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Synthesis and Biological Evaluation of Novel GSK-3β Inhibitors as Anticancer Agents

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A series of isoxazol-indolin-2-one was designed for GSK-3β inhibitors as novel anticancer agents based on their binding mode analysis in GSK-3β crystal structure. Total 21 compounds were synthesized and evaluated for their inhibitory activity against two tumor cell lines (DU145 and HT29). Most of the synthesized compounds were potent with above 80% inhibitory activity at 100 μM, and several compounds were examined for inhibitory activity against GSK-3β. Among them, **15(Z)** (R_1=H, R_2=3-Cl-phenyl) was most active with 78% inhibition of tumor cell line (HT29) at 20 μM and 72% inhibition of GSK-3β at 20 μM.

Key Words : Glycogen synthase kinase 3β inhibitor, Isoxazol-indolin-2-one, Anticancer

Introduction

Protein phosphorylation is a crucial mechanism to regulate cell biology, and its abnormalities induce many diseases. For this reason, pharmacological inhibitors of kinase and phosphatases have become a major interest in drug discovery. Glycogen synthase kinase 3 (GSK-3) is one of the most elaborated enzyme by its function in the regulation of glycogen synthesis and in determination of cell's fate. GSK-3 consists of two known genes GSK-3α and β, which bear highly homogenous catalytic domain and different ability area for transcription and translation in some tissue.[1] GSK-3β plays an crucial role in cellular events and has been consistently implicated in many diseases, including Alzheimer's disease, Parkinson's disease and diabetes.[2] Over the past decade, several outstanding results demonstrated that GSK-3β signaling pathway is an important mediator to tumor suppression by multiple levels of various down-regulating oncoproteins.[3] According to the reports, GSK-3β inhibitor leads to antiproliferation of tumor cells in breast cancer, as well as prostate and colon cancer,[4] and such inhibitory profiles are then of value to develop a new cancer target. Until now, the mode of action of GSK-3β inhibitor was not found out clearly, but the relationship between GSK-3β inhibitor and tumor suppression was proved by published reports. The apoptosis of tumor cell by GSK-3β inhibitor is supported by three hypotheses. First, it is known that GSK-3β is usually over expressed in cancer cell and it is implicated in NF-κB, transcription factor, which is participated in cell proliferation.[5] In fact, inhibition of GSK-3β in tumor cell results in reducing NF-κB transcriptional activity and leads to cell apoptosis. Second, GSK-3β inhibitor is tightly associated with the activation of tumor necrosis factor related apoptosis-inducing ligand (TRAIL) and this corporation reduces tumor cell proliferation.[6] Finally, p53 is a tumor suppressor protein and involved in preventing cancer. When cell damage signal is passed, the secretion of p53 is started and negatively regulated by GSK-3β, consequently the effect of GSK-3β inhibitor leads to tumor cell apoptosis.[7]

GSK-3β and various ligands were crystallized,[1] and its overall shape consists of a large N-terminal beta sheet and a C-terminal shaping alpha helix. The ATP binding pocket, which located between these two lobes, was shaped up by Arg96, Arg180 and Lys205 where the phosphate group of the primed substrate and the pseudo-substrate binds. The hydrogen bond interactions of ATP competitive inhibitors with D133 and V135 were shown in crystal structures of GSK-3β, this result shows that it is crucial element to design novel scaffold as GSK-3β inhibitors. A number of reported potent GSK-3β inhibitors were configured small and planar molecules, having five-membered ring as maleimide moiety.[8] From the binding mode analysis of GSK-3β inhibitors, two hydrogen bonds at hinge region of active site are crucial for inhibitory activities. An isoxazol-indolin-2-one moiety was designed and docked into the active site of GSK-3β (PDB code, 1Q5K). As can be seen in Figure 1, important residues such as D133 and V135 in hinge region show H-bond interaction with an isoxazol-indolin-2-one moiety, and R141 additionally contacts with piperazine moiety of ligand. This result suggests that the structure bearing an isoxazol-indolin-2-one moiety can be a good inhibitor in the active site of GSK3β.

In this work, isoxazol-indolin-2-one derivatives were synthesized as GSK-3β inhibitors and they were evaluated for inhibitory activities against tumor cell lines of prostate cancer (DU145) and colon cancer (HT29). The selected compounds

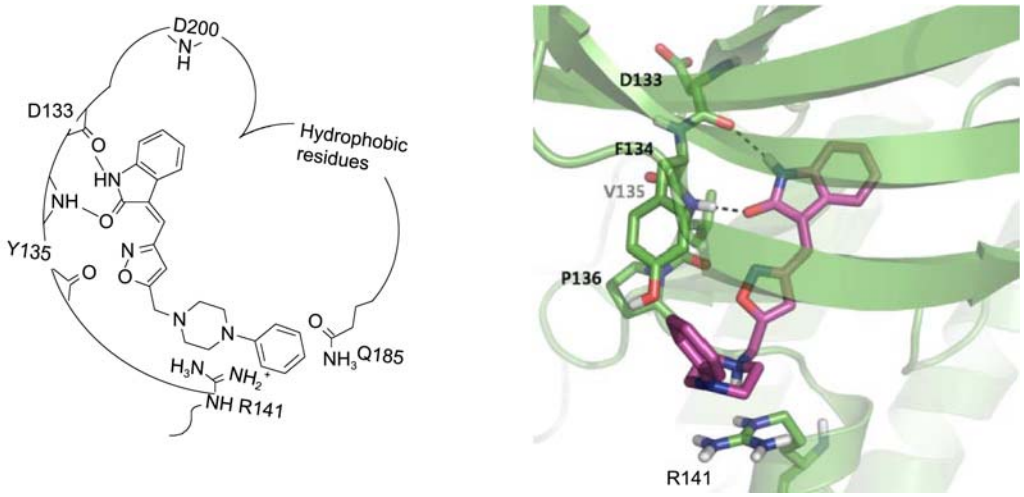


Figure 1. Docking images of designed compound in GSk-3β active site.

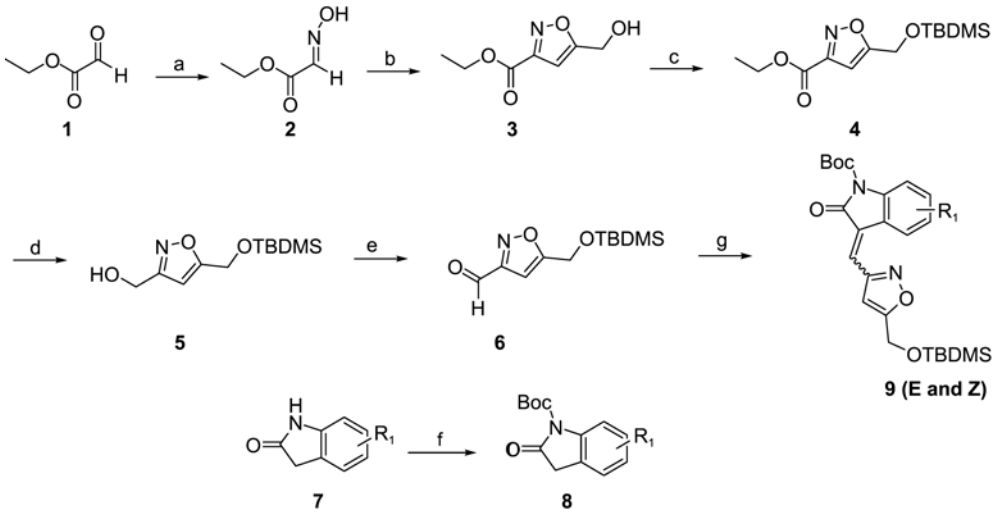
were tested for the inhibition of enzyme activities against GSK-3β.

Experimental

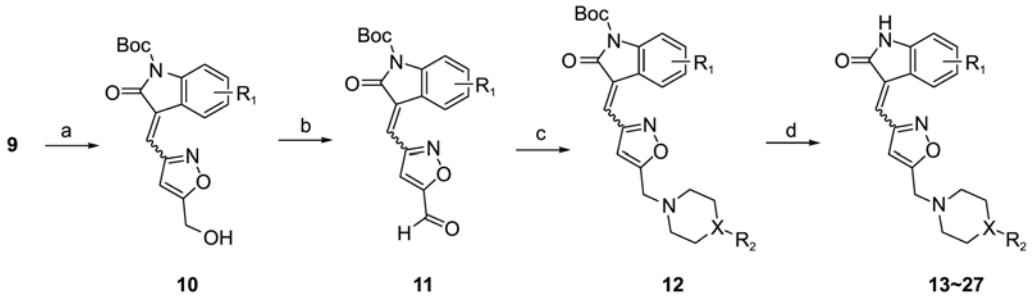
The synthetic strategy of isoxazol-indolin-2-one derivatives was depicted in Scheme 1. Aldol-type coupling was used as constructive step between indoline fragment and isoxazole carbaldehyde moiety. For the synthesis of isoxazolyle carbaldehyde fragment, oxime intermediate **2** was obtained by treatment of ethyl formylformate with hydroxylamine hydrochloride in basic condition. Isoxazole ring formation was carried out by two steps. Addition of *N*-chlorosuccimide (NCS) formed 2-chloro-hydroxyimino ester, and then the reaction with propagyl alcohol led to an isoxazolyl methyl alcohol **3** successively.

Careful addition of TEA to oxymoyl chloride is necessary to avoid the formation of nitrile oxide which is dimerized rapidly and reproduces starting material. This reversible reaction caused low yield of desired compound. To avoid undesirable side reaction, TEA was used in dilute solution and it was added slowly. The primary alcohol **3** was protected with TBDMS-Cl, and ethyl ester group was converted to alcohol with DIBAL-H and PCC oxidation of the alcohol **5** afforded an aldehyde **6**. After coupling step between indolin-2-one and intermediate **6**, desired product was obtained but its poor solubility interrupts purification; therefore, N-Boc protection was necessary for the improvement of the physical property of indolin-2-one. *N*-protected indolin-2-one **8** was coupled with **6** in ethyl alcohol reflux. E and Z isomers of **9** were separated by column chromatography on silica gel, and E isomer was obtained as major product.

Deprotection of intermediate **9** with TBAF yielded the alcohol **10**, followed by oxidation to give aldehyde intermediate **11**. Aldol-type coupling between **11** and substituted phenylpiperazine afforded the intermediate **12**. Finally, *N*-deprotection in acidic condition was carried out and the synthesis of this series was accomplished after column

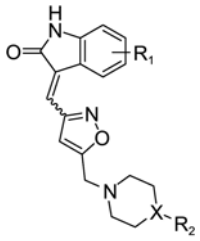


Scheme 1. Reactions and conditions. a) pyridine, $NH_2OH.HCl$, 70%; b) i) NCS, CH_2Cl_2 ii) TEA, propagyl alcohol, CH_2Cl_2, 61%; c) TBDMS-Cl, imidazole, CH_2Cl_2, 98%; d) DIBAL-H, CH_2Cl_2, 98%; e) PCC, CH_2Cl_2, 72%; f) $(Boc)_2O$, Na_2CO_3, THF, 88%; g) **8**, piperidine, EtOH, reflux, 3 h, 53%.



Scheme 2. Reactions and conditions. a) 1 N TBAF, THF, 44%; b) DMP, CH_2Cl_2, 90%; c) phenylpiperazine, $NaBH(OAc)_3$, 4 Å molecular sieve, CH_2Cl_2, 44%; d) 1 N HCl in MeOH, CH_2Cl_2, 75%.

Table 1. The cytotoxicity (%) of synthesized compounds



Entry	R_1	R_2	X	DU145			HT29		
				100 μM	20 μM	4 μM	100 μM	20 μM	4 μM
13(E)	H	Phenyl	N	78.36	34.57	8.49	86.70	63.68	9.42
13(Z)	H	Phenyl	N	74.00	31.52	11.64	86.01	84.55	9.80
14(E)	H	2-Cl-Phenyl	N	75.19	19.60	9.97	85.84	45.17	6.96
14(Z)	H	2-Cl-Phenyl	N	85.32	44.39	14.64	88.87	76.36	9.63
15(E)	H	3-Cl-Phenyl	N	76.79	41.49	15.41	87.40	76.69	17.20
15(Z)	H	3-Cl-Phenyl	N	77.64	58.47	19.79	87.15	78.42	15.61
16(E)	H	2-OMe-Phenyl	N	83.58	53.81	17.70	87.01	79.65	15.67
16(Z)	H	2-OMe-Phenyl	N	83.45	44.43	15.74	86.78	70.37	8.28
17(E)	H	3-OMe-Phenyl	N	71.76	40.89	11.23	86.16	74.39	11.88
17(Z)	H	3-OMe-Phenyl	N	71.32	43.42	12.14	84.17	69.20	10.17
18(E)	H	3-Me-Phenyl	N	79.27	40.42	15.58	86.98	68.66	8.87
18(Z)	H	3-Me-Phenyl	N	78.92	58.11	15.75	86.73	76.00	5.99
				100 μM	25 μM	6.25 μM	100 μM	25 μM	6.25 μM
19(E)	5-Cl	2-Cl-Phenyl	N	80.20	7.67	–2.33	83.05	–1.42	–2.53
20(E)	5-Cl	3-Cl-Phenyl	N	85.27	58.83	12.32	87.41	67.68	8.70
21(E)	5-Cl	4-Cl-Phenyl	N	65.46	32.00	14.19	71.90	54.54	12.83
22(E)	5-Cl	2-F-Phenyl	N	81.40	19.99	3.35	84.76	16.93	-2.39
23(E)	5-Cl	Methyl	N	79.00	36.82	17.78	81.35	50.12	26.08
24(E)	5-F	3-F-Phenyl	N	69.14	62.67	15.79	76.39	67.81	12.67
25(E)	H	3-F-Phenyl	N	81.56	30.55	10.54	85.80	48.93	11.29
26(E)	5-Cl	3-F-Phenyl	N	84.32	33.69	6.49	86.76	58.22	9.65
27(E)	5-Cl	-	C	78.04	39.23	21.07	82.23	58.87	17.96
Doxorubicin				62.79			60.89		

chromatography. Total 21 final compounds were synthesized and they were evaluated for cytotoxicity in two tumor cell lines. The characteristic feature and the biological activity of title compounds were shown in Table 1.

Results and Discussion

The cytotoxicity of the synthesized compounds were evaluated against two tumor cell lines (DU145: human prostate cancer cell and HT29: human colon adenocarcinoma cell) at various concentration and Doxorubicin was employed as a reference. The result indicated that isoxazol-indolin-2-one derivatives showed good activity by over 80% at 100 μM on average, and were slightly more active for HT29 compared to DU145. Among them, **13(Z)** showed highest inhibitory activity at 20 μM against HT29 (84.55%).

Table 2. Inhibitory activity of selected compounds against GSK-3β

Compound	(%, 20 μM)	Compound	(%, 20 μM)
13(E)	13	**17(E)**	ND
13(Z)	15	**17(Z)**	26
14(E)	38	**18(E)**	4
14(Z)	33	**18(Z)**	ND
15(E)	4	**20(E)**	8
15(Z)	72	**23(E)**	10
16(E)	ND	**25(E)**	25
16(Z)	17	[a]**AR-A014418**	29

[a]**AR-A014418** (*N*-(4-methoxybenzyl)-*N*′-(5-nitrothiazole-2-yl))urea was tested at 1 μM. ND: Not determined

The compounds **14(Z)**, **15(E**, **Z)** and **16(E)** exhibited good inhibitory activity of 76-80% at 20 μM against HT29. Regarding to the effect of substituents, the nature or positional feature of R_1 and R_2 did not play an important role in inhibitory activity.

It was found that, whether it was E or Z, there were no significant differences in activity, because the potency is related to simply indolin-2-one, not to the structural conformation of isoxazol. The most stable conformation of substituents was arranged by free rotation and they offered similar range of activities. For 5-halogen substituted indol-2-one derivatives, these compounds effectively inhibited the proliferation of cell as previous results with over 80% at 100 μM except **21(E)** and **24(E)**, but the activity at 25 μM was slightly reduced. Among **19(E)**, **20(E)** and **21(E)**, which have chlorine on phenylpiperazine, **20(E)** having *meta*-chloro was most potent at 20 μM and 100 μM. It was notable that the compound **24(E)** bearing 5-F at R_1 and 3-F at R_2 was most potent at 25 μM with 62.67% (DU145) and 67.81% (HT29).

Fifteen compounds were selected for inhibitory activities against GSK-3β, but generally showed poor results. Among them, compound **15(Z)** (R_1=H, R_2=3-Cl-phenyl), which was one of the active compound in cytotoxicity, showed 72% of activity. When compared to E isomer, Z isomer was more active, however it was not the constant tendency for all isomer couples, for example, **14** (**E**/**Z**) and **18** (**E**/**Z**).

In summary, we designed and synthesized a series of isoxazol-indoline-2-one derivatives for GSK-3β inhibitor as anticancer agent. They were evaluated for inhibitory activity against tumor cell lines and the kinase. The cytotoxicity was potent with over 80% inhibition for most of synthesized compounds, and among them, compound **15(Z)** showed the best inhibitory enzyme activity (72%). Isoxazol-indoline-2-one analogues were potent inhibitor for GSK-3β to treat cancer and further study will continue.

Experimental Section

General. All prepared compounds were identified by NMR spectroscopy, obtained from Bruker Avance 75 MHz, 300 MHz or 400 MHz spectrometer, using the residual solvent peak in $CDCl_3$ (7.24 ppm for ^{1}H and 77.16 ppm for ^{13}C) or DMSO-d_6 (2.50 ppm for ^{1}H and 39.52 ppm for ^{13}C). Chemical shifts are given in ppm, and *J* values in hertz. Analytical thin layer chromatography (TLC) analyses were performed on Merck Silica Gel 60 F_{254} (0.25 mm) plates, and column chromatography was carried out on Silica Gel 60 (0.063-0.200 mm) on Merck. All reagents were obtained from commercial supplies and used without further purification. Dichloromethane (DCM) and Tetrahydrofuran (THF) were distilled from calcium hydride/benzophenone and sodium hydride/benzophenone, respectively, under nitrogen atmosphere.

3-((5-((4-Phenylpiperazin-1-yl)methyl)isoxazol-3-yl)-methylene)indolin-2-one (13).

13(E): yield 66%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.71 (d, 1H, *J* = 7.7 Hz), 8.35 (s, 1H), 7.45 (s, 1H), 7.34-7.25 (m, 3H), 7.08 (t, 1H, *J* = 7.6 Hz), 6.96-6.86 (m, 4H), 6.48 (s, 1H), 3.86 (s, 2H), 3.26 (t, 4H, *J* = 4.6 Hz), 2.76 (t, 4H, *J* = 4.9 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.9, 169.8, 158.2, 151.1, 142.2, 131.6, 131.4, 129.1, 127.3, 122.6, 121.2, 120.0, 119.9, 116.2, 109.9, 106.0, 53.2, 52.9, 46.1.

13(Z): yield 8%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (s, 1H), 7.59 (s, 1H), 7.56 (d, 1H, *J* = 7.8 Hz), 7.51 (s, 1H), 7.33-7.24 (m, 3H), 7.11-7.06 (m, 4H), 6.95-6.92 (m, 2H), 6.89-6.85 (m, 2H), 3.85 (s, 2H), 3.25 (t, 4H, *J* = 4.8 Hz), 3.75 (t, 4H, *J* = 5.0 Hz).

3-((5-((4-(2-Chlorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (14).

14(E): yield 18%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.72 (d, 1H, *J* = 7.7 Hz), 7.78 (s, 1H), 7.44 (s, 1H), 7.35 (t, 2H, *J* = 8.0 Hz), 7.23 (s, 1H), 7.12-7.07 (m, 2H), 7.01 (t, 1H, *J* = 7.7 Hz), 6.89 (d, 1H, *J* = 7.7 Hz), 6.49 (s, 1H), 3.88 (s, 2H), 3.14 (s, 4H), 2.80 (s, 4H).

14(Z): yield 66%, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.63 (s, 1H), 7.60 (s, 1H), 7.56 (d, 1H, *J* = 7.5 Hz), 7.36 (dd, 1H, *J* = 7.8, 1.4 Hz), 7.30 (s, 1H), 7.24-7.20 (m, 1H), 7.11-7.04 (m, 2H), 7.02-6.96 (m, 1H), 6.89 (d, 1H, *J* = 7.7 Hz) 3.86 (s, 2H), 3.13 (s, 4H), 2.78 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 167.0, 159.0, 149.1, 140.3, 130.6, 130.5, 128.7, 127.6, 123.9, 123.7, 122.4, 120.5, 120.4, 109.8, 106.5, 53.4, 51.0.

3-((5-((4-(3-Chlorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (15).

15(E): yield 50%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.70 (d, 1H, *J* = 7.7 Hz), 8.51 (s, 1H), 7.45 (s, 1H), 7.35-7.26 (m, 1H), 7.17 (t, 1H, *J* = 8.0 Hz), 7.08 (t, 1H, *J* = 7.0 Hz), 6.92 (s, 1H), 6.89 (d, 1H, *J* = 3.7 Hz), 6.81 (t, 2H, *J* = 8.7 Hz), 6.48 (s, 1H), 3.85 (s, 2H), 3.25 (t, 4H, *J* = 4.6 Hz), 2.74 (t, 4H, *J* = 4.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.8, 169.7, 158.2, 152.1, 142.2, 134.9, 131.6, 131.4, 130.0, 127.3, 122.6, 121.2, 119.9, 119.5, 115.9, 114.0, 109.9, 106.0, 53.4, 48.6, 29.7.

15(Z): yield 39%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (s, 1H), 7.59 (s, 1H), 7.56 (d, 2H, *J* = 7.7 Hz), 7.31 (dd, 1H, *J* = 7.7, 0.9 Hz), 7.16 (t, 1H, *J* = 8.1 Hz) 7.08 (t, 1H, *J* = 7.6 Hz), 6.88-6.76 (m, 4H), 3.84 (s, 2H), 3.24 (t, 4H, *J* = 4.8 Hz), 2.73 (t, 4H, *J* = 5.0 Hz).

3-((5-((4-(2-Methoxyphenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (16).

16(E): yield 62%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.71 (d, 1H, *J* = 7.6 Hz), 8.55 (s, 1H), 7.45 (s, 1H), 7.37-7.29 (m, 1H), 7.14-6.84 (m, 6H), 6.48 (s, 1H), 3.88 (s, 2H), 3.86 (s, 3H), 3.15 (s, 4H), 2.81 (s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.9, 169.8, 158.2, 152.2, 142.3, 141.0, 131.6, 131.3, 127.63, 123.1, 122.6, 121.3, 121.0, 120.0, 118.2, 111.1, 109.8, 106.0, 55.3, 53.2, 53.1, 50.5.

16(Z): yield 20%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 (s, 1H), 7.79 (s, 1H), 7.59 (s, 1H), 7.56 (d, 1H, *J* = 7.5 Hz), 7.31-7.29 (m, 1H), 7.10-6.91 (m, 4H), 6.86 (d, 2H, *J* = 7.9 Hz), 3.86 (s, 2H), 3.84 (s, 3H), 3.14 (s, 4H), 2.80 (s, 4H).

3-((5-((4-(3-Methoxyphenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one.

(17E): yield 47%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (d, 1H, *J* = 7.7 Hz), 8.54 (s, 1H), 7.48 (s, 1H), 7.33 (t, 1H, *J* = 7.4 Hz), 7.16 (t, 1H, *J* = 8.1 Hz), 7.06 (t, 1H, *J* = 7.7 Hz), 6.88 (d, 1H, *J* = 7.7 Hz), 6.54 (dd, 1H, *J* = 8.1, 1.6 Hz), 6.46-6.41 (m, 3H), 3.89 (s, 2H), 3.78 (s, 3H), 3.23 (s, 4H), 2.73 (s, 4H).

3-((5-((4-*m*-Tolylpiperazin-1-yl)methyl)isoxazol-3-yl)-methylene)indolin-2-one (18).

18(E): yield 56%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.07 (d, 1H, *J* = 7.7 Hz), 7.56 (s, 1H), 7.35-7.28 (m, 1H), 7.17 (t, 1H, *J* = 7.5 Hz), 7.07 (t, 1H, *J* = 7.6 Hz), 6.90 (d, 1H, *J* = 7.7 Hz), 6.79-6.70 (m, 3H), 6.48 (s, 1H), 3.85 (s, 2H), 3.25 (t, 4H, *J* = 4.3 Hz), 2.76 (t, 4H, *J* = 4.6 Hz), 2.33 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.9, 169.1, 158.2, 151.1, 142.3, 138.8, 131.6, 131.4, 129.0, 127.2, 122.6, 121.2, 120.9, 119.9, 117.1, 113.3, 109.9, 106.0, 100.7, 100.4, 53.2, 53.0, 49.2, 21.7.

18(Z): yield 33%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.80 (s, 1H), 7.60 (s, 1H), 7.56 (d, 1H, *J* = 7.6 Hz), 7.18-7.26 (m, 1H), 7.16 (t, 1H, *J* = 7.4 Hz), 7.08 (t, 1H, *J* = 7.6 Hz), 6.86 (d, 1H, *J* = 7.7 Hz), 6.76-6.68 (m, 3H), 3.84 (s, 2H), 3.24 (t, 4H, *J* = 4.4 Hz), 2.75 (t, 4H, *J* = 4.7 Hz), 2.32 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.3, 166.9, 159.0, 151.2, 140.3, 138.8, 131.4, 130.5, 130.4, 128.9, 123.9, 122.7, 122.2, 120.8, 120.5, 117.0, 113.3, 109.7, 106.5, 106.0, 53.2, 46.2, 21.7.

5-Chloro-3-((5-((4-(2-chlorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (19E): yield 61%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.43 (d, 1H, *J* = 1.9 Hz), 7.96 (s, 1H), 7.48 (s, 1H), 7.37 (dd, 1H, *J* = 8.2, 1.4 Hz), 7.34-7.30 (m, 1H), 7.26-7.21 (m, 1H), 7.06 (dd, 1H, *J* = 8.1, 1.4 Hz), 7.02-6.96 (m, 1H), 6.83 (d, 1H, *J* = 8.2 Hz), 6.48 (s, 1H), 3.88 (s, 2H), 3.14 (s, 4H), 2.81 (s, 4H).

5-Chloro-3-((5-((4-(3-chlorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (20E): yield 39%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47 (s, 1H), 7.35-7.27 (m, 1H), 7.17 (t, 1H, *J* = 8.1 Hz), 6.89-6.76 (m, 4H), 6.48 (s, 1H), 3.86 (s, 2H), 3.26 (t, 4H, *J* = 4.8 Hz), 2.74 (t, 4H, *J* = 5.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 169.1, 157.9, 152.1, 140.4, 134.9, 131.1, 130.6, 130.0, 128.1, 127.5, 122.6, 121.3, 119.5, 115.5, 114.0, 110.6, 106.1, 53.1, 52.8, 48.6, 29.7, 29.3.

5-Chloro-3-((5-((4-(4-chlorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (21E): yield 51%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.81 (s, 1H), 7.90 (s, 1H), 7.47 (s, 1H), 7.30 (s, 4H), 6.82 (d, 1H, *J* = 8.2 Hz), 6.52 (s, 1H), 6.06 (s, 1H), 3.97 (s, 2H), 3.34 (s, 2H), 2.89 (s, 2H), 2.60 (s, 2H).

5-Chloro-3-((5-((4-(2-fluorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (22E): yield 50%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.24 (s, 1H), 7.47 (s, 1H), 7.10-7.03 (m, 2H), 6.97 (t, 3H, *J* = 6.6 Hz), 6.83 (d, 1H, *J* = 8.2 Hz), 6.49 (s, 1H), 3.88 (s, 2H), 3.17 (s, 4H), 2.79 (s, 4H).

5-Chloro-3-((5-((4-methylpiperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one.

(23E): yield 46%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.80 (s, 1H), 7.35-7.27 (m, 1H), 7.17 (t, 1H, *J* = 8.1 Hz), 6.89-6.76 (m, 4H), 6.48 (s, 1H), 3.86 (s, 2H), 3.26 (t, 4H, *J* = 4.8 Hz), 2.74 (t, 4H, *J* = 5.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 169.0, 157.9, 140.4, 131.0, 130.6, 128.0, 127.5, 122.6, 121.3, 110.5, 106.0, 61.8, 54.7, 53.0, 52.5, 45.6.

5-Fluoro-3-((5-((4-(3-fluorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (24E): yield 40%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (dd, 1H, *J* = 9.6, 2.6 Hz), 7.57 (s, 1H), 7.47 (s, 1H), 7.24-7.16 (m, 1H), 7.08-7.01 (m, 1H), 6.84 (q, 1H, *J* = 4.2 Hz), 6.68 (dd, 1H, *J* = 8.2, 2.1 Hz), 6.63-6.52 (m, 2H), 6.47 (s, 1H), 3.86 (s, 2H), 3.26 (t, 4H, *J* = 4.8 Hz), 2.74 (t, 4H, *J* = 5.0 Hz).

3-((5-((4-(3-Fluorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one.

(25E): yield 48%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.74 (s, 1H), 8.53 (d, 1H, *J* = 7.7 Hz), 7.36 (s, 1H), 7.30 (d, 1H, *J* = 7.6 Hz), 7.17 (t, 1H, *J* = 7.9 Hz), 7.02 (t, 1H, *J* = 7.6 Hz), 6.89 (d, 2H, *J* = 9.6 Hz), 6.74 (s, 1H), 6.70 (t, 1H, *J* = 3.3 Hz), 6.55-6.50 (m, 1H), 3.84 (s, 2H), 3.20-3.16 (m, 4H), 2.61-2.58 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.5, 168.8, 164.9, 162.5, 159.2, 153.1, 144.2, 132.1, 131.9, 130.8, 126.7, 122.0, 121.0, 119.4, 111.3, 110.4, 107.1, 105.2, 105.0, 102.3, 102.1, 52.4, 48.0.

5-Chloro-3-((5-((4-(3-fluorophenyl)piperazin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (26E): yield 53%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 8.64 (d, 1H, *J* = 2.1 Hz), 7.44 (s, 1H), 7.38 (dd, 1H, *J* = 8.3, 2.2 Hz), 7.17 (t, 1H, *J* = 7.9 Hz), 6.93 (s, 1H), 6.90 (d, 1H, *J* = 8.3 Hz), 6.75-6.69 (m, 2H), 6.54-6.49 (m, 1H), 3.85 (s, 2H), 3.18 (t, 4H, *J* = 4.4 Hz), 2.59 (t, 4H, *J* = 4.7 Hz). ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.7, 168.5, 165.3, 162.1, 158.6, 153.2, 153.0, 143.0, 131.6, 131.0, 130.8, 130.6, 126.4, 125.9, 122.5, 121.0, 111.8, 111.3, 107.4, 105.3, 105.0, 102.4, 102.1, 52.4, 48.0.

5-Chloro-3-((5-((piperidin-1-yl)methyl)isoxazol-3-yl)methylene)indolin-2-one (27E): yield 51%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.81 (d, 1H, *J* = 1.8 Hz), 8.08 (s, 1H), 7.47 (s, 1H), 7.30 (d, 1H, *J* = 2.0 Hz), 6.83 (d, 1H, *J* = 8.2 Hz), 6.44 (s, 1H), 3.77 (s, 2H), 2.52 (s, 4H), 1.68-1.61 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7, 169.1, 157.8, 140.3, 131.0, 130.5, 128.0, 127.5, 122.6, 121.6, 110.5, 105.9, 61.8, 54.3, 53.8, 25.8, 23.8.

Materials and Methods for Bioassays.

***In vitro* Cytotoxicity:** Cytotoxic activities of all compounds were investigated using the MTT assay. Human colon

adenocarcinoma (HT-29) and human prostate cancer (DU-145) were provided from Korean Cell Line Bank. All cell lines were grown in RPMI 1640 (Gibco BRL) supplemented with 10% (v/v) heat inactivated Fetal Bovin Serum (FBS) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells (3×10^3 cells/well) were seeded into 96-well plate. Samples of various concentrations were added to each well in duplicate, and then incubated at 37 °C with 5% CO_2 for two days such that time cells were in the exponential phase of growth at the time of drug addition. 15 μL of the Dye solution (Promrga, CellTiter96) was added to each well, and then incubated at 37 °C for up to 4 hours in a humidified, 5% CO_2 atmosphere. After incubation, 100 L of the Solubilization Solution/Stop Mix (Promrga, CellTiter96) was added to each well and allowed to stand overnight in a sealed container with a humidified atmosphere at room temperature to completely solubilize the formazan crystals. The optical density was measured using a microplate reader (Versamax, Molecular Devices) with a 570 nm wavelength.

GSK-3β Activity: An FITC-labeled eIF2B substrate peptide (FITCRRRRAAEELDSRAGS(PO_4)PQL) synthesized by Peptron (Daejeon, Korea) was used as a substrate for the GSK-3β activity assay. Kinase reaction was performed using 0.25 μL of GSK-3β (100 μg/mL; Cell Signaling Danvers, MA, USA) in 25 μL of 4 mM MOPS, pH 7.2, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM EDTA, 1 mM DTT in the presence of 100 μM ATP and 10 μM FITC-labeled substrate. After incubation for 10 min at room temperature, the reaction was terminated by addition of 25 μL of 100% cold ethanol. The quenched reaction solution was then analyzed by capillary electrophoresis (CE) on a Beckman P/ACE 5000 CE-LIF system (Beckman-Coulter, Fullerton, CA, USA) equipped with a 488 nm line of a 3 mW argon-ion laser. The emission signal was collected through a built-in 520 ± 10 nm filter, and all measurements were performed 20 ± 0.1 °C in a liquid-cooled capillary cartridge. Fused-silica capillaries of a total length of 27 cm and 50 μm inner diameter (Beckman-Coulter) were used by pretreating with 1 N NaOH and rinsing with deionized water for 5 min each. Between runs, the capillary was sequentially rinsed with 1 N NaOH, deionized water and running buffer (44.5 mM tris(hydroxymethyl)aminomethane, 44.5 mM boric acid, 1.25 mM EDTA, 0.5 M NDSB-195, and 0.1% Tween 20, pH 8.3) for 2 min each. Samples were injected to the capillary by pressure of 0.5 psi for 1 s, and separations were carried out by applying +10.8 kV. Data collection was accomplished by the P/ACE software, followed by analysis with the Origin software (OriginLab, Northamton, MA, USA).

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