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Novel GSK-3 inhibitors with improved cellular activity

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Abstract—A novel series of [1-(1*H*-benzimidazol-7-yl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl] arylhydrazones was synthesized and shown to potently inhibit glycogen synthase kinase-3 (GSK-3). In light of detailed structure–activity relationships and structural knowledge of the GSK-3 binding pocket, a benzimidazole substituent was incorporated onto the pyrazolopyrimidine core resulting in improved potency over previous analogs. More importantly, these derivatives show low nanomolar efficacy for stimulating glycogen synthesis in vitro and therefore may be useful in the treatment of type 2 diabetes mellitus.

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed serine–threonine kinase known to phosphorylate glycogen synthase.[1] Glycogen synthase (GS) functions as the rate-limiting enzyme for glycogen formation and is inactivated upon phosphorylation by GSK-3.[2] Since GSK-3 is constitutively active in vivo, GS typically exists in the inactive state. Insulin, signaling via the PI-3 kinase/PKB pathway, inhibits GSK-3, resulting in GS activation, and subsequent formation of glycogen.[3] The conversion of plasma glucose into glycogen has been shown to be decreased in the liver and skeletal muscle of diabetic patients.[4] Analysis of skeletal muscle from type 2 diabetic humans showed GSK-3 activity and expression levels were significantly higher than those in healthy subjects.[5] Therefore, small molecules that mimic the action of insulin by inhibiting GSK-3 may be useful for the treatment of type 2 diabetes mellitus.

A number of publications have emerged describing molecules that inhibit GSK-3.[6] Several compounds have also been reported to stimulate glycogen synthesis in vitro[6a,b] as well as lower plasma glucose in diabetic animals.[7] We recently described a novel class of pyrazolopyrimidine analogs that inhibit GSK-3 (Fig. 1). Chemical modifications to this template yielded detailed structure–activity relationships (SAR) as well as compound **1A**, which potently inhibits GSK-3 ($pIC_{50} = 8.2$).[8] Combining the SAR data with structural information gained from the X-ray crystal structure of GSK-3β,[9] we have designed a second-generation compound set with increased potency. More importantly, these derivatives show significant improvements in cellular efficacy for stimulating glycogen synthesis in vitro as compared to our earlier series.

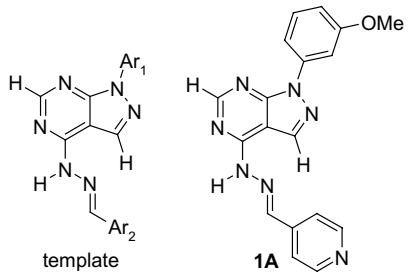

Figure 1. Pyrazolopyrimidine template.

The binding orientation for the pyrazolopyrimidine series was originally based on in silico docking of compound **2A** into a model of the ATP-binding pocket generated from the crystal structure of GSK-3β. As shown in Figure 2, the pyrazolopyrimidine makes two hydrogen-bond contacts to the hinge region of the enzyme through the N–H of the hydrazone and the N-5 nitrogen of the pyrimidine core. The hydrazone is oriented toward the opening of the binding pocket where the aryl group Ar_2 interacts with water and possibly the

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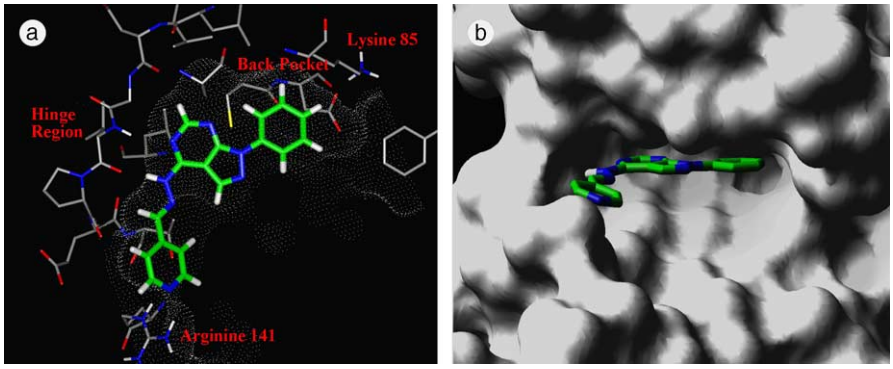
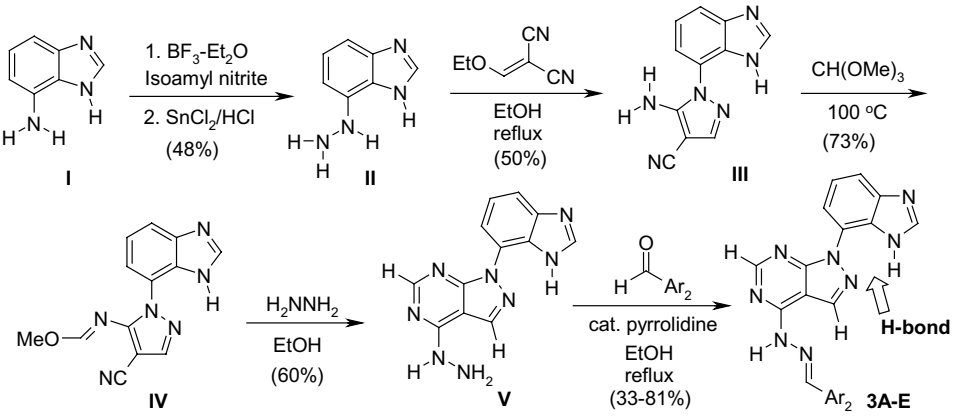


Figure 2. Proposed binding of compound **2A** into the GSK-3 ATP-binding pocket.

positively charged arginine (Arg 141). As expected, a variety of polar substituents are tolerated on Ar_2 as illustrated by compounds **1A**–**E**, **2A**–**C**, and **3A**–**E**.

The aryl group Ar_1 of the pyrazolopyrimidine protrudes deeper into the enzyme toward a lysine residue (Lys 85). In silico models and X-ray crystal data show this ring occupies a narrow region within the enzyme and needs to adopt a nearly coplanar geometry with the pyrazolopyrimidine core for optimal binding. Therefore, we hypothesized that introduction of substituents that favor the co-planar arrangement of Ar_1 with the pyrazolopyrimidine core would result in optimal potency. The desired planar geometry might be achieved via formation of an intramolecular hydrogen bond between a hydrogen-bond donor on the aryl ring and the nitrogen at the 2-position of the pyrazole core. Ultimately we decided that benzimidazole-containing analogs such as **3** (Scheme 1) would serve as good substrates to test this idea. This ring system also places another heavy atom in the narrow region of the enzyme that may enhance binding (Fig. 3). In addition, the distal nitrogen atom in the benzimidazole ring might interact as a hydrogen-bond acceptor with the positively-charged Lys 85 in a similar manner as the –OMe substituent.

The novel benzimidazole–pyrazolopyrimidine derivatives **3A**–**E** were prepared as depicted in Scheme 1 from 4-amino-benzimidazole, which is readily obtained in two steps from commercially available 3-nitrophenylenediamine. Treatment of aniline **I** with BF_3–Et_2O and isoamyl nitrite produced the intermediate diazonium salt that was subsequently reduced to the aryl hydrazine **II**. A solution of **II** and ethoxymalononitrile in refluxing ethanol gave the pyrazole-adduct **III**, which upon heating with trimethyl *ortho*-formate yielded the imino-ether **IV**. Formation of the pyrazolopyrimidine core was affected via a Dimroth rearrangement by heating **IV** in the presence of excess hydrazine monohydrate in ethanol to give the desired intermediate **V**.[10] The final target compounds **3A**–**E** were synthesized via condensation of **V** with various aldehydes and a catalytic amount of pyrrolidine in refluxing ethanol.[11]



Scheme 1. Syntheses of benzimidazole containing pyrazolopyrimidines.

Table 1. Enzyme and cellular potency of pyrazolopyrimidines

Compounds	$Ar_1 =$	$Ar_2 =$	pIC_{50}[a]	EC_{50}[b,c] (μM)	%Max[c,d]	Cell permeation (nM/s)
1A	3-OMe-phenyl	4-Pyridyl	8.2	0.22 (±0.03)	37 (±2)	127
1B	3-OMe-phenyl	*p*-F-phenyl	8.1	Inactive	Inactive	5
1C	3-OMe-phenyl	*p*-C(O)NH$(CH_2)_2NMe_2$-phenyl	7.8	4.00 (±0.51)	26 (±2)	15
1D	3-OMe-phenyl	*p*-SO_2Me-phenyl	8.6	0.31 (±0.04)	19 (±0)	168
1E	3-OMe-phenyl	*p*-CO_2H-phenyl	7.5	1.88 (±0.49)	46 (±2)	65
2A	Phenyl	4-Pyridyl	7.0	1.22 (±0.16)	48 (±3)	99
2B	Phenyl	*p*-F-phenyl	6.2	NT	NT	5
2C	Phenyl	*p*-C(O)NH$(CH_2)_2NMe_2$-phenyl	7.7	NT	NT	98
3A	Benzimidazole	4-Pyridyl	8.4	0.11 (±0.01)	45 (±3)	212
3B	Benzimidazole	*p*-F-phenyl	8.2	0.23 (±0.06)	57 (±4)	92
3C	Benzimidazole	*p*-C(O)NH$(CH_2)_2NMe_2$-phenyl	8.8	2.33 (±0.38)	76 (±2)	15
3D	Benzimidazole	*p*-SO_2Me-phenyl	8.3	0.24 (±0.07)	54 (±3)	61
3E	Benzimidazole	*p*-CO_2H-phenyl	8.3	2.73 (±0.23)	42 (±2)	17

[a] Concentration, which inhibits 50% of the activity of GSK-3β.
[b] Concentration, which elicits 50% of the maximal response.
[c] Values are means of three experiments.
[d] Maximum glycogen produced by the compound as a percentage of the maximum glycogen produced by 1 μM insulin.

Table 1 highlights the potency of GSK-3 inhibition (pIC_{50}) and glycogen synthesis stimulation (EC_{50}) of the various analogs. The potency of the compound was determined via a scintillation-proximity assay (SPA) in which the ability of the compound to inhibit the phosphorylation of a synthetic GS peptide by GSK-3β was measured.[12] As hypothesized, the benzimidazole-containing analogs **3A**–**E** were quite potent in inhibiting GSK-3 with pIC_{50}'s > 8. In all examples studied, the benzimidazole series (see examples **3A**–**C**) led to a significant (greater than 10-fold) improvement in potency as compared with the unsubstituted phenyl derivatives (examples **2A**–**C**[8]). In addition, it appears the benzimidazoles **3A**–**E** were also more potent than the corresponding 3-methoxyphenyl compounds **1A**–**E**[8] though several are not statistically significant.

We next looked at the effect of these compounds on the synthesis of ^{14}C-containing glycogen from ^{14}C-labeled glucose in a rat skeletal muscle cell line (L6 cells).[13] The cellular efficacy of each compound was derived from a 10-point dose–response curve and reported as the effective concentration that elicits the half-maximal response (EC_{50}). The %max refers to the maximal glycogen produced in response to the compound expressed as a percentage of the maximal glycogen synthesized in response to a fully efficacious dose of insulin (1 μM). In this assay, compound **3A** ($EC_{50} = 0.11$ μM) showed a 10-fold improvement in efficacy as compared to the unsubstituted analog **2A** ($EC_{50} = 1.22$ μM), which is in good agreement with the relative potencies of these two compounds for inhibition of GSK-3β (**3A** is 25-fold more potent than **2A**). The maximal amount of glycogen produced in response to these two compounds was essentially the same, ca. 45% of the maximal insulin response. None of the compounds synthesized were able to achieve 100% of the insulin value and analog **3C** gave the highest response at 76%. This is not surprising considering that insulin likely stimulates other signaling pathways, which also increase glycogen synthesis such as upregulating glucose transport and inhibiting glycogen breakdown.

The benzimidazole derivatives **3A**–**D** also showed better cellular responses for both EC_{50} and %max values as compared to the 3-methoxyphenyl compounds **1A**–**D**, even though the respective potencies in the enzyme inhibition assay are similar. The most dramatic case was observed for the analogs in which $Ar_2 =$ 4-fluorophenyl (**1B** and **3B**). Although both compounds are equipotent against the enzyme (pIC_{50}'s ~ 8.1), the 3-methoxyphenyl derivative **1B** showed no effect on stimulating glycogen synthesis, whereas the benzimidazole **3B** produced a robust response ($EC_{50} = 0.23$ μM). The large difference in observed efficacy might be a result of the ability of each compound to cross the cell membrane. Since GSK-3 is an intracellular kinase, compounds must enter the cell to affect glycogen synthesis. We used a MDCK cell line assay in an attempt to qualitatively estimate a compound's ability to permeate the cell.[14] As shown in Table 1, compound **1B** gave a very low permeation value (5 nM/s) and it is likely that this compound does not readily enter the cell. Interestingly, replacement of the 3-methoxyphenyl group with the benzimidazole moiety (compound **3B**) led to a significant increase in the permeation value (92 nM/s). Therefore we propose the large difference in efficacy between compounds **1B** and **3B** is a result of their respective abilities to cross the cell membrane. In general, for compounds with similar pIC_{50} values for GSK-3 inhibition and MDCK values >20 nM/s, there is a consistent degree of efficacy for glycogen production as illustrated among compounds **1A**, **1D**, **3A**, **3B**, and **3D**. These compounds all have pIC_{50} values in the range of 8.2–8.6, MDCK values >20 nM/s, and EC_{50} values between 0.11 and 0.31 μM. For compounds with MDCK values <20 nM/s, the cellular efficacy was relatively weak (EC_{50} >1 μM) regardless of enzyme potency. For example, **3A** and **3E** both contain a benzimidazole and are equipotent at GSK-3. But while **3A** shows good cellular efficacy, **3E** does not ($EC_{50} = 2.73$ μM). Again, based on the MDCK data, it appears that cell permeation may account for the significant difference in efficacy since **3E** yielded a low permeation value (17 nM/s) and **3A** was quite high (212 nM/s). Therefore, within this compound class we believe MDCK data is a useful tool in predicting cell permeation and cellular efficacy.

In conclusion, we have used previous SAR data and structural knowledge of the GSK-3 binding pocket to design a novel set of GSK-3 inhibitors. The benzimidazole substituent forms an intramolecular hydrogen bond to the N-2 nitrogen of the pyrazole core, resulting in a coplanar geometry that allows enhanced binding to the kinase active site and increased potency as compared to the unsubstituted-phenyl analogs. In addition, the benzimidazole compounds demonstrate improved cellular efficacy in stimulating glycogen synthesis over previous series. We also found that within this set of compounds, MDCK data is a useful tool in predicting cell permeation and compound efficacy. The in vivo effects of these compounds in animal models of type 2 diabetes will be reported shortly.

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11. Compound purity was determined to be >95% by both ^{1}H NMR and LC/MS spectroscopy; the spectral data were consistent with the reported structure.
12. GSK-3β was assayed in 96-well microtitre plates at a final concentration of 20 nM in 100 mL HEPES at pH 7.2 containing 10 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 0.3 mg/mL heparin, 2.8 μM peptide substrate (Biotin—Ahx-AAAKRREILSRRP-S(PO_3)YR-amide), 2.5 μM ATP and 0.2 μCi/well [γ^{33}P]-ATP. After 40 min, the reaction was stopped by addition of 100 mM EDTA and 1 mM solution in 100 mM HEPES followed by a solution of diluted streptavidin coated SPA beads (Amersham) in PBS to give a final concentration of 0.25 mg of beads per assay well. The wells were counted on a Packard TopCount NXT microplate counter.
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