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Identification of small molecules that inhibit GSK-3β through virtual screening

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ABSTRACT

Glycogen synthase kinase-3β (GSK-3β) is involved in glycogen metabolism, neuronal cell development, osteoblast differentiation. Small molecule inhibitors of GSK-3β have various therapeutic potential for the treatment of diabetes type II, bipolar disorders, stroke and chronic inflammatory disease.

To identify GSK-3β inhibitors with novel scaffold from chemical library, we primarily screened out putative inhibitors through computer modeling and subsequently evaluated the inhibitory activity of selected compounds against GSK-3β by in vitro Z'-LYTE™ assay. A series of compound KRMs strongly inhibited phosphorylation of its substrate with IC_{50} value of approximately 0.5 μM. Also, we demonstrated that KRM-189 and KRM-191 competed with ATP for GSK-3β, leading to decreased V_{max} and constant K_m with increasing concentrations of ATP as determined from Lineweaver–Berk equation. Moreover, they showed the selectivity for GSK-3β over other kinases with IC_{50} values of 2 to 10 μM or more Incubation of cells with KRM-191 with highly selective and potent inhibitory activity caused accumulation of β-catenin, downstream of GSK-3β signaling pathway, indicating that small molecule can prevent degradation of β-catenin via GSK-3β inhibition. Our results suggest that modeling in combination with in vitro assays can be used for the identification of selective and potent inhibitors.

Glycogen synthase kinase-3 (GSK-3) is a cytoplasmic serine–threonine kinase and exists in two highly homologous forms, GSK-3α and GSK-3β.[1] Especially, GSK-3β plays a key role in signaling pathway transmitted by insulin or Wnt, which has been implicated in glucose homeostasis, remodeling of bone mass or developmental process of the embryo.[2] GSK-3β is constitutively active in resting cells and treatment of cells with agents, such as insulin and lithium chloride (LiCl), is shown to cause GSK-3 inactivation through a PI 3-kinase (PI3-K)-dependent mechanism. PI3-K-induced activation of PKB/Akt results in phosphorylation of Ser21 on GSK-3α and Ser9 on GSK-3β, therefore leading to the inhibition of GSK-3 activity. The phosphorylated N-terminus becomes a primed pseudosubstrate that occupies the positive binding pocket and the active site of the enzyme and acts as a competitive inhibitor for true substrates. Several known GSK-3 substrates participate in a wide network of cellular processes, including glycogen metabolism, transcription, translation, cytoskeletal regulation, intracellular vesicular transport, cell cycle progression, and apoptosis. Phosphorylation of these substrates by GSK-3β usually has an inhibitory effect. Arg96 is shown to be a crucial component of the positive pocket that binds primed substrates. Small molecule inhibitors that fit in the positively charged pocket of the kinase domain of GSK-3β are useful for selectively inhibiting primed substrates. Therefore, intervention of GSK-3β might be a useful target to the treatment and prevention of diabetes, Alzheimer and osteoporosis. Up to date, a few compounds are known to inhibit directly its enzyme. Lithium chloride has a specific inhibitory activity in vitro and in intact cells although millimolar concentration of IC_{50} is limited to therapeutic use. Besides, small molecules such as bisindole or aniline maleimides, kenpaullone, indirubin, or the marine natural product hymenialdisine have been reported as GSK-3β inhibitors.[3] All the small molecules under development inhibit in a competitive manner with ATP and as a result, show no selectivity over a wide variety of protein kinases. In this presentation, we found out a scaffold structure of thiadiazolinone (TDZD) that might be a putative inhibitor of GSK-3β through running computer modeling, and furthermore synthesized TDZD derivatives to address moiety of which could be attributing to their inhibitory activity. Addition of some groups to parent chemical improved potently inhibitory activity against GSK-3β in enzyme assay. It also inhibited enzyme by competing with ATP, but has the selective inhibition for GSK-3β over other serine/threonine kinases. In a bioassay measuring β-catenin accumulation as a result of GSK-3β inhibition, it showed that β-catenin was still accumulated in the total cell extracts as did insulin or lithium chloride in this experiment. Taken together, computer modeling will provide a useful tool for the primary identification of scaffolding structure fitted into binding pocket of enzyme with known structure.

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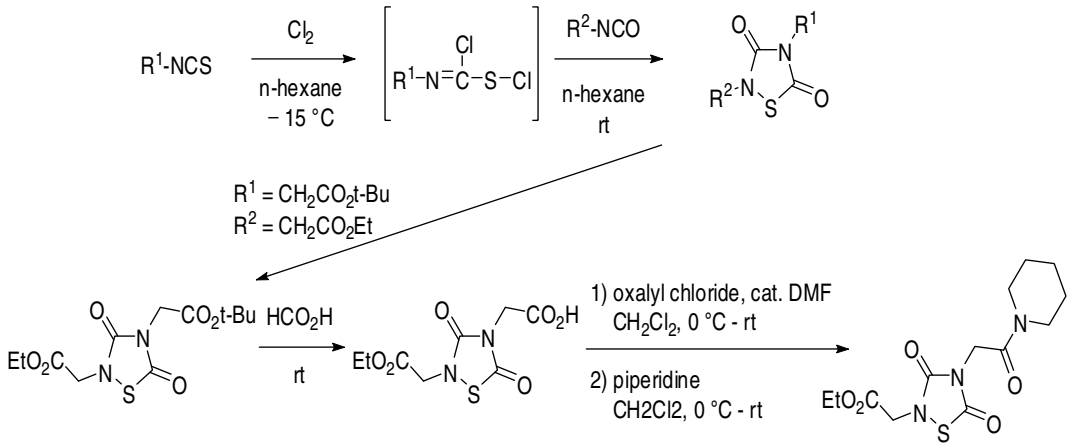


Scheme 1.

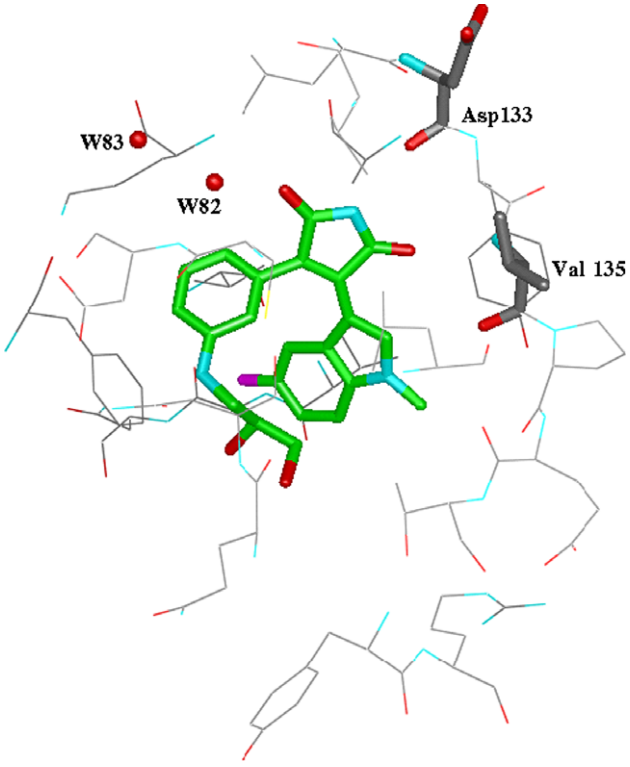


Figure 1. The residues within 5A around the bound-ligand are shown in 1R0E.pdb.

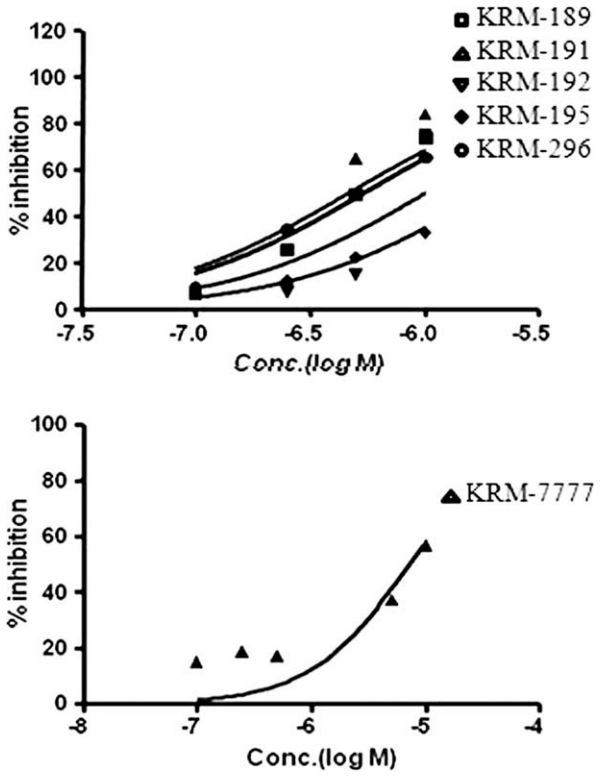


Figure 2. Inhibition curves of each compound were obtained from the incubation of enzyme with various concentrations of it, respectively. Inhibitory potency was compared with each other for % inhibition at 10 μM and IC_{50}.

Table 1
Docking scores for potent GSK-3β inhibitors selected from chemical library.

Compound	Docking score
KRM-191	6.81
KRM-296	6.8
KRM-192	6.77
KRM-195	6.57
KRM-189	3.97

To virtually screen out putative ligands that are capable of acting as inhibitors of GSK-3β from in-house chemical library, we employed SurflexDock[4] method interfaced with Sybyl 7.3.1.[5] SurflexDock[4] is a new docking methodology that combines Hammerhead's empirical scoring function[6] with a molecular similarity method to generate putative alignment of ligands. It[4] employs an idealized active site ligand (called a protomol) as a target to generate putative alignments of molecules or molecules fragments. These putative poses are achieved using the Hammerhead scoring function.[6]

Initially, in-house library including 170 compounds with TDZD fragment as a small molecule library, synthesized from diverse isocyanates and isothiocyanates via known method[7] as shown in Scheme 1,[8] was docked into binding sites of GSK-3β. Before performing docking, all compounds were minimized using Cerius2[9] suite of programs and the conserved water molecule (W82 as shown in Fig. 1) was also specified near Thr138 side chain atom and Asp200 backbone atom, which can play a crucial role in inhibitor binding.

In an attempt to find out small molecules having an inhibition activity against GSK-3β using SurflexDock,[4] PDB ID 1R0E as a

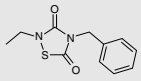
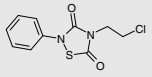
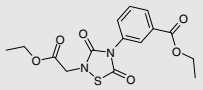
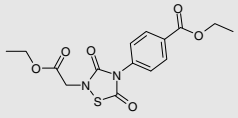
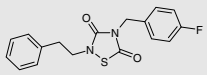
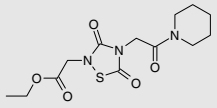
complex reference structure was adopted. Using active site extracted from 1R0E as PDE ID, we generated a protomol as an idealized active site ligand. SurflexDock's protomols utilize CH_4, C=O and NH fragments. Protomol construction was based on protein residues proximal to the native ligand and on parameter settings to produce a small and buried docking target (SurflexDock parameters: proto_thresh 0.6 and proto_bloat 0). Each docking of putative ligands returned up to 50 scored poses, with the score consisting of a nominal affinity score.[6] Through SurflexDock method, we chose the pose with best score from multiple docking of the same ligand. Out of 170 compounds, five compounds were finally selected after considering a structural diversity and docking score. The best pose of the chosen ligands and their resulting scores are shown in Table 1.

To further investigate inhibitory potency of some derivatives with thiadiazolidinones (TDZD) chosen through computer modeling for the inhibitors of GSK-3β, we performed Z'-LYTE™ kinase assay,[11] in which ratiometric fluorescence transfer between substrate and product was reproducibly determined. Inhibition of GSK-3β by each compound was shown in Figure 2 by plotting relative inhibition of enzyme at increasing doses of compound. IC_{50} values that are indicative of inhibitory activity of each compound were summarized in Table 2. In vitro enzymatic data showed that five compounds had their distinctive IC_{50} values with a similar inhibitory potency at a fixed concentration. Scaffold with benzyl and ethyl group as substituents exhibited low IC_{50} value, similar to value reported previously.[3] Derivatives with different combination of substituent groups had different IC_{50} values ranging from 0.5 to 2 μM.

To delineate the inhibitory mechanism of compounds with most potent inhibitory activity under investigation (KRM-189 and KRM-191), kinetic experiment was carried out under different concentrations of ATP and TDZD derivative. Double-reciprocal plot of kinetic profiles was shown in Figure 3a and b. Lineweaver Burk plot suggests that either KRM-189 or -191 was demonstrated to be a typical competitive inhibition against substrate ATP, indicating that chemical derivatives share binding pocket of enzyme with ATP. With increasing concentrations of ATP, it displayed intersection of same V_{max} and different K_m values. A parent compound (KRM-7777) has been reported to inhibit GSK-3β selectivity over other protein kinases independent of ATP concentration. Therefore, TDZD with some modification might shift inhibition mode from noncompetitive to competitive while improving the inhibitory competency.

Due to its involvement of GSK-3β in multiple pathways as described above, selectivity of GSK-3β inhibition is one of a considerable important factor in the development of inhibitors for therapeutic applications. Since GSK-3β is supposed to be phylogenetically most close to cyclin dependent kinases (CDKs),[12] it is compelling to achieve selectivity against CDKs. Moreover, structural and functional similarities between GSK-3β and casein kinase 2 (CK2) were suggested on phylogenetic trees.[13] PKA or PKC

Table 2
Summary for the inhibitory potency against GSK-3β and the selectivity of TDZD derivatives for GSK-3β over other kinases with reference to KRM-7777. Some other serine/threonine kinases were tested with chemicals to inhibit potently GSK-3β. All the reaction conditions were almost similar with exception of substrates suitable for respective enzyme, and were carried out according to the instructions provided by manufacturer.

Compound	Structure	% inhibition/IC_{50}									
		GSK-3β		CSNK2		PKA		PKC,α		CDK1	
		At 10[a]	IC_{50}[b]	At 100[c]	IC_{50}	At 100[c]	IC_{50}[d]	At 100[c]	IC_{50}[d]	At 100[c]	IC_{50}[d]
KRM-7777		65	7149	30		30		88	13.8	15	
KRM-189		74	548	17		35		84	5.8	0	
KRM-191		84	467	21		87	2.2	89	5.3	18	
KRM-192		75	1012	36		81	6.9	78	48.3	12	
KRM-195		80	1863	31		20		51	38.7	20	
KRM-296		65	539	21		75	17	93	4.1	15	
LiCl		71	25	42		6		0		36	

[a] % inhibition at 10 μM concentration.
[b] IC_{50} unit is nM.
[c] % inhibition at 100 μM concentration.
[d] IC_{50} unit is μM.

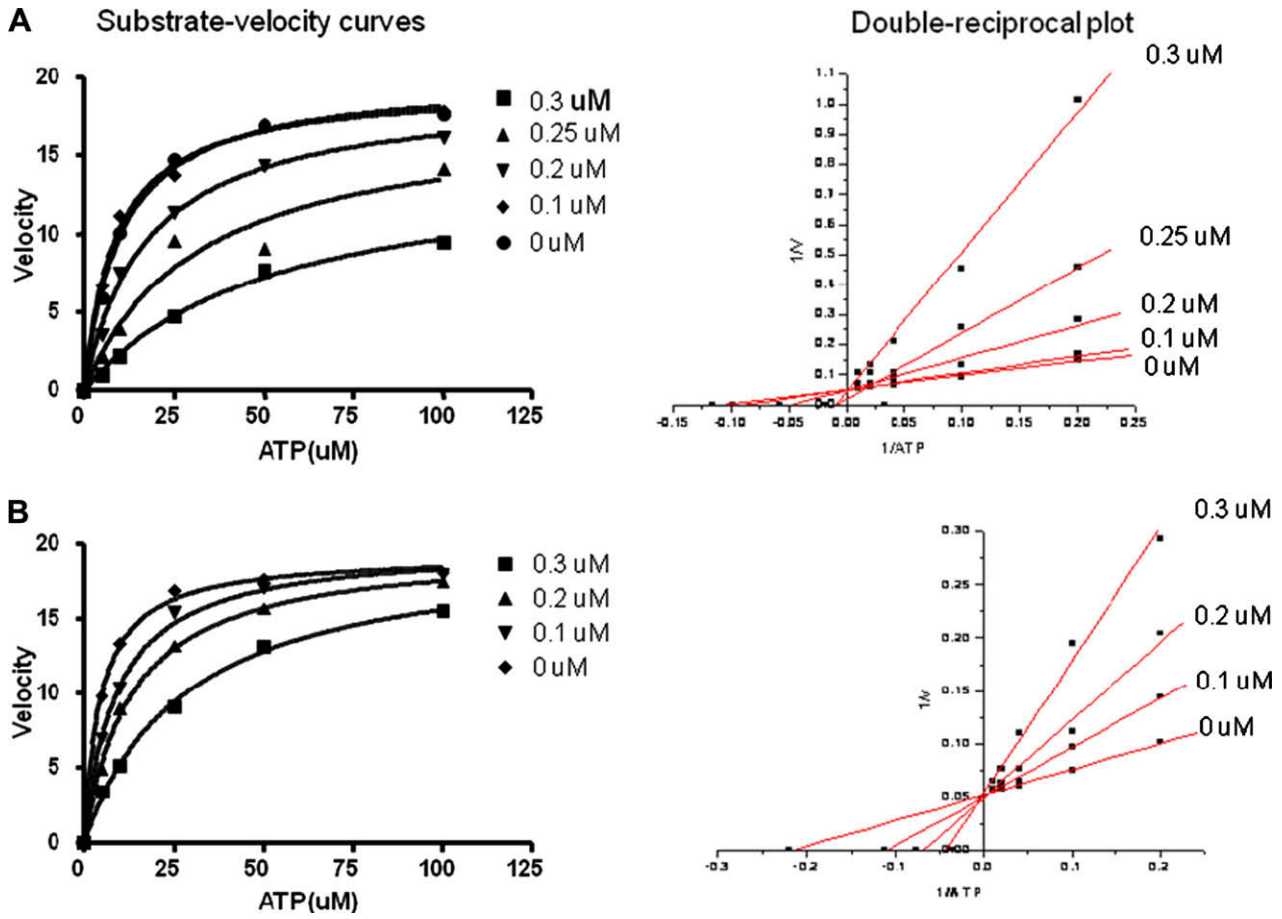


Figure 3. The inhibitory potency of TDZD derivatives against GSK-3β. Compound selected through computer modeling was subjected to enzyme reaction to get enzyme velocity with substrate and double-reciprocal plot with increasing concentrations of KRM-189 (a) and KRM-191 (b) at various doses of ATP.

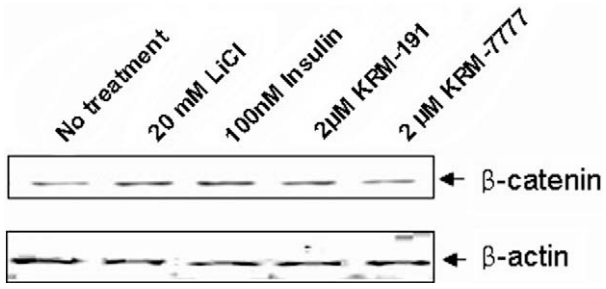


Figure 4. Effect of GSK-3β inhibitors on accumulation of β-catenin in C_2C_{12} myoblast. Myoblasts plated at a density of 5×10^5 were exposed to chemicals at 2 μM for 3 h. Cells harvested were lysed to obtain total proteins and then subjected to SDS–PAGE for immunoblot with antibody raised against β-catenin. As an internal standard, β-actin was detected by probing blotted membrane with anti β-actin.

belongs to be different from superfamily including GSK-3β on the human kinome tree. Therefore, selective inhibition of TDZD derivatives against GSK-3β was examined over other related kinases using Z'-LYTE substrate. In our inhibition data, unexpectedly, neither CDK2 nor CK2 were affected at even high concentrations of chemical derivatives whereas two serine/threonine kinases distinct from GSK-3β such as PKC and PKA were susceptible to inhibition by TDZD derivatives. Two potent compounds were 10-fold more selective for GSK-3β over PKC and PKA and 2 order more selective over CSNK2 and CDK1.

To further examine downstream event of GSK inhibition in the intact cells, β-catenin accumulation following GSK-3β inhibitor treatment was followed with reference to lithium chloride and insulin (Fig. 4). β-Catenin was continuously degraded under normal differentiation, resulting in lower level. Well-known GSK-3β inhibitor lithium chloride (LiCl) as well as insulin, which activate insulin signaling to indirectly inactivate GSK-3β, has been suggested to protect β-catenin from proteasome degradation. This biological data shows that in vitro activity of KRM-191 is reflected to β-catenin accumulation in cells left treated with KRM-191.

Taken together, this report exhibits one typical example of drug development through docking virtual screening leading to potent inhibitors. After well-defined target and optimized algorithm package are established, introduction of in silico modeling into drug discovery and development pipeline will enrich the fraction of drug-like compounds in library, resulting in the reduction of biological testing in lead discovery. This process has already been included in drug discovery pipeline of pharmaceutical company.

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