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Identification of novel scaffold of benzothiazepinones as non-ATP competitive glycogen synthase kinase-3β inhibitors through virtual screening

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ABSTRACT

Glycogen synthase kinase-3β (GSK-3β) is an important serine/threonine kinase that has been proved as a key target for neurodegenerative diseases and diabetes. Up to date, most of known inhibitors are bound to the ATP-binding pocket of GSK-3β, which might lead widespread effects due to the high homology between kinases. Recently, some of its non-ATP competitive inhibitors had been confirmed having therapeutical effects owing to their high selectivity. This finding opens a new pathway to study hopeful drugs for treatment of these diseases. However, it is still a challenge nowadays on how to efficiently find non-ATP competitors. Here, we successfully discovered a novel scaffold of benzothiazepinones (**BTZs**) as selective non-ATP competitive GSK-3β inhibitors through virtual screening approach. A 3D receptor model of substrate binding site of GSK-3β was constructed and applied to screen against drug-like Maybridge database through Autodock program. **BTZ** compounds were top ranked as efficient hits and were then synthesized for further screening. Among them, the representative compound **4j** showed activity to GSK-3β (IC_{50}: 25 μM) in non-ATP competitive mechanism, and nearly no inhibitory effect on other 10 related protein kinases. Overall, the results point out that **BTZ** compounds might be useful in treatment of Alzheimer's disease and diabetes mellitus as novel GSK-3β inhibitors. It also suggests, on the other hand, that virtual screening would provide a valuable tool in combination with in vitro assays for the identification of novel selective and potent inhibitors.

Glycogen synthase kinase-3β (GSK-3β) is an important serine/threonine protein kinase and has been confirmed playing a critical role in two serious diseases as type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD). In PI3K pathway, GSK-3β controls many important functions conducted by insulin, such as glycogen synthesis, transport and uptake of glucose, gluconeogenesis and adipogenesis. As two important substrates of GSK-3β, both insulin receptor substrate-1 (IRS-1) and glycogen synthase (GS) mediate the main process of glycogen metabolism in insulin signaling acting in upstream and downstream, respectively. Once GSK-3β is abnormally much active, GS activity will be inhibited directly and distinctly, and then the normal process of glycogen synthesis will slow down, which consequently lead to the elevation of the blood sugar concentration.[1] The inhibition of IRS-1 occurs at the same time and lets the response ability of the receptors to insulin be markedly reduced.[2] On the other hand, GSK-3β is also the key enzyme in AD cascade by regulating both the total tau level and its phosphorylation. Highly active GSK-3β not only exceedingly phosphorylates protein tau but also leads to the overproduction of β-Amyloid (Aβ) by phosphorylation of amyloid precursor protein (APP) in the meantime.[3] Among the whole process, increased production of Aβ is the most critical point since Aβ can in return further activate GSK-3β. The higher activation of GSK-3β will then abnormally speed up the phosphorylation of tau to a large extent. As the result, excessive phosphorylated protein tau accumulates to form AD.[4] It has been proved that the accumulation of oligomeric Aβ and hyper-phosphorylation of tau is just the major factors to induce the damages of cholinergic neurons and the representative cognitive impairments in AD patients.[5]

Nowadays more and more evidences have been provided that GSK-3β inhibitors are very powerful in treatment of T2DM and AD. They have some advantages for treating T2DM. GSK-3β inhibitors generally have good hypoglycemic effect, and usually produce many other positive effects, such as promoting insulin release, stimulating glucose uptake and improving the sensitivities of cells to insulin.[6] Besides, they normally lead to few of the following events as weight gain, accumulation of fat tissue, and increase of blood triglyceride and cholesterol content.[7] As for treating AD, GSK-3β inhibitors also show a lot of positive effects. They are very effective to reduce the production of Aβ,[8] prevent cells from death and thus alleviate external behavior damages to some extent.[9] And the most encouraging thing is that the close internal link between

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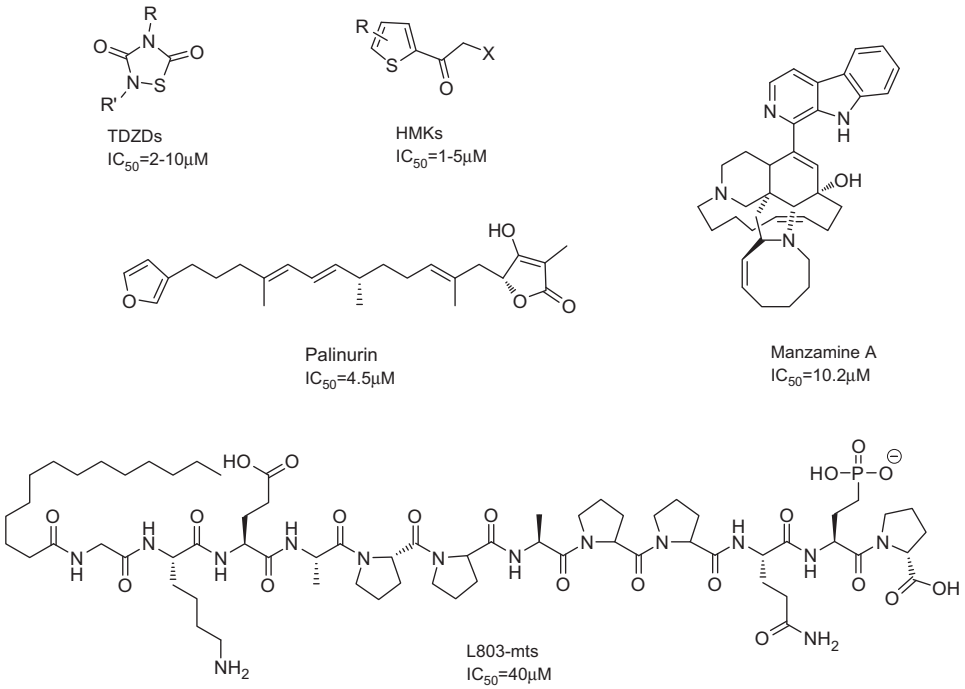


Figure 1. Structures of reported non-ATP competitive GSK-3β inhibitors.

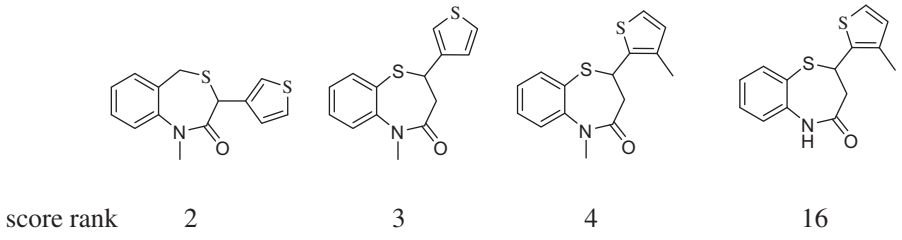


Figure 2. Hits of benzothiazepinones analogues by virtual screening.

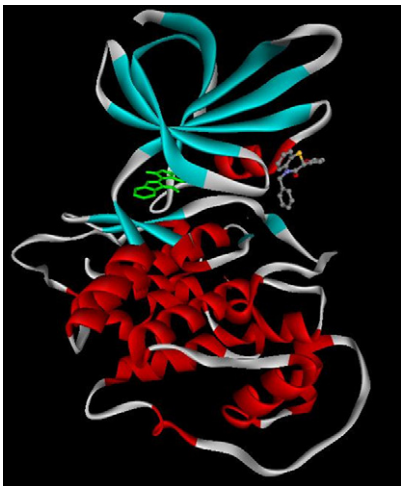


Figure 3. The docked **4h** and BIO in the binding pocket. The molecule in green stick is the ATP-competitive ligand BIO; that colored in atom type is the compound **4h**.

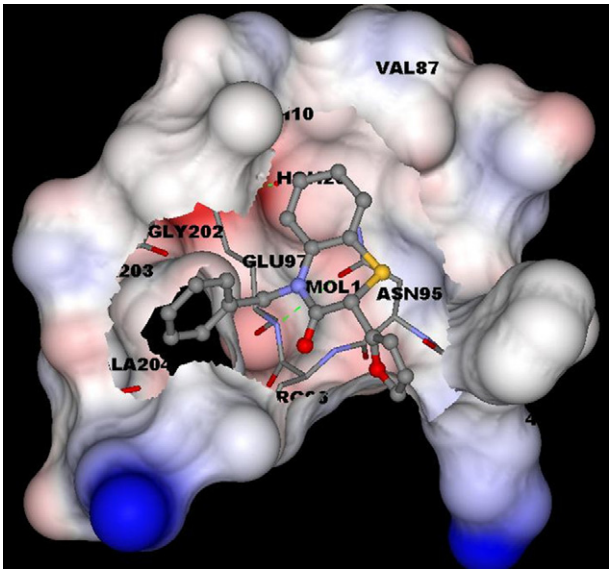
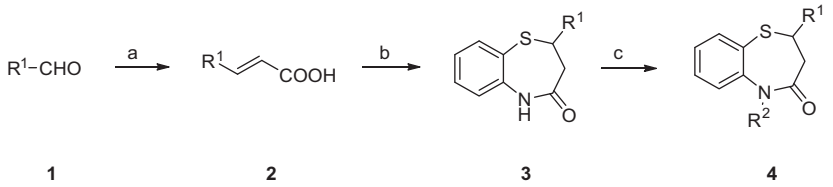


Figure 4. Amino acid residues with solvent-accessible surface involved in substrate binding pocket within 6 Å are shown. The compound **4h** was in ball-and-stick model, and the amino acid residues were in line form. The H-bond interactions between titled compound and the receptor were shown in green dashed line.



1 2 3 4

Scheme 1. Reagents and conditions: (a) $CH_2(COOH)_2$, pyridine, piperidine, 2 h, reflux, 69–91%; (b) 2-aminobenzenethiol, 4 Å molecular sieve, 6 h, 180 °C, 25–66%; (c) R^2X, X = Cl, Br, I, NaH, DMF, 0.5 h, −10 °C, 11–86%.

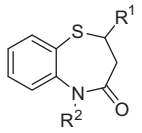
the pathogeneses of T2DM and AD is being revealed and becoming more and more clear in recent years.[10] That may partly explain why AD has even been referred to type 3 diabetes.[11] So, if it is finally identified that GSK-3β inhibitors truly have a kind of unusual collaborative effects on these two diseases, it is really an exciting progress just like killing two birds with one stone.

However, though GSK-3β inhibitors have so many good activities as mentioned above, the risk for side effects really exists and cannot be ignored. As many kinase inhibitors are, the most known GSK-3β inhibitors act to the enzyme's ATP-binding site,[12] which is highly conserved in more than 500 kinases. As a result, these ATP competitive inhibitors sometimes should offer adverse effects in a potential chronic treatment.[13] So it is quite an encouraging advance that non-ATP competitive GSK-3β inhibitors be developed, which paves a new way for getting more promising drugs to T2DM and AD[7a]. During the past ten years, five families of non-ATP competitive GSK-3β inhibitors have been reported (Fig. 1). Among them, thiadiazolidinones (TDZD) is the first one reported by Martinez et al. at 2002[14] and following confirmed to have both good selectivity and excellent therapeutical effects on AD.[15] Tideglusib (NP-12), a member of TDZD, is undergoing Phase II b clinical trials both on AD and orphan tauopathy in Europe now and it is also the only one as new GSK-3β inhibitor under clinical trials so far in the world. Another family is halomethylketones (HMK), but its future of being developed into a real drug has been denied since their inhibitory activity is very strongly irreversible to enzyme.[16] In addition, the small peptide L803-mts, a kind of substrate competitive inhibitor, has proven to be effective in vivo on T2DM and neurological diseases.[2] Lately two marine natural products of alkaloid manzamine A and sesquiterpene palinurin are reported to well decrease tau phosphorylation as cell permeable non-ATP competitive inhibitors,[17] but the further studies for them were greatly limited by their chemical structures.

So, it is still urgent to explore more GSK-3β inhibitors acting as non-ATP competitive with high selectivity. There are at least two key problems to be concerned about for such type of inhibitors. The first one is the application of appropriate methods for finding such type of leads. The widely used high throughput screening (HTS) is yet not an appropriate tool for finding non-ATP competitive compounds because the affinity of binding to the ATP outside area is mostly weaker than in ATP-binding site, and the activities of such type of inhibitors are generally as low as micromolar level, thus the potential hits are very easily to be abandoned off in screening.[18] Actually, no one of the non-ATP competitive inhibitors mentioned above is found through HTS. The other problem is about how to keep the action mode in chemical modifications, which is due to the fact that the action mode of non-ATP type would often shift to ATP competitive in structure modification. All the TDZD analogues modified by Kang's group had been verified to be ATP competitive inhibitors rather than expected non-ATP competitive ones in his case.[19]

As a computational counterpart to HTS, virtual screening (VS) is developed lately to make up for the deficiencies. VS approach has some obvious advantages in hits finding such as fast and good cost-effective.[20] A number of new inhibitors have already been found by this approach. However, to our best knowledge, there is not yet any report on discovering GSK-3β inhibitors with non-ATP competitive action mode through VS up to now.

Table 1
GSK-3β inhibitory activities of **BTZs**



Compound	R^1	R^2	IC_{50} (μM)[a]
4a	2-Thienyl	H	830
4b	2-Thienyl	Me	480
4c	2-Thienyl	Et	>100
4d	2-Thienyl	^{i}Pr	>100
4e	2-Thienyl	Bn	47.5
4f	2-Furyl	Me	>100
4g	2-Furyl	^{i}Pr	>100
4h	2-Furyl	Bn	77.2
4i	2-Furyl	3-Cl-Bn	27.0
4j	Ph	2-NO_2-Bn	25.0
TDZD-8[b]			1.4

[a] IC_{50}, the mean value of at least two separate determinations, each determination were means of triplicate experiments.
[b] **TDZD-8**, the first reported non-ATP competitive GSK-3β inhibitor, was used as reference compound in this study.

Here, we successfully discovered a novel scaffold of benzothiazepinones (**BTZs**) as selective non-ATP competitive GSK-3β inhibitors through virtual screening approach. First, a 3D receptor model of substrate binding pocket of GSK-3β was particularly constructed and applied to screen against drug-like compounds library. As the result several **BTZ** compounds were top ranked as the effective hits, and some their analogues were then synthesized. Among them, the representative compound **4j** acted in a non-ATP competitive manner to GSK-3β with IC_{50} value of 25 μM, which is better than L803-mts. Moreover, it nearly did not show any inhibition on other 10 related protein kinases involved in our tests.

The computational search was performed using receptor structure-based screening by docking with Autodock 3.0.5.[21] Of its three different search algorithms, the Lamarckian Genetic algorithm (LGA) was finally used to do the docking since the other two (simulated annealing and genetic algorithm) showed less efficient in preliminary experiments. LGA is an optimization algorithm for docking flexible ligands into protein binding sites to explore the full range of ligand conformational flexibility with the rigid protein,[20] utilizing Lamarckian notation that an adaptations of an individual to its environment can be inherited by its offspring. The grid maps representing the ligand were calculated with Auto grid. The dimensions of the grid were $60 \times 60 \times 60$ grid points with a spacing of 0.375 Å between the grid points and centered on the ligand. The X-ray crystal structure of GSK-3β kinase (PDB code: 1UV5) was selected as the model of the receptor and the supposed binding pocket of TDZD composed of Arg 96, Lys 205 and Tyr 216, as screening receptor model since there are still no

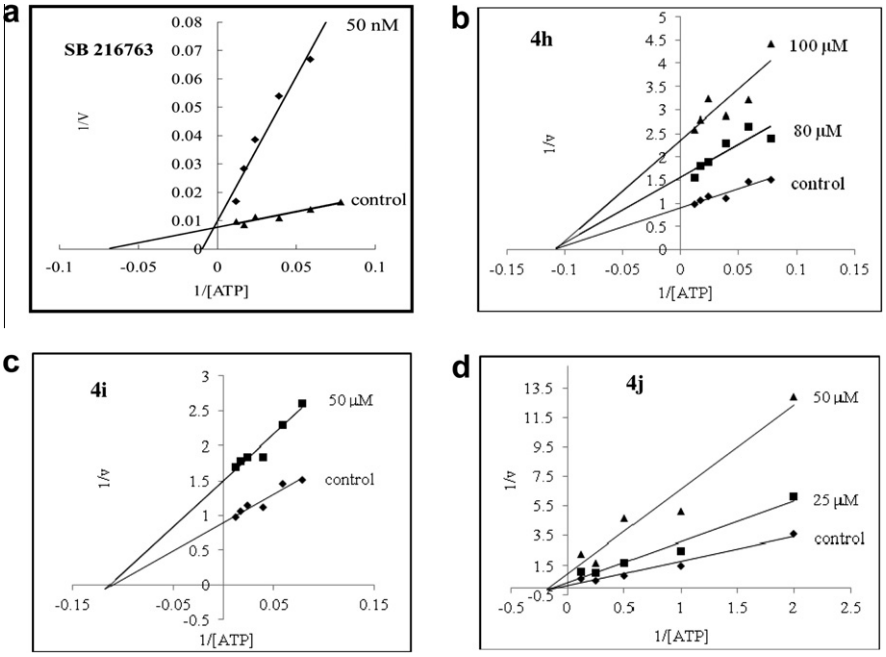


Figure 5. Double-reciprocal plot of kinetic data about assays of GSK-3β protein kinase activity at different concentrations of **SB 216763**, a known ATP competitive GSK-3β inhibitor as reference inhibitor (a), and **BTZ** inhibitors **4h**, **4i** and **4j** (b, c and d, respectively). ATP concentrations in the final reaction mixture varied from 0.5 to 8 μM. The concentration of GS-2 was kept constant in all of experiments at 6.25 μM. Compound concentrations were depicted in the plot.

any reports on the crystal complex of non-ATP competitive inhibitors to GSK-3β, and TDZDs are still the best ones so far. Maybridge database was prefiltered before screening according to the rules of lead-likeness.

These jobs were run on a Thinkpad 64-bit 32-processor LINUX cluster and totally 150,000 drug-like molecules were submitted to be screened. The average docking cost about 25 min. For all dockings, one hundred independent runs with step sizes of 0.2 Å for translations, and 5 Å for orientations and torsions, an initial population of random individuals with a population size of 150 individuals, a maximum number of 1.5×106 energy evaluations, maximum number of generations of 37,000, an elitism value of 1, and a number of active torsion of 9 were used. All other run parameters were kept at their default settings. Final docked conformations were clustered using a tolerance of 2 Å root mean square deviations (RMSD). After carefully visual inspection of their poses, the top 20 compounds greatly matched were identified as reasonable hits from 100 top dockings ranked with the lowest binding free energy.

Among them, **BTZ** analogues aroused our great interest due to their high-rankings and novel structure as GSK-3β inhibitor (Fig. 2). It was noteworthy that if there was no methyl group attached at the nitrogen atom, the ranking of hit sharply dropped to 16th. This result suggested that the substituents at nitrogen atom might have great impact on the activity or selectivity of the molecule. In order to verify this assumption, various alkyl and aryl groups as methyl, isopropyl and benzyl were introduced to the nitrogen atom of **BTZ**, respectively. Besides, the furyl group was also replaced by a thienyl group as its bioisostere.

Before making the synthesis, the binding affinities of these designed compounds were checked using the VS model. One known ATP-competitive compound BIO was also docked to this model as a reference.[22] In the result, these designed **BTZ** compounds could indeed bind to the substrate area while BIO bound to the ATP site (Fig. 3). In a typical sample, the binding affinity of compound **4h** was predicted to be about -7.82 kcal mol^{-1}, and its inhibitory activity to be about 1.86 μM. In this docking study, the predicted active cavity was composed of four amino acid residues as Phe67, Phe93, Arg 96, Lys205, which is slightly different to the VS model (Fig. 4).

Table 2
Inhibitory activity (% inhibition) of compound **4j** (100 μM) against several protein kinases

Protein kinases	% Inhibitory	Protein kinases	% Inhibitory
Flt-1[a]	21.8	ErbB2[b]	0
KDR[a]	9.9	ErbB4[b]	0
PDGFR-β[a]	−133.3	EPH-A2[c]	0
RET[a]	55.2	Abl[c]	74.4
EGFR[b]	0	RON[d]	0

[a] **Su11248** as reference inhibitor for Flt-1 (87.1% inhibitory), KDR (89.7% inhibition) and PDGFR-β (82.1% inhibition).
[b] **BIBW2992** as reference inhibitor for EGFR (86.9% inhibition), ErbB2 (79.4% inhibition) and ErbB4 (82.8% inhibition).
[c] **Dasatinib** as reference inhibitor for EPH-A2 (83.1% inhibition), Abl (90.0% inhibition).
[d] **PD173074** as reference inhibitor for RON (93.4% inhibition).

Finally, ten derivatives of 2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one were successfully synthesized through three steps as Knoevenagel reaction, cyclization and N-alkylation (Scheme 1).[23]

Kinase-Glo™ luminescent kinase assays were performed to investigate inhibitory potency of these synthesized **BTZs.**[24] IC_{50} values of each compound were summarized in Table 1. It could be seen from these data that the bulk aryl moieties at 5-position of **BTZ** might be very important for remaining the inhibitory activity. Introduction of benzyl moiety to that site makes the activity increased (**4e**, **4h**, **4i** and **4j**), while compounds with hydrogen atom or alkyl substituents at 5-position almost show no activity (**4a**, **4b**, **4c** and **4d**).

To delineate the inhibitory mechanism of compounds with most potent inhibitory activity (**4h**, **4i** and **4j**), kinetic experiments were carried out under different concentrations of ATP and tested compounds. Double-reciprocal plot of kinetic profiles are shown in Figure 5. All of three compounds are demonstrated to be a typical non-competitive inhibition against phosphorylation substrate ATP

according to these data, indicating that **BTZ** derivatives do not share binding pocket of enzyme with ATP. With increasing concentrations of ATP, the compounds display intersection of same Km and different Vmax values. Of them, the representative compound **4j** was further assayed against a panel of other 10 related kinases and showed high selectivity to GSK-3β over these enzymes (Table 2).

As summarized, we built a supposed non-ATP binding pocket as screening receptor model and employed it to virtually screen out potential ligands against GSK-3β. In this approach, Autodock program was used to perform the screening from a drug-like chemicals library, and the hits were expected to be capable of acting as non-ATP-competitive mechanism. Successfully a novel scaffold of **BTZ** was synthesized and confirmed by kinetic analysis to be indeed a kind of non-ATP competitive inhibitor of GSK-3β. The representative compound **4j** shows inhibitory activity against GSK-3β with an IC_{50} value of 25 μM, and little inhibition on other 10 related protein kinases. These results suggest **BTZ** compounds might have expectative selectivity over enzymes. Since GSK-3β inhibitors with that kind of structure have never been reported before, these **BTZ** compounds are much promising candidates worthy of further studies due to the advantages of non-ATP mechanism for activity and selectivity. On the other hand, our work has also showed that VS approach would be a useful tool in primarily search for novel scaffolding structures especially when HTS is not suitable to finding specific ligands.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.043.

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24. Polgár, T.; Baki, A.; Szendrei, G. I.; Keseruu, G. M. *J. Med. Chem.* **2005**, *48*, 7946. The measurement of GSK-3β inhibition was performed in assay buffer using transparent 96-well plates according to the Kinase-Glo assay method of Baki. In a typical assay, 4 μL of different concentration compound of interest (dissolved in DMSO) was diluted by 14 μL of assay buffer, and 2 μL (20 ng) of enzyme solution were added to each well followed by 20 μL of assay buffer containing 12.5 μM substrate and 4 μM ATP. After 30 min of incubation at 30 °C, the enzymatic reaction was stopped with 40 μL of Kinase-Glo reagent. Glow-type luminescence was recorded after 10 min. The activity is proportional to the difference of the total and consumed ATP. The inhibitory activities were calculated on the basis of maximal activities measured in the absence of inhibitor. The IC_{50} value was defined as the concentration of each compound that reduces 50% the enzymatic activity with respect to that without inhibitors..