enhancers [8]. GSK-3 β plays a key role in the canonical Wnt pathway, where its activity is required for β -catenin degradation. Accumulation of β -catenin can lead to the uncontrolled cell growth and proliferation [9] and the tumor-like growth of embryonic stem cells [10]. Due to the activatory role of GSK-3 inhibition in Wnt pathway it was initially supposed that the pharmacological inhibition of GSK-3 would be carcinogenic, but more thorough analysis of different experimental data has revealed that it is not true in many cases, and in many other cases GSK-3 inhibition does lead to anti-cancer effect [11]. A number of concepts of GSK-3 inhibitors application in cancer treatment were introduced, but none of them reached the clinic. Examples of successful anti-cancer repositioning of well-known GSK-3 inhibitors initially targeted against diabetes or Alzheimer's disease allows one to suggest that there is no specific structural requirements making a GSK-3 inhibitor anti-cancer, or at least they are not known. Many reports are appearing in medicinal chemistry literature, establishing the importance of GSK-3 in anti-cancer agent design, and with each new report the rationale behind the interconnections of GSK-3 and cancer becomes more clearly evident.

SCOPE OF THIS REVIEW

A vast amount of information regarding GSK-3 as a drug target is available. The status of preclinical and clinical development of GSK-3 inhibitors was reviewed by Martinez *et al.* [12-14]. The number of molecules for which GSK-3 inhibition data are reported is estimated to be about 2000 for the molecules published in the journals; even more molecules can be found only in patents. Analysis of the patent data and, in particular, the data on chemotypes and substructures present in GSK-3 inhibitors, was recently performed by Phukan *et al.* [15]. Several excellent reviews were published in recent years summing up the information on available strategies of GSK-3 inhibitors application in cancer chemotherapy [16-18]; in

[16] the role of GSK-3 in tumorigenesis is thoroughly discussed, while an extensive analysis of studies of GSK-3 inhibitors as anti-cancer agents in different cancer types is given in [17]. In this review we are highlighting the data from *in vitro* and *in vivo* experiments published during the last two years paying special attention to the studies where unexpected data were obtained. Wide therapeutic potential and availability of various X-ray structures make the design of GSK-3 inhibitors very attractive for pharmaceutical companies and academic researchers. Dozens of articles are published in this field every year, and in this review we summarize mostly the studies specifically devoted to the design of anticancer GSK-3 inhibitors as well as strategic approaches to the design of GSK-3 inhibitors in general.

GENERAL REMARKS: GSK-3 STRUCTURE, FUNCTION AND REGULATION

Glycogen synthase kinase 3 is represented in the human organism by two isoforms, α and β , with β 2 splice variant available for the latter one [19,20]. The longer (51 kDa) GSK-3 α isoform is encoded in chromosome 19q13.2, the shorter (47 kDa) GSK-3 β gene is located in 3q13.33. These genes are not redundant: for example, they play different roles in the regulation of transcription [21], and expression levels in various tissues are different for the isoforms [22]. GSK-3 α (-/-) mice are characterized by the serious heart dysfunction [23], and GSK-3 β (-/-) mice die *in utero* [24]. The isoforms share high homology of the catalytic domains (97%), and α isoform bears much longer glycine-rich N-terminal tail than β isoform [19]. Majority of ATP-competitive GSK-3 inhibitors do not possess the isoform specificity.

Three regulatory phosphorylation sites were identified in GSK-3. The main site of phosphorylation is located at the N-terminal tail (α Ser21/ β Ser9), phosphorylation of this site leads to the inhibition of constitutive GSK-3 activity. The most common kinase affecting this site is Akt (also known as protein kinase B) [25], which participates in insulin pathway and memory regulation [26]. Dephosphorylation of GSK-3 on this residue with the aim to activate the kinase is usually performed by protein phosphatase PP1 [27]. Both Akt and PP1 were suggested as potential targets for the situations when GSK-3 activation should play a positive therapeutic role (see the section 'Role of GSK-3 inhibition in cancer: recent findings').

Autophosphorylation of GSK-3 on Tyr216 (β isoform numbering is used if not specified explicitly) leads to the activation of the kinase function. Nevertheless, the activity gain upon phosphorylation does not exceed 100%, and non-phosphorylated form of GSK-3 is still active enough to perform the catalytic function

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[28,29]. This site of phosphorylation is important mostly for the fine tuning of the kinase activity.

The third site of GSK-3 regulatory phosphorylation is located at the Thr390 residue belonging to the C-terminal tail, which is phosphorylated by p38 MAP kinase to inhibit GSK-3. It can be suggested that mechanism of such inhibition is similar to the mechanism of autoinhibition by pSer9 [30].

GSK-3 catalytic domain structure was thoroughly studied by X-ray crystallography: 31 structures of human GSK-3 β were available in the Protein Data Bank [31] on January 06, 2012. Among them the structures without ligands, with ATP analogues, with ATP-competitive inhibitors, and complexes with FRAT and Axin peptides are present. N- and C-terminal tails are disordered in all these structures, but short peptides containing Ser9 residue were co-crystallized with Akt. The catalytic domain of GSK-3 adopts the common kinase fold [32] with large and small lobes connected with the hinge (Fig. 1). ATP binding site is located between the lobes, and adenine moiety of ATP forms hydrogen bonds with the hinge backbone [33,34].

A commonly accepted mechanism of autoinhibition by Ser9 phosphorylation was suggested based on these X-ray studies. Substrate selectivity profile is very specific for GSK-3; this kinase strongly prefers pre-phosphorylated ('primed') substrates bearing consensus sequence (S/T)XXX[p(S/T)], where (S/T) is the site of phosphorylation by GSK-3, X is any amino acid residue, and p(S/T) is the priming site [35]. The primed phosphate binds in the positively charged site between the activation loop and loop C formed by Arg96, Arg180, and Lys205 [36,37]. Interaction of pSer9 with this site does not allow the substrate to bind, being the reason for autoinhibition of GSK-3 [25,37]. Putative binding modes for non-ATP-competitive inhibitors were suggested in the vicinity of this binding site, allowing to explain their inhibition mechanism.

Allosteric binding sites were explored on the surface of GSK-3 β by means of pocket analysis [38], and a putative binding site was detected for an inhibitor characterized by the non-competitive interaction with both ATP and peptide substrate. The binding site is located in the large lobe, and the key interactions are putatively formed by the inhibitor with Arg209 and Thr235 residues.

Lithium ions inhibit GSK-3 [39] by competition with magnesium [40,41]. The mechanism of this process was thoroughly studied by molecular dynamics and QM/MM simulations. The change of Mg²⁺ to Li⁺ was suggested to compromise the phosphoryl transfer mechanism [42], whereas other alkali metal ions lacking GSK-3 inhibitory properties (Na⁺, K⁺ and Rb⁺) cannot reside in this position and do not form stable interactions with the kinase [43] (location of this Mg²⁺ is shown in Fig. 1A).

GSK-3 plays a crucial role in the Wnt pathway, which defines the development of eukaryotic organisms. Consequently, orthologues of this kinase should be present in these organisms. It was shown that GSK-3 inhibition with 6-bromo-5-methylindirubin-3'-oxime induces apoptosis-like death in *Leishmania donovani* parasites [44]. Available sequences of parasite GSK-3s were subjected to homology modelling that revealed the possibility for design of selective anti-parasite drugs working in a similar way [45]. The selectivity can be achieved due to the presence of parasite-specific substitutions in the ATP binding sites of these enzymes.

ROLE OF GSK-3 INHIBITION IN CANCER: RECENT FINDINGS

Aberrancies of GSK-3 functioning were observed in several cancer types. Generally, there are two mutually exclusive aberrancies: (1) overinhibition of GSK-3 via phosphorylation at Ser9, and (2) overexpression of active GSK-3. The general strategy to treat the first aberrancy is inhibition of upstream kinases, most common

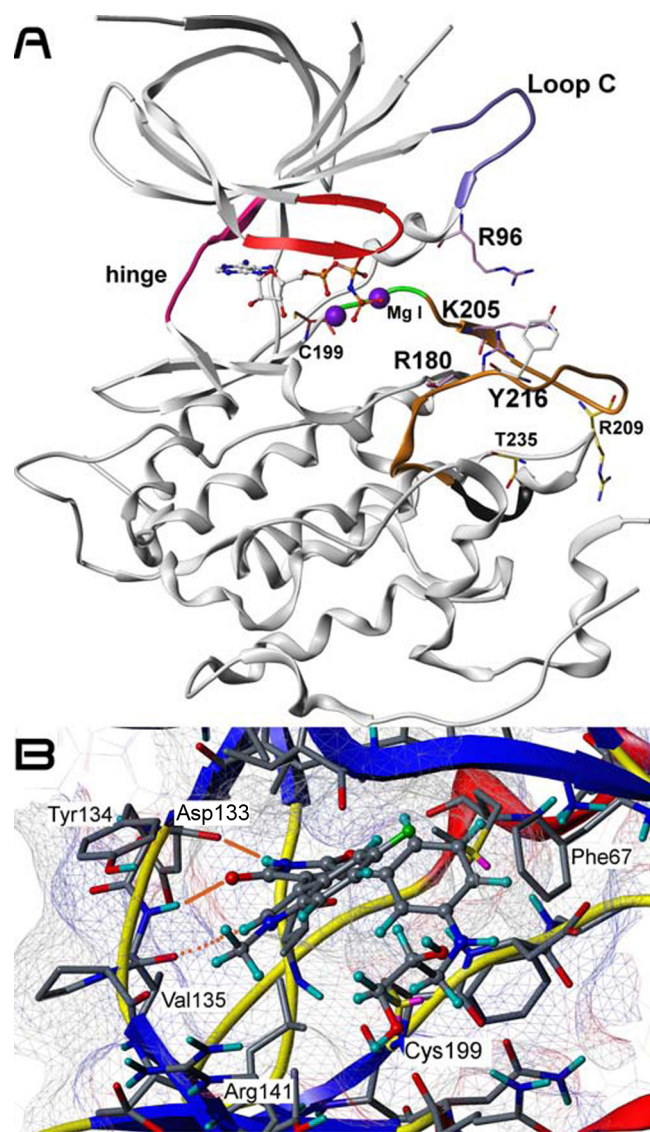


Fig. (1). X-ray structure of GSK-3 β . **A**) Overview of the catalytic domain structure (PDB ID 1PYX [34]). Non-hydrolyzable ATP analogue (AMPPNP) is shown as the balls-and-sticks model, important residues are shown as sticks (see text for discussion), and Mg²⁺ atoms are shown as balls. Glycine loop is colored red, DFG (Asp-Phe-Gly) motif is green, activation loop is orange, and APE (Ala-Pro-Glu) motif is black. **B**) Binding mode of an inhibitor in the ATP binding site (PDB ID 1R0E [103]). NH \cdots O hydrogen bonds are shown as orange lines, CH \cdots O hydrogen bond is shown as a dotted line. (The color version of the figure is available in the electronic copy of the article).

of them being Akt (Fig. 2). For example, 3-chloroacetylindole **1** inhibits Akt allosterically and lowers Ser9 phosphorylation of GSK-3 β , leading to suppression of colon cancer growth *in vivo* [46]; tumorigenic factor of colorectal cancer is downregulation of tumor suppressor 15-prostaglandin dehydrogenase by β -catenin [47]. Several series of substrate-mimetic inhibitors of Akt (e.g., **2**) were constructed based on GSK-3 β sequence, showing IC₅₀ values for Akt in micromolar range [48-50]. Reviews of anti-cancer therapeutics targeting Akt are available [51,52].

Activation of GSK-3 β by lowering the level of its Ser9 phosphorylation can also be achieved by selective inhibition of protein kinase C β , which can phosphorylate both Akt and GSK-3 β . This strategy may be used for tumor suppression in acquired immunodeficiency syndrome-related non-Hodgkin lymphoma (NHL), where

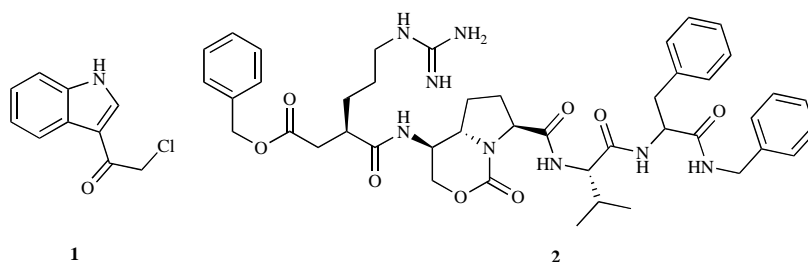


Fig. (2). Akt inhibitors.

stimulation of GSK-3 β has pro-apoptotic effects [53]. In an aggressive form of NHL, anaplastic large cell lymphoma, fusion kinase NPM-ALK is expressed, which inhibits GSK-3 β via PI3K/Akt pathway [54]. PPAR γ antagonists may also play a GSK-3 activating role in Burkitt's lymphoma, a kind of highly malignant NHL [55]. Another way of pSer9-GSK-3 β dephosphorylation leading to lowering of β -catenin level was realized with the help of methylseleninic acid in esophageal squamous cell carcinoma cells [56].

Differentiation-sensitive malignant glioma cell lines (C6, U87-MG) are characterized by overexpression and overactivation of GSK-3 β but not GSK-3 α , whereas in differentiation-resistant ones (U251, T98G) the expression of GSK-3 β is low. The enzyme controls the differentiation of the cells of these brain tumors. Induction of the GSK-3 β expression or expression of S9A mutant GSK-3 β restores the sensitivity of differentiation-resistant cells and can be used as a therapeutic paradigm in malignant brain tumors [57]. A thorough review of GSK-3 role in brain tumors and their treatment is available [58].

Grade of malignancy in breast cancers is strongly correlated with securin accumulation upon GSK-3 β inactivation [59]. Securin is a chaperone protein regulating activation of separase, which in turn regulates segregation of chromosomes to opposite ends of the cell during mitosis. Phosphorylation of securin by GSK-3 β (but not by GSK-3 α) leads to its degradation, and increased expression of securin is observed in several cancers along with inactivation of GSK-3 β [59].

The oncogenic factor of ovarian endometrioid carcinoma is MSX2, a member of homeobox protein family. The accumulation of this protein in the ovarian cancer cells is caused by activation of Wnt pathway, and use of GSK-3 β inhibitor SB216763 (**3**; Fig. 3) was shown to cause the increase of MSX2 expression [60].

Inactivation of GSK-3 β by Ser9 phosphorylation was found to be correlated with downregulation of mismatch repair gene PMS2 in nasopharyngeal carcinoma tissues [61]. It was suggested that GSK-3 activation may be a potential strategy for the future therapy of this carcinoma.

Controversial data are available on GSK-3 β role in melanoma treatment. The enzyme phosphorylates p53 tumor suppressor protein and activates its transport from nucleus and degradation in proteasomes. Multi-kinase inhibitor sorafenib (**12**; Fig. 4) activates GSK-3 via inhibition of upstream kinases and thus alters intracellular distribution of p53 to induce the programmed cell death (cell lines A375 and SKMEL5) [62]. At the same time, the use of highly selective GSK-3 β inhibitor DW12 as well as SB216763 and LiCl induces p53-dependent apoptosis in human WM793 melanoma cells [63]. Such controversy can be attributed to differences in the experiment design and/or the use of certain cell lines.

Several cancer types are characterized by overexpression of active GSK-3. Apparently the most studied of them is prostate cancer, particularly in the castration-resistant form. In this form of cancer, GSK-3 modulates the activity of androgen receptor (AR), and the inhibition of the enzyme with ATP-competitive inhibitors

(e.g., SB216763) downregulates AR to suppress AR-mediated cell growth *in vitro* [64] and *in vivo* [65]. Interestingly, another GSK-3 competitive inhibitor AR-A014418 (**4**) showed AR activation in GSK-3 independent manner in the same study [64]. Non-ATP-competitive GSK-3 inhibitors (lithium chloride, TDZD-8 (**5**), and L803-mts (**7**)) were tested in mouse xenograft models of prostate cancer and led to a significant inhibition of the tumour growth [66].

A large body of evidence exists on anti-cancer activity of GSK-3 inhibitors in pancreatic cancers [67]. Selective GSK-3 β inhibitor manzamine A (**6**) [68] was shown to reduce the metastatic potential of AsPC-1 pancreatic cancer cells and to sensitize them to TRAIL-induced apoptosis [69]. This result was obtained on the cell line, and it was not established whether GSK-3 inhibition is a real driving force of such effect. Another recent study showed very promising results of GSK-3 β inhibition by SB216763, which lead to a specific induction of apoptosis in the pancreatic cancer cells (PANC1 and MIA PaCa-2) *via* JNK pathway activation leaving the non-transformed pancreatic cells intact [70]. Inhibition of GSK-3 with AR-A014418 also may sensitize PANC1 cells to gemcitabine, a standard pancreatic cancer chemotherapy drug, via modification of Rb/E2F pathway but not through NF- κ B inhibition [71,72]. Similarly to other cancer types, GSK-3 β activation via gene silencing of galectin-3 induces β -catenin degradation and suppresses the cancer cell migration and tumor growth in PNC1, AsPC-1 and BxPc-3 pancreatic cancer cell lines [73]. A series of benzofuran-3-yl-(indol-3-yl)maleimides was designed and successfully inhibited the proliferation of pancreatic cancer cells [74].

Increase of cyclin-dependent protein kinase inhibitor p21 level leads to the suppression of the bladder tumorigenesis caused by deletion of *Pten* gene. The inhibition of GSK-3 β and, to a lesser extent, GSK-3 α , led to the induction of p21 in a β -catenin independent manner [75]. The best effect in inhibition of the urothelial carcinoma cell proliferation was achieved using a combination of GSK-3 inhibitor SB216763 and PI3-kinase inhibitor LY294002 (**13**), since the activity of PI3-kinase/Akt pathway inhibits GSK-3 [75].

Inhibition of GSK-3 β by CHIR99021 (**11**), initially designed as anti-diabetic compound, suppresses the visfatin-induced proliferation of hepatoma cells HepG2 [76]. Inhibition of GSK-3 also suppresses obesity-induced liver tumorigenesis [77]. Obesity may also induce the elevation of tumor necrosis factor α , which suppresses the activity of GSK-3 β in colon and may result in Wnt-mediated tumorigenesis [78]. Interestingly, pro-apoptotic role of GSK-3 inhibition by lithium chloride was also shown in colorectal cancer cells [79]. Thus, GSK-3 inhibitors may perform as a double-action drugs, because type II diabetes mellitus is also strongly related to obesity; this double action could be useful in the treatment of both obesity-induced diabetes and tumors.

Human renal cell carcinoma (RCC) is characterised by aberrant accumulation of GSK-3 β in nucleus leading to cancer cell proliferation and survival via nuclear factor κ B (NF- κ B)-mediated expression of Bcl-2 and XIAP. Pharmacological inhibition of GSK-3 β by AR-A014418, SB216763 and TDZD-8 decreased the viability of

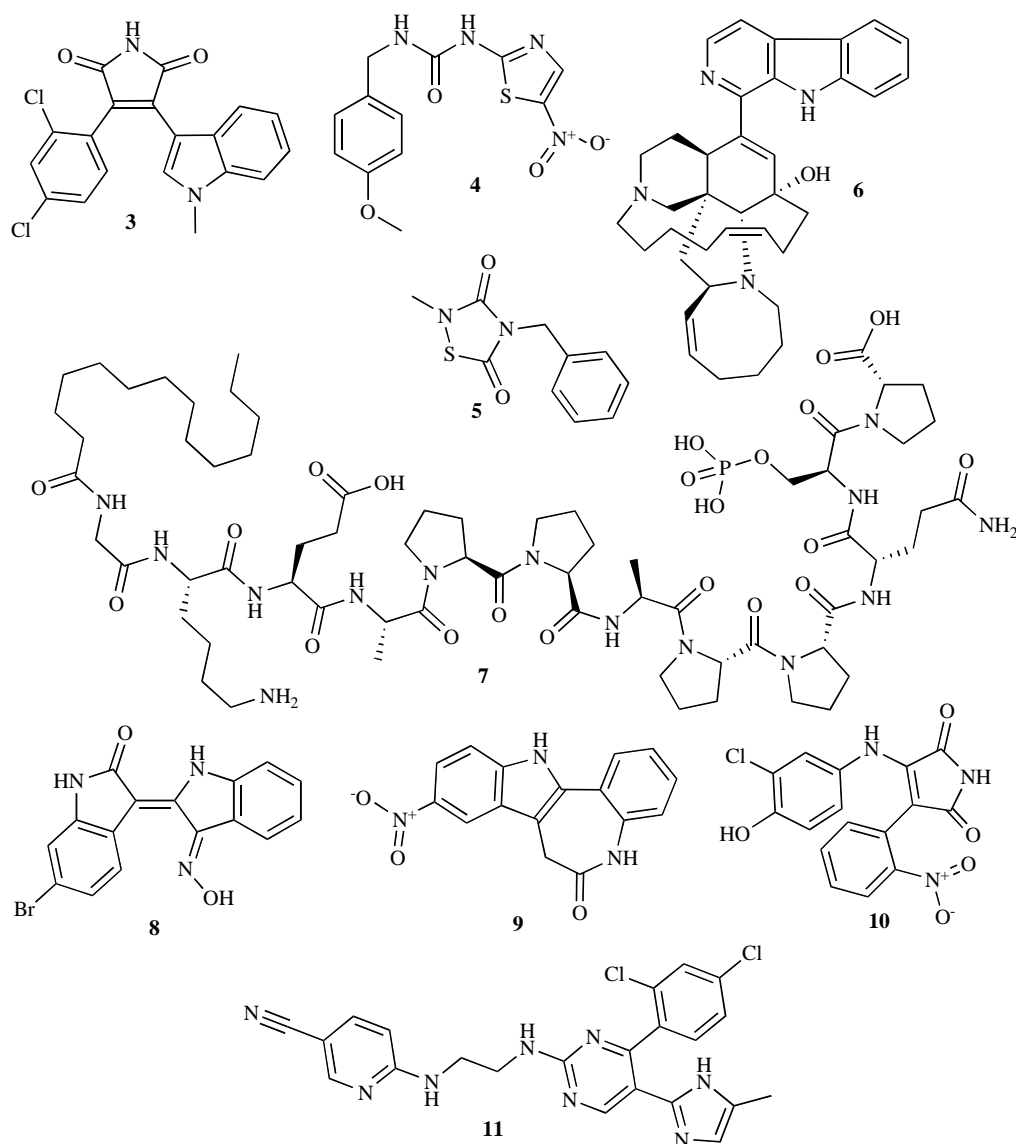


Fig. (3). Standard and widely used GSK-3 inhibitors.

cancer cells in dose- and time-dependent manner [80]. Analogous data were obtained for the same inhibitors in chronic lymphocytic leukemia B cells [81]. Synergistic effects were revealed for the combination of AR-A014418 and Docetaxel (**14**), chemotherapeutic drug with limited effect on RCC when administered alone, allowing one to suggest that such combination can be promising in the course of the treatment of human RCC [80]. Another way of GSK-3 β regulation in RCC may be re-expression of miR-199a miRNA [82].

The role of GSK-3 in leukemia was also studied rather well. Initially it was shown that GSK-3 inhibitors (SB216763, 6-bromoindirubin-3'-oxime (BIO, **8**), and alsterpaullone **9**) show inhibitory effects on specific leukemia cell lines characterised by mutations of the proto-oncogene *MLL* (mixed lineage leukemia) [83]. Later it was shown that different inhibitors (SB216763, SB415286 (**10**), and BIO) suppress cell growth in various leukemia cell lines, acute myeloid leukemia, acute lymphoblastic leukemia, and myelodysplastic syndrome [84]. BIO was also tested in an animal model of leukemia and showed >75% increase of survival time with no signs of tumor formation [84]. This effect is not unanticipated, because

indirubin is the main active ingredient of traditional Chinese plant mixture Danggai Longhui Wan, which can treat chronic myelocytic leukemia [85]. Such effect was attributed to the inhibition of cyclin-dependent kinases [85], but in later studies on other leukemias it was shown that in these cases GSK-3 inhibition is the main driving force of antiproliferative activity [83]. Induction of apoptosis in leukemic cells treated with SB415286 or lithium was shown to be mediated by depolarization of mitochondrial membrane and dephosphorylation of Bcl-2 [86,87]. Another mechanism of apoptosis induction in leukemic cells is related to the indirect repression of c-Myb function due to the inhibition of GSK-3 [88].

Stimulation of the inhibitory phosphorylation of GSK-3 may be an alternative strategy when direct inhibition of the enzyme is ineffective or needed to be avoided. One way of such stimulation is the inhibition of protein phosphatase PP1, which dephosphorylates pSer9-GSK-3 β . For example, antifungal antibiotics tautomycin **16a** and tautomycetin **16b** were shown to suppress the growth of medullary thyroid cancer cells via this mechanism [89]. Upregulation of the active form of Akt by rutin **15** and flavonoids from

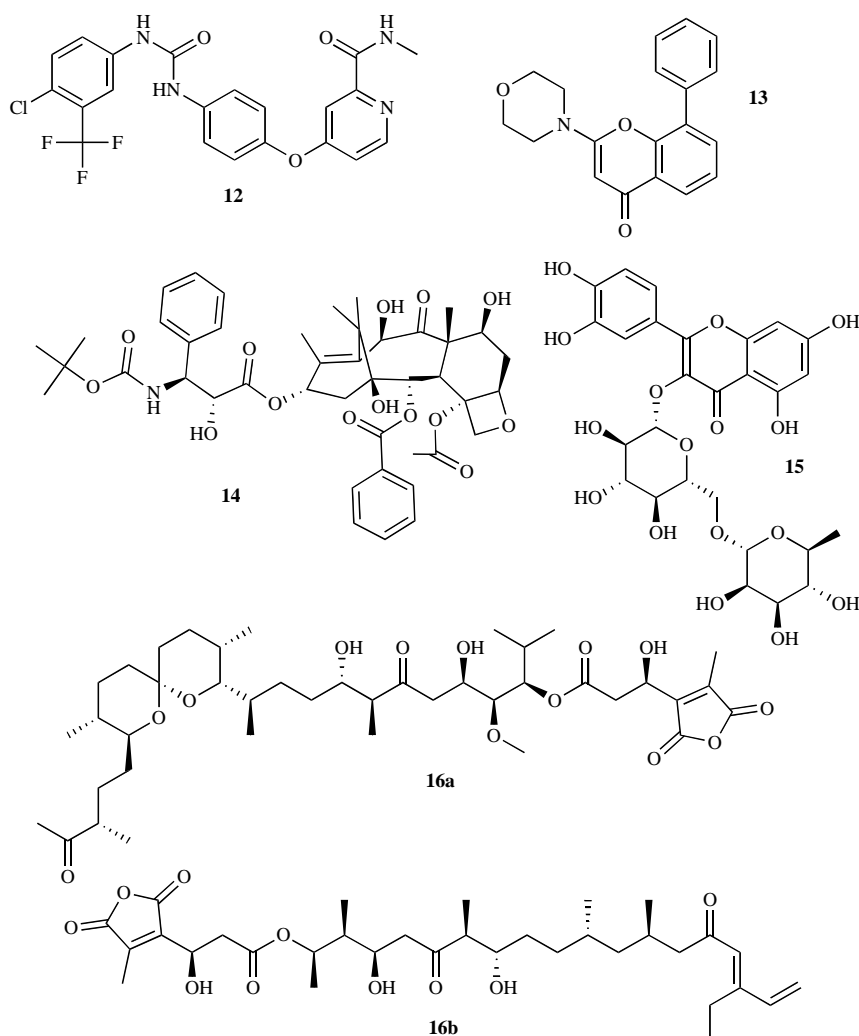


Fig. (4). Synergists of GSK-3 inhibitors (12-14); indirect inhibitors of GSK-3 (15-16).

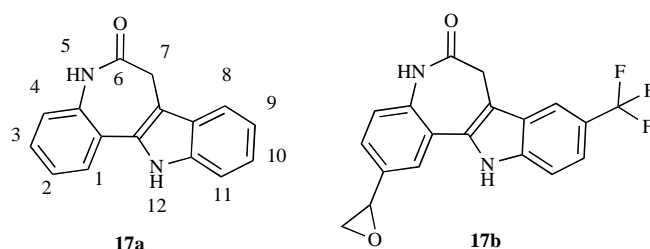


Fig. (5). Paullones.

Hammada scoparia extract also leads to the increased phosphorylation of GSK-3 β and apoptosis of adherent leukemic cells [90].

A large body of evidence exists on the positive role of GSK-3 inhibition in cancer treatment. Availability of seemingly controversial data suggests the need for a thorough study of the tumor before the choice of treatment, and in a number of specific tumors GSK-3 inhibitors will have the curative properties. Thus, GSK-3 inhibition represents a vital strategy in cancer treatment.

DESIGN OF GSK-3 INHIBITORS

The clinical status of many GSK-3 inhibitors was reviewed earlier [12-14]. The situation with the standard tools for GSK-3

studies did not change since those times dramatically, and most *in vitro* and *in vivo* studies are still performed using SB216763, AR-A014418, TDZD-8 and other compounds mentioned in the previous section. Clinical studies of GSK-3 inhibitors are underway, but lithium remains the only GSK-3 inhibitor approved for the use in humans [3, 12-14]. Design of newer compounds is aimed to achieve better therapeutic applicability and efficacy of molecules.

Availability of a number of different sites on the surface of GSK-3 makes possible the design of inhibitors with different mechanisms of action. The most common approach is the design of ATP-competitive inhibitors. Several successes were achieved in the design of substrate-competitive inhibitors; irreversible binding was

recently revealed for one of such inhibitors. Very recently, purely allosteric inhibitors were identified, which act in non-ATP-competitive and non-substrate-competitive manner.

ATP-COMPETITIVE INHIBITORS OF GSK-3

ATP-competitive inhibitory activity against GSK-3 was shown for the molecules belonging to various unrelated classes. Typical representatives of such molecules were mentioned in the previous section; a variety of other molecules were also suggested.

Three classes of molecules can be called “classic” GSK-3 inhibitors as they were known before wide studies of this enzyme and were initially targeted to other kinases. 2,3-Bis-arylmaleimides (**18**; Fig. 6) were designed twenty years ago as simplified analogues of staurosporine (**24**; Fig. 6), acting as protein kinase C inhibitors [91,92], and their inhibitory activity against GSK-3 was found seven years later [93]. Indirubins (Fig. 8) were identified in plant extracts [85] and initially positioned as inhibitors of cyclin-dependent kinases (CDKs); GSK-3 inhibitory activity of these compounds was published two years later [94]. Paullones (**17a**; Fig. 5) were identified during screening of NCI compounds against CDKs [95], and antiproliferative activity was initially shown for paullones only as the result of CDK inhibition [96]. Introduction of

epoxide sidechains increases antiproliferative activity of paullones and makes GSK-3 inhibition more effective (e.g., **17b**) [97].

Anticancer activity of bis-arylmaleimides has been the reason for thorough exploration of this scaffold. Substitution of single aryl moiety to more flexible arylalkylaminomoiety has been shown to be a viable strategy for the design of inhibitors; one hit identified in such a way (IM-12, **19**) showed substantial Wnt pathway activating properties [98]. Substitution of maleimide moiety to pyrazolone led to a spectacular selectivity profile of the resulting molecule **20** [99]; though, it was not tested in cancer cell lines.

Computer-aided molecular design of bis-arylmaleimides meets several problems lying in the conformational space of these molecules. As opposed to staurosporine, two conformations are usually available for these molecules, which we refer to as “folded” and “extended”, the latter one being energetically less favourable. In the folded conformation π - π interactions are present between the aromatic substituents, whereas the extended conformation is roughly planar and only slight van-der-Waals interactions appear between the aromatic substituents. X-ray studies show that extended conformation can be stabilized only in conformationally restricted molecules like PDK1 inhibitor LY333531 (**21**; PDB ID 1UU3) [100], whereas for macrocyclic (**22**; PDB ID 2OW3) [101] or non-

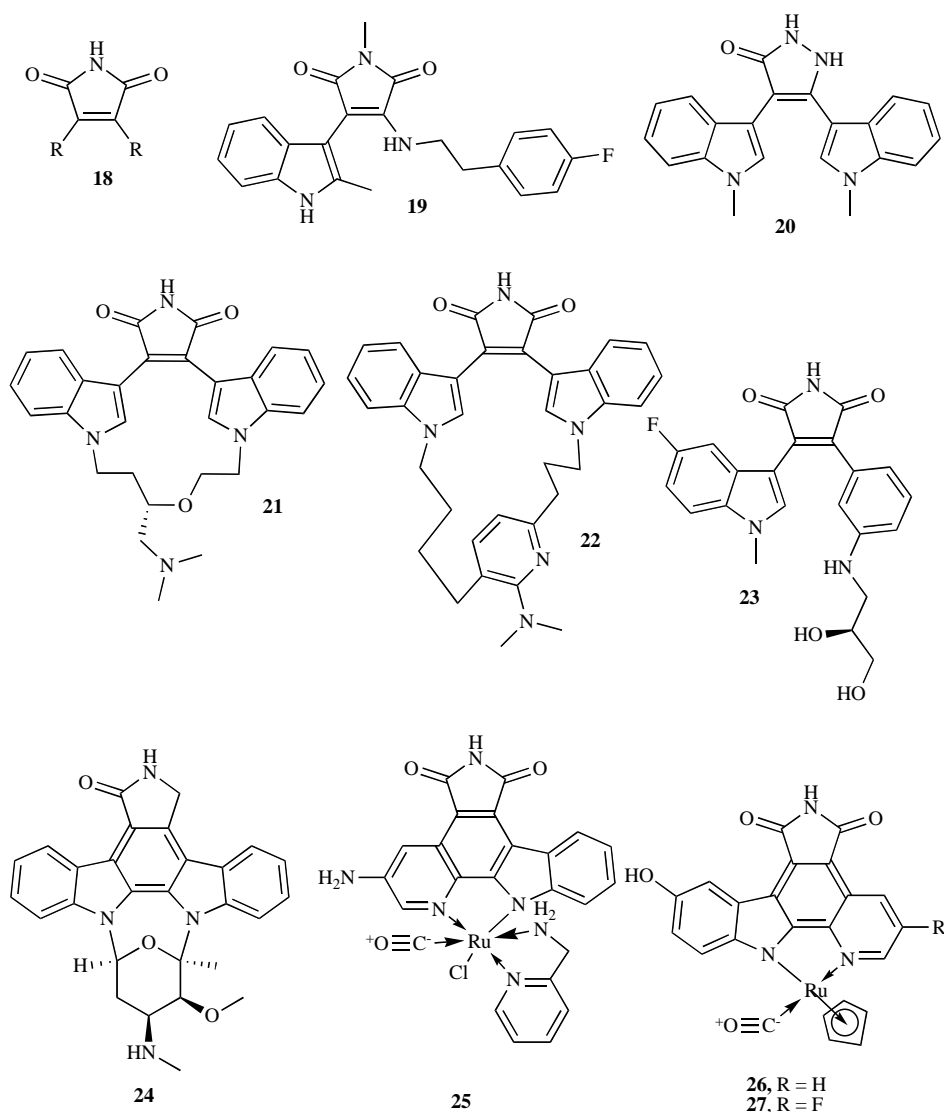


Fig. (6). Bis-arylmaleimides and related compounds.

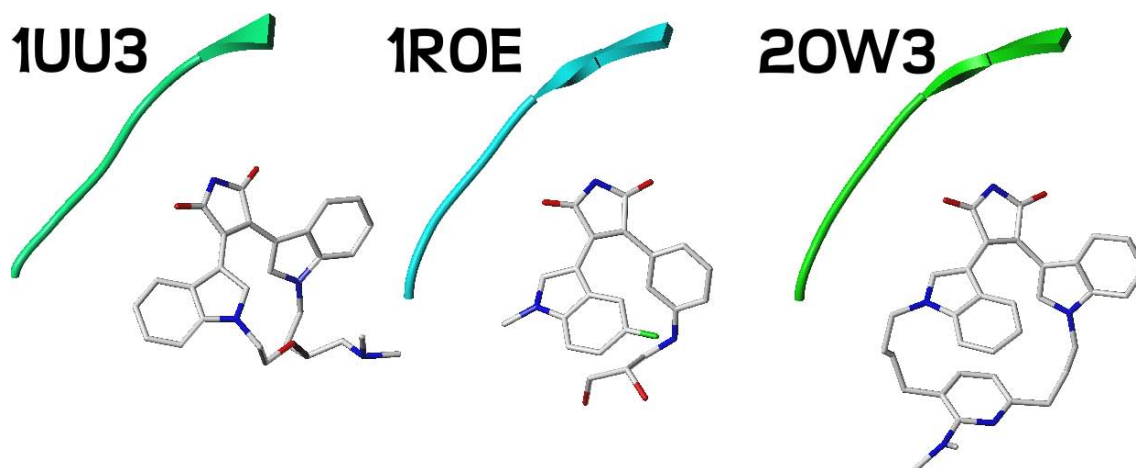


Fig. (7). Superposition of representative kinase-bound maleimide ligands aligned by hinge backbone (shown in cartoon representation). We refer to 1UU3 ligand conformation as «extended» and 1R0E/2OW3 conformation as «folded». Note that in the folded conformation six-membered ring of the indole on the left forms π - π interactions with the aromatic moiety on the right.

cyclic molecules the folded conformation is more favourable (**23**; PDB ID 1R0E) [102] (Fig. 7). When 3D structures of molecules are generated or docked, it is the usual situation when extended conformation is generated instead of the folded one due to the high barrier of conformational transition [103-105]. This situation may be not very dramatic in QSAR studies, even in 3D-QSAR case, because only the presence and relative positions of functional groups influence the model quality substantially. Nevertheless, in docking studies the conformation of the ligand is vitally important, and rationalization of docking-based data, for example, in 3D-QSAR analysis based on different predicted binding modes, allows to distinguish between them and choose the one that better describes the quantitative binding data [106]. Another problem that may appear in QSAR modelling of maleimide derivatives is their pseudosymmetry: two similar substituents are attached to the equivalent attachment points of the maleimide moiety, and dissimilarities may be counted wrong if wrong orientation is chosen for the molecule. To overcome these difficulties, Baskin *et al.* developed the learned symmetry concept, where copies of compounds with permuted substituents were introduced into the training set to increase the quality of models [107].

An elegant approach to the design of selective GSK-3 inhibitors was developed based on the use of stable organometallic compounds as bioisosteres of staurosporine **24**, a well-known multi-kinase inhibitor. The aliphatic cycle of staurosporine can be substituted to octahedral metal complex, leading to several advantages: easier synthetic accessibility, larger substitution variability, large number of possible stereoisomers, ability to design small rigid globular structures organised around the metal centre [108]. Such compounds, named octasporines, opened an easy way to the design of highly selective inhibitors: rather subtle structure changes allowed the design of very selective inhibitors of specific kinases. Strikingly, the Λ -OS1 (**25**) is the inhibitor of GSK-3 α with 7-fold selectivity to GSK-3 β , and no structural basis for such selectivity can be suggested due to the large distance between ATP-binding site and substitution sites differing these two isoforms [108]. Anticancer activity due to GSK-3 β inhibition was found for closely related compounds: DW12 (**26**) induces apoptosis in human melanoma cells [62] and colon carcinoma cells [109], and NP309 (**27**) also induces apoptosis in Burkitt-like lymphoma (BJAB cells), vincristine-, and daunorubicin-resistant leukemia cells [109].

Studies of indirubin scaffold (**28**; Figure 8) were much less diversity-oriented and mostly included introduction of small substituents into the core of the molecules. The most general

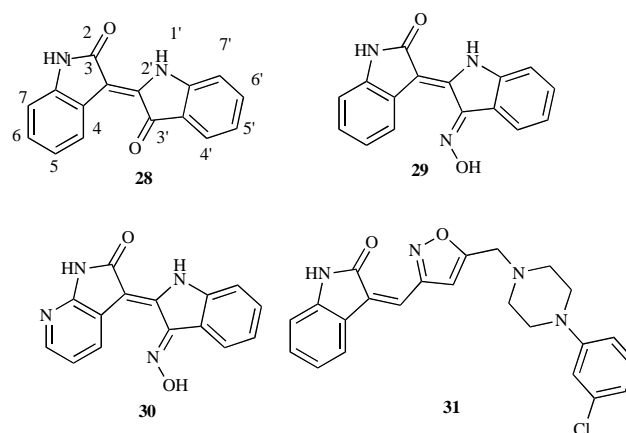


Fig. (8). Indirubins.

substitution was the introduction of 3'-oxime group (**29**), and various analogues of indirubin-3'-oxime (e.g., **8**) are widely used as standard GSK-3 inhibitors in many high-throughput screening as well as *in vitro* or *in vivo* studies [110-112]. Low selectivity of many indirubins against CDKs can often be a stumbling block, but evidences exist that dual inhibition of these kinases may be therapeutically useful [113]. Interestingly, the close relative of indirubins, 7-azaindirubin-3'-oxime **30** does not show inhibitory activity on any of tested CDK or GSK-3 β , but does possess antiproliferative activity, putatively not related to kinase inhibition [114].

The idea to “split” indirubin molecule into two parts and to use only one of them in further studies was employed to design a novel series of isoxazol-indolin-2-one derivatives (e.g., **31**) [115]. The anticancer activity of these compounds was shown in prostate and colon cancer cell lines.

Several GSK-3 inhibitors were identified after filtering databases of compounds through simple pharmacophore models [116, 3]. Classical medicinal chemistry optimization of leads performed at pharmaceutical companies allowed them to develop such advanced inhibitors as CHIR99021 (**11**) [3]. These studies played an important role in the generation of knowledge about GSK-3 inhibitors, and their result was brilliant in the course of availability of only general data as input. Such strategy of scaffold-oriented synthesis is still used, and novel lead compounds are being identified (e.g., **32**; Fig. 9) [117], and medicinal chemistry optimization of hits

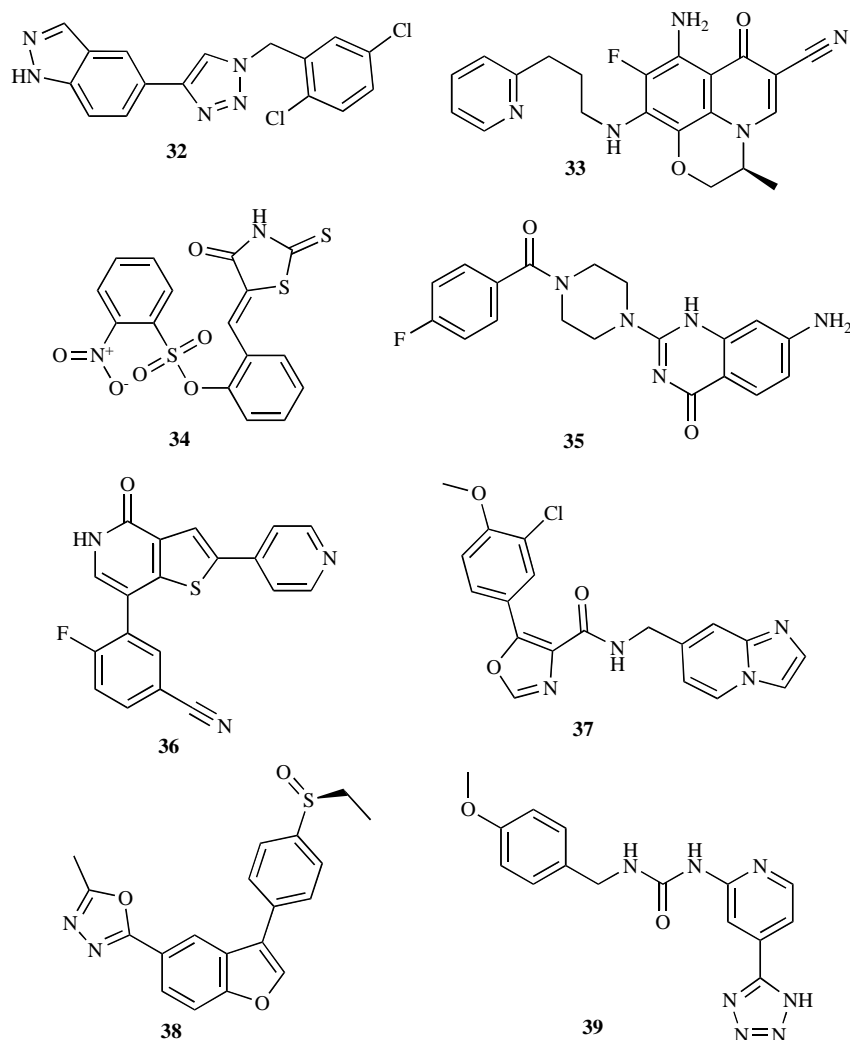


Fig. (9). Novel GSK-3 inhibitors.

allows to find molecules with picomolar activity (e.g., **33**) [118, 119].

More advanced 3D pharmacophore construction techniques (HipHop pharmacophore model supported by a recursive partitioning tree) were used for the identification of novel inhibitors with micromolar activity via virtual screening (e.g., **34**); no further development was reported [120]. Fragment-based design of molecules fitting the 3D pharmacophore model (Catalyst implementation) revealed a number of putative inhibitors (e.g., **35**), but no experimental testing of these molecules was performed [121].

High-throughput screening of large databases of compounds revealed a number of valuable hits. Among the newest ones are 2-(4-pyridyl)thienopyridinones **36**, which showed unusual binding modes after co-crystallization with GSK-3 β : they formed a sole hydrogen bond with the hinge Val135 backbone NH via their pyridine moiety [122]. Another hydrogen bond was formed with a water molecule in the vicinity of Asp200 belonging to DFG motif. Substitution of pyridyl moiety to other hinge-binding groups led to deterioration of the selectivity profile of compounds.

The CH \cdots O hydrogen bonds are quite common in protein-ligand complexes [123] but usually underrepresented in the computer-aided drug design software. Such bonds are present in at least a half of available X-ray structures of GSK-3 complexes with inhibitors. They were utilized in a design strategy to choose the best

heterocyclic moiety, and the best binding energy was achieved for a compound with such hydrogen bond [124]. The CH \cdots O hydrogen bonds are routinely present in many novel GSK-3 inhibitors such as 1,3-oxazoles **37** [125], 1,3,4-oxadiazoles **38** [126,127], and 1-aryl-3-benzylureas (**4**, **39**) [128,129], and represent a certain problem for the docking-based design and optimization of compounds [130].

Generation of a large body of experimental data on GSK-3 inhibitors allowed us to apply cheminformatics methods to the analysis of these data. Whereas most studies rely on the analysis of a series of structurally related compounds, availability of data on structurally unrelated ones opens the possibility for generalizations. We have analyzed the performance of different virtual screening techniques and performed a large-scale virtual screening campaign to identify novel scaffolds potentially able to inhibit GSK-3 [131].

Two peculiarities highlight this study among others: (1) the use of a large dataset of true inhibitors and true non-inhibitors (decoys), where substantial amount of decoys were experimentally shown not to inhibit the target protein [132], and (2) the use of three ideologically different virtual screening methods [133]: molecular docking [130], pharmacophore modelling, and one-class classification (OCC) [134]. The set of inhibitors consists of 1685 molecules published up to August 2010 and was manually curated and checked for data quality and consistency (1226 inhibitors and 209 decoys were used in [130] and [134], then the database was slightly ex-

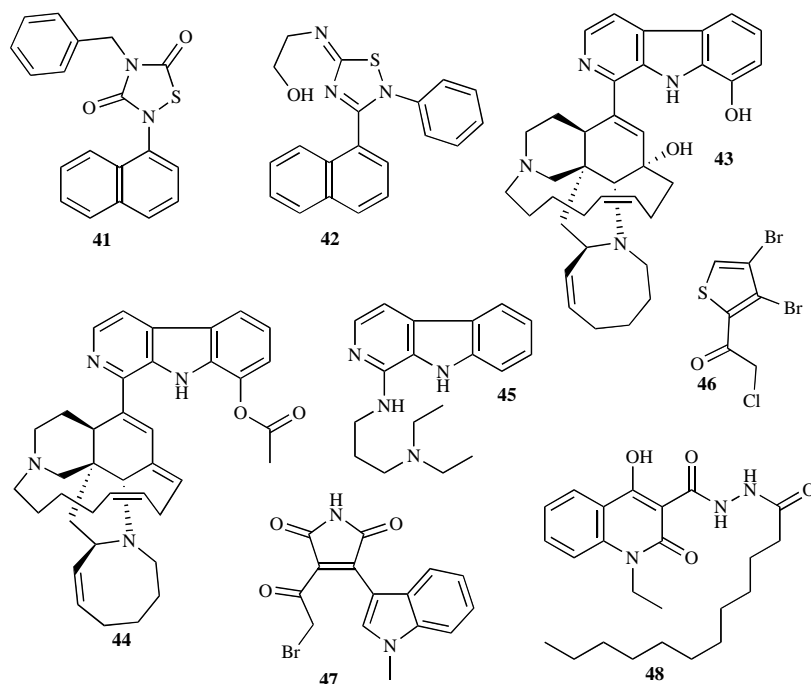


Fig. (11). Non-ATP-competitive GSK-3 inhibitors.

The most developed non-ATP-competitive GSK-3 inhibitor tidesglusib (**41**; Fig. 11) (now at phase II clinical trials as anti-Alzheimer's drug candidate) belongs to the class of thiazolidinones (TDZD). Compounds of this class were initially synthesized as potassium channel openers [148] and then were repurposed as GSK-3 β inhibitors revealing the non-ATP-competitive mechanism of action [149]. The most known of these compounds is TDZD-8 (**5**), now commonly used as a standard selective GSK-3 inhibitor. The initial hypothesis of TDZD binding suggested the interaction with the priming phosphate binding site via the formation of hydrogen bonds and stacking of the aryl group with the Tyr216 sidechain [149]. This theory was later adjusted with the help of molecular docking [150] and pocket analysis [38], expanding the definition of TDZD binding site to the cavity between activation loop, glycine loop and loop C. It was later shown that the presence of carbonyl groups is not crucial for TDZDs and TDZD-like compounds such as ITDZs (**42**) and that the latter compounds inhibit GSK-3 β in a substrate-competitive manner [151,152]. Good brain permeability properties were shown for these compounds, making them applicable for AD treatment.

Another strategy was employed for the design of substrate-competitive inhibitors based on the substrate-mimicking peptides. In this strategy the need for priming phosphate in the substrate was employed; the substrate sequence was modified to achieve the best inhibitory properties. Peptide L803 (sequence KEAPPAPPQ[pS]P), derived from heat shock factor-1 sequence, has shown the optimal inhibitory activity, and myristoylation of this peptide was performed to improve the cellular permeability [153]. The resulting peptide L803-mts has gained substantial popularity as a standard GSK-3 inhibitor for *in vitro* and *in vivo* tests. The binding mode of L803-mts (**7**) was studied by peptide docking and molecular dynamics simulations [154,155], suggesting the interaction of phosphoserine with the priming phosphate binding site and placing the resting part of the peptide in the same cavity between activation loop, glycine loop, and loop C, as suggested for TDZDs. The analysis of interactions in this binding mode had prepared the ground for the design of more efficient peptide inhibitor L803F (sequence KEAPPAPPQ[pS]PF) [156].

Manzamine A (**6**) and related compounds from manzamine class represent the third well-studied class of non-ATP-competitive GSK-3 β inhibitors [157]. These weird molecules are synthesized in marine sponges by bacteria [158] and possess a wide spectrum of biological activity; a number of semisynthetic modifications (**43**, **44**) were suggested for these alkaloids [68,159-163] as well as simplified analogues inspired by manzamine scaffold [164-167]. Simplification of this scaffold (**45**) leads to the lack of kinase inhibitory activity; both β -carboline and polycyclic ircular moiety are required for this activity. The binding mode of manzamines was studied by docking [159] and by molecular dynamics simulation [168]; it was suggested that the most favorable site of interaction is located in the same hydrophobic cavity as the interaction site of TDZDs and inhibitory peptides, despite the interaction with the priming phosphate binding site is less probable due to the absence of highly polar moieties in the manzamine molecule. Introduction of hydroxyl group into the β -carboline core (**43**) leads to lower IC_{50} of such derivatives [68], allowing one to suggest that this group may bind in the positively charged phosphate binding site.

IRREVERSIBLE INHIBITORS OF GSK-3

Irreversible character of GSK-3 inhibition was recently shown for tidesglusib, but this irreversibility seemingly is not defined by covalent binding of the inhibitor [169]. The most developed strategy of covalent kinase inhibitors design suggests the exploitation of reactivity of cysteine residues located in the ATP binding pocket [170,171]. Such strategy was successfully used for the design of GSK-3 inhibitors due to the presence of Cys199 residue [172-174]; the designed inhibitors fall into different classes from simple thienylhalomethylketones (**46**) to more advanced structures based on the maleimide scaffold (**47**).

PURELY ALLOSTERIC INHIBITORS OF GSK-3

Recent studies based on the pocket analysis technique revealed a binding site for purely allosteric inhibitor VP0.7 (**48**), whose inhibition of GSK-3 does not comply with ATP-competitive nor substrate-competitive kinetics [37]. The binding mode of this compound is defined by sandwiching of the aromatic ring between

Arg209 and Thr235. This binding site opens the possibilities for the design of novel inhibitors and suggests one more possible mechanism for manzamine binding, however further validation and testing are required for this promising cavity.

CONCLUDING REMARKS

The role of glycogen synthase kinase 3 in cancer is rather controversial, but definite evidences were obtained that in certain conditions the inhibition of this kinase provides well-defined anticancer effects. Complicated profile of GSK-3 inhibitors' action makes them not easily applicable in the current state of science, but further design of more advanced inhibitors and development of personalized medicine will attract more attention to this field. Different strategies were used for the design of GSK-3 inhibitors, and still there are strategies underrepresented in this field, such as the search for purely allosteric inhibitors or the design of bivalent inhibitors, which consist of ATP-competitive and substrate-competitive inhibitors connected with a linker [175]. The great amount of knowledge accumulated in this field allows researchers not to wander in darkness, but to use it as the guiding stars.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

AD	=	Alzheimer's Disease
ATP	=	adenosine triphosphate
Bcl-2	=	B-cell lymphoma 2
BEDROC	=	Boltzmann-enhanced discrimination of ROC
BIO	=	6-bromoindirubin-3'-oxime
CDK	=	cyclin-dependent kinase
DUD	=	directory of useful decoys
FRAT	=	frequently rearranged in advanced T-cell lymphoma
GSK-3	=	glycogen synthase kinase 3
ITDZ	=	5-imino-1,2,4-thiadiazole
JNK	=	c-Jun N-terminal kinase
MAP	=	mitogen-activated protein
MLL	=	mixed lineage leukemia
MSX2	=	muscle segment homeobox 2
NCI	=	National Cancer Institute
NF- κ B	=	nuclear factor κ B
NHL	=	non-Hodgkin lymphoma
NPM-ALK	=	Nucleophosmin/anaplastic lymphoma kinase fusion
OCC	=	one-class classification
PDB	=	Protein Data Bank
PKD1	=	pyruvate dehydrogenase lipoamide kinase isozyme 1
PI3K	=	phosphoinositide 3-kinase
PMS2	=	postmeiotic segregation increased 2
PP1	=	protein phosphatase 1

PPAR γ	=	peroxisome proliferator-activated receptor γ
Pten	=	phosphatase and tensin homolog
QM/MM	=	quantum mechanics/molecular mechanics
QSAR	=	quantitative structure-activity relationships
Rb/E2F	=	retinoblastoma protein/E2F transcription factor complex
RCC	=	renal cell carcinoma
ROC	=	receiver operating characteristic
TDZD	=	thiadiazolidinones
TRAIL	=	tumor necrosis factor-related apoptosis-inducing ligand
XIAP	=	X-linked inhibitor of apoptosis protein

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