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Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors

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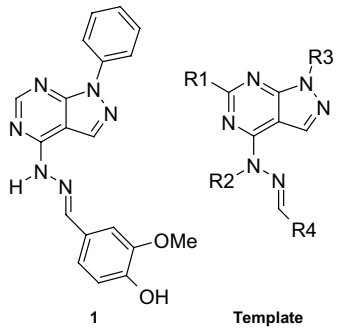
Abstract—A series of [1-aryl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl]arylhydrazones were discovered as novel inhibitors glycogen synthase kinase-3 (GSK-3). Based on initial modeling a detailed SAR was constructed. Modification of the interior binding aryl ring (Ar_1) determined this to be a tight binding region with little room for modification. As predicted from the model, a large variety of modifications could be incorporated into the hydrazone aryl ring. This work led to GSK-3 inhibitors in the low nano-molar range.

Glycogen synthase kinase-3 (GSK-3) is a serine–threonine kinase ubiquitously expressed in all tissues and was originally identified as one of five protein kinases that phosphorylate glycogen synthase.[1] The rate-limiting step for glycogen formation is determined by the enzyme glycogen synthase (GS) and GS activity is modulated by GSK-3 via phosphorylation.[2] Signaling via the PI-3 kinase/PKB pathway, insulin inhibits GSK-3, resulting in GS activation and subsequent formation of glycogen.[3] Recent analysis has shown GSK-3 activity and expression levels were significantly higher in the skeletal muscle from Type 2 diabetic humans than those in healthy subjects.[4] Suggesting aberrant GSK-3 activity may contribute to diabetes. Therefore, small molecules that mimic the action of insulin by inhibiting GSK-3 represents a new approach for the treatment of Type 2 diabetes. GSK-3 is also known to phosphorylate the microtubule associated protein tau,[5] the hyperphosphorylation of which is an early event in neurodegenerative conditions such as Alzheimer's disease (AD). Inhibition of GSK-3 with potent selective small molecules has been shown to protect primary neurons from death induced by reduced PI-3 kinase activity.[6] Therefore inhibitors of GSK-3 may have utility in the treatment of AD.

Recently, a number of publications have emerged describing molecules that inhibit GSK-3.[7] Stimulation of glycogen synthesis in vitro as well as lowering of plasma glucose in diabetic animals by GSK-3 inhibitors has been reported.[8] We recently identified a novel class of pyrazolopyrimidine derivatives from a high throughput screen that inhibit GSK-3 (Fig. 1). The original screening hit **1** had a $pIC_{50} = 7.0$ for inhibition of GSK-3 and was determined to bind in a competitive manner with ATP.[9] Since this template represented a novel GSK-3 inhibitor, we proceeded to investigate this series of compounds. Herein we report on the binding mode and SAR of these GSK-3 inhibitors.

Our initial SAR around this template is shown in Table 1. Replacement of H at R1 with chloro or phenyl (**2**, **3**) results in a complete loss of activity. Likewise replacement of H at R2 with methyl results in a complete loss of activity. Replacement of the phenyl at R3 with hydrogen



1 Template

Figure 1. Pyrazolopyrimidine template.

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Table 1. Inhibition of GSK-3beta

Compound	R1	R2	R3	R4	pIC_{50}[a]
1	H	H	Ph	–Ph–(3-OMe, 4-OH)	7.0
2	**Cl**	H	Ph	–Ph–(3-OMe, 4-OH)	<5
3	**Ph**	H	Ph	–Ph–(3-OMe, 4-OH)	<5
4	H	**Me**	Ph	–Ph–(3-OMe, 4-OH)	<5
5	H	H	**H**	–Ph–(3-OMe, 4-OH)	5.5
6	H	H	***i*-Pr**	–Ph–(3-OMe, 4-OH)	5.6
7	H	H	**Bn**	–Ph–(3-OMe, 4-OH)	<5
8	H	H	Ph	**–4-pyridyl**	7.0

[a] Log of concentration, which inhibits 50% of the activity of GSK-3.

or isopropyl (**5**, **6**) results in weakly active compounds where a benzyl (**7**) at this position results in a complete loss of activity. Lastly replacement of the 3-methoxy-4-hydroxyphenyl group at R4 with a 4-pyridyl (**8**) group gives a compound of equal activity.

Based on the result with compounds **2**, **3**, and **4**, we reasoned that the hydrazone hydrogen and the proximal pyrimidine nitrogen were hydrogen bonding to the hinge region of the ATP binding pocket. In silico docking[10] gave the structure shown in Figure 2, in which the hydrazone hydrogen interacts with the carbonyl of valine 135 and the pyrimidine nitrogen interacts with the NH of valine 135. Although not a hydrogen bond in the formal sense, a potential third interaction could be occurring between the C2 hydrogen of the pyrimidine ring and the backbone carbonyl of the aspartate 133 residue. This model projects the R4 group toward the solvent front and the R3 group deeper into the binding pocket. The loss of activity with compounds **5**, **6**, and **7** suggest that the binding of the R3 phenyl ring is tight. With this binding mode of the template in mind, our approach was to set R1 and R2 as hydrogen and explore the SAR of the R3 (Ar_1) and R4 (Ar_2) positions.

Scheme 1 outlines the general synthesis of the template. The Ar_1 group was set at the start of the synthesis, with the starting aryl-hydrazines (**I**), which upon treatment with ethoxymethylenemalonitrile in refluxing ethanol gave the desired 1-aryl-4-cyano-5-aminopyrazoles (**II**) in good yields. Condensation with aqueous formic acid and subsequent treatment with $POCl_3$ gave the chloro-pyrimidine core (**III**). Displacement of the chloride with hydrazine and final condensation with Ar_2 aldehydes gave the desired products (**IV**).[11] NMR and X-ray analysis revealed that only the ***E*** hydrazone isomer was formed.

Initially we set Ar_2 as 4-pyridyl and examined the effect of substitution on the phenyl ring at Ar_1 (Table 2). The addition of *ortho*-substituents, –Me or –OMe (**9** and **12**, respectively), led to a decrease in activity as compared to the unsubstituted analog (**8**). *ortho*-Substitution in Ar_1 with –Me or –OMe likely results in twisting of the aryl ring out-of-plane with the pyrazolopyrimidine core, suggesting that the preferred binding configuration is co-planar. In addition, there appears to be little room at the back of the binding pocket since *para*-substitution of Ar_1 (**11** and **14**) dramatically weakened potency. Interestingly, the *meta*-OMe substituent (**13**), but not the

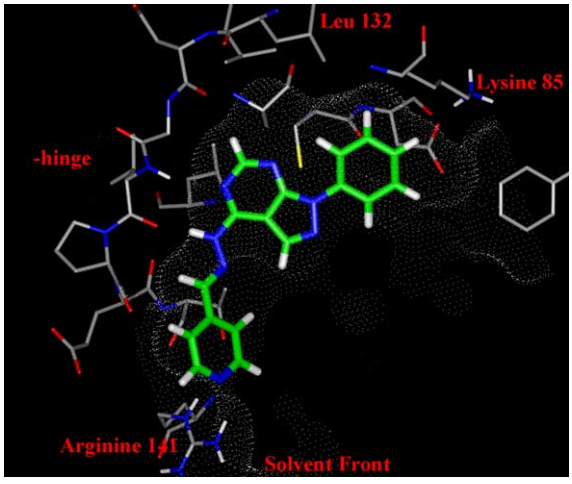
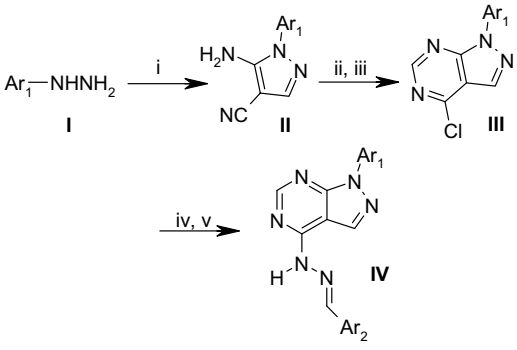


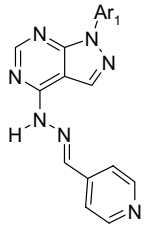
Figure 2. Binding of pyrazolopyrimidines to GSK-3.



Scheme 1. Reagents and conditions: (i) ethoxymethylenemalonitrile, EtOH reflux, 4 h; (ii) 88% formic acid, reflux, 24 h; (iii) $POCl_3$, DMF (cat.) reflux 4 h; (iv) NH_2NH_2–H_20, EtOH, 45 °C 24 h; (v) Ar_2CHO, EtOH, pyrrolidine (cat.) reflux 5 h.

meta-Me analog (**10**), led to a 10-fold improvement in potency ($pIC_{50} = 8.2$) as compared to the unsubstituted phenyl derivative **8** ($pIC_{50} = 7.0$). Therefore we examined other substituents at the *meta*-position (compounds **15**–**23**). Since the *meta*-OMe analog **13** had improved potency over the unsubstituted analog **8**, we first looked at other alkoxy derivatives. Surprisingly, the addition of the extra methylene in the *meta*-OEt group (**15**) significantly decreased potency relative to the *meta*-OMe ($pIC_{50} = 7.0$ vs 8.2, respectively). Replacing the *meta*-OMe with the electron-withdrawing $–OCF_3$ (**16**) also led to a dramatic drop in potency. Similarly, both the

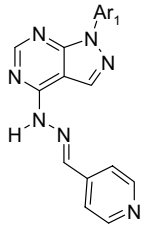
Table 2. Inhibition of GSK-3beta

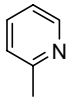
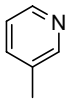
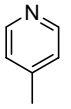
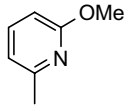


Compound	Ar_1	pIC_{50}[a]
Initial SAR	Substituted phenyl	
8	H	7.0
9	*o*-Me	6.8
10	*m*-Me	6.8
11	*p*-Me	<4.5
12	*o*-OMe	5.6
13	*m*-OMe	8.2
14	*p*-OMe	6.0
m-Substitution	Substituted phenyl	
15	*m*-OEt	7.0
16	m-OCF_3	6.5
17	*m*-NH*n*Pr	5.4
18	*m*-NH(CH_2)cyclopropyl	5.7
19	*m*-NHAc	6.8
20	*m*-NH(CO)*n*Pr	6.2
21	*m*-F	6.5
22	*m*-Br	6.6
23	*m*-NO_2	<4.5

[a] Log of concentration, which inhibits 50% of the activity of GSK-3.

Table 3. Inhibition of GSK-3beta



Compound	Ar_1	pIC_{50}[a]
24		5.9
25		7.5
26		8.5
27		7.4
28		7.4

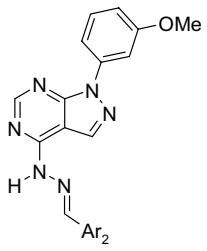
[a] Log of concentration, which inhibits 50% of the activity of GSK-3.

electron-donating amine analogs (**17**, **18**) and the electron-withdrawing amides (**19**, **20**) substantially lost potency relative to compound **13**. Substitution at the *meta*-position with the relatively small but electron-withdrawing substituents –F or $–NO_2$, analogs **21** and **23**, respectively, also weakened potency as compared to compound **13**. From this data set, it appears that electron donating, sterically small substituents are favored at the *meta*-position. For example, *meta*-OMe is optimal for potency but not *meta*-OEt or *meta*-N(H)R, which are perhaps too sterically large. In addition, the *meta*-Me or *meta*-F substituents (examples **10** and **21**, respectively), are disfavored since they are weakly electron donating compared to methoxy. SAR data within this series support that Ar_1 is binding in a narrow pocket of the enzyme, in which co-planarity of the phenyl ring with the pyrazolopyrimidine is optimal. In addition, closer examination of the model suggests that the electronegative oxygen atom of the *meta*-methoxy group may interact with the electropositive side chain of the lysine 85 residue. This modeling does not suggest a formal hydrogen bond, but the relative proximity would support an electrostatic interaction.

With Ar_2 still set as the 4-pyridyl, we examined the effects of replacing the phenyl group of compound **8** with heterocycles (Table 3). Replacement of the phenyl with a 2-pyridyl (**24**) group results in a large loss of activity, possibly due to a disfavoring of co-planarity of the pyridyl ring with the pyrazolopyrimidine core. Addition of a 6-OMe substituent to the 2-pyridyl ring restores activity (**27**), but is still less active than the *meta*-methoxyphenyl containing compound (**13**). We reasoned that the lone pair of the 2-pyridyl is repulsed by either of the neighboring nitrogen lone pairs of the pyrazolopyrimidine core, thus disfavoring a co-planar arrangement of these ring systems. The 3-pyridyl (**25**) is modestly more potent then the phenyl, where the 4-pyridyl (**26**) is substantially more potent. The reason for the increase in potency of the 4-pyridyl is not clear, but as stated above for the *meta*-methoxyphenyl group, an interaction with the electropositive lysine 85 side chain could play a role.

Next we turned to examining the effect of substituents on the hydrazone aryl ring (Ar_2). As stated above, the proposed binding model orientates the Ar_2 group toward the solvent front of the enzyme. We therefore predicted that a large variety of groups would be allowed at Ar_2. Since the Ar_2 group is installed in the last step of the synthesis we were able to prepare a large number of compounds. The *meta*-methoxyphenyl was retained as the Ar_1 substituent, and the various Ar_2 groups prepared are shown in Table 4. As expected a large variety of groups were tolerated at Ar_2. Replacement of the 4-pyridyl of compound **13** with an unsubstituted phenyl ring (**29**) did not affect the activity, suggesting that the pyridyl nitrogen does not make any significant contacts with the enzyme. It is of interest to examine what decreased activity in Table 4. Addition of an *ortho* substituent (**30**, **33**) resulted in a decrease in

Table 4. Inhibition of GSK-3beta

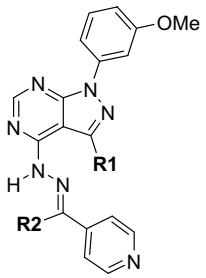


Compound	Ar_2	pIC_{50}[a]
Initial SAR	Substituted phenyl	
29	H	8.0
30	*o*-OMe	<4.6
31	*m*-OMe	7.6
32	*p*-OMe	7.5
33	*o*-F	6.9
34	*m*-F	8.1
35	*p*-F	8.1
36	3,4-diF	7.8
37	*m*-NH_2	8.0
38	*p*-NH_2	8.6
39	*m*-SO_2Me	8.4
40	*p*-SO_2Me	8.6
p-Alkoxy	Substituted phenyl	
41	*p*-OH	7.9
42	*p*-O-allyl	6.8
43	*p*-O(4-F)Bn	5.6
44	*p*-$O(CH_2)_2NMe_2$	8.2
p-Amines	Substituted phenyl	
45	*p*-NEt_2	5.6
46	*p*-CH_2NMe_2	8.3
47	*p*-CH_2NHEt	8.4
48	*p*-NHAc	7.9
49	*p*-NHSO2Me	7.8
50	*p*-$NH(CO)(CH_2)_2NMe_2$	8.3
p-Carbon	Substituted phenyl	
51	*p*-Me	<4.5
52	*p*-Ph	<4.5
53	*p*-CN	7.8
54	*p*-CO_2H	7.5
55	*p*-$C(O)NH(CH_2)_2NMe_2$	7.8
56	*p*-$C(O)NH(CH_2)_2SO_2Me$	8.1
57	*p*-$SO_2NH(CH_2)_2NMe_2$	8.0

[a] Log of concentration, which inhibits 50% of the activity of GSK-3.

activity, where *meta* and *para* substitution is generally allowed. Nonpolar substituents in the *para*-position can lead to a large decrease in activity, such as with 4-Me, 4-phenyl, 4-allyloxy, and 4-(4-fluorobenzyloxy) (**51**, **52**, **42**, and **43**, respectively). It is likely that these larger hydrophobic residues are projecting into the aqueous environment outside the binding pocket. Although, at best, modest improvements in potency were realized with changes at Ar_2 (**38**, **40**), other parameters of the molecules could be effected. Improvements in solubility and cellular efficacy were realized with some of the modifications. For example, in a cell based glycogen accumulation assay, in which the effect of the compound on ^{14}C-glucose incorporation into glycogen was measured, compound **13** was 10-fold more potent that compound **29**, even though their activity at the enzyme was very similar.[12]

Table 5. Inhibition of GSK-3beta



Compound	R1	R2	pIC_{50}[a]
13	H	H	8.2
58	Me	H	8.0
59	Et	H	7.9
60	*N*-Pr	H	8.0
61	*i*-Pr	H	7.5
62	H	Me	7.2
63	Me	Me	5.2

[a] Log of concentration, which inhibits 50% of the activity of GSK-3.

We next looked at the effect of substituents at the 3-position of the pyrazole ring, and at the hydrazone carbon, as shown in Table 5. Incorporation of small *n*-alkyl group in the 3-position of the pyrazole ring (R1) has little effect on the activity of the compounds.[13] With R1 as *iso*-propyl a slight decrease in activity was observed. From the model, groups at this position would project into an empty region of the enzyme-binding pocket. Due to the branching of the *iso*-propyl group it is possible that an unfavorable steric interaction arises with the hydrazone group. Substitution at R2 with a methyl group results in a significant, but not complete loss of activity (**62**). It is interesting that compound **63** in which a methyl group has been incorporated at both R1 and R2 is nearly devoid of activity.

In conclusion, we have described the binding mode and SAR of a novel class of GSK-3 inhibitors. The binding mode, initially proposed by in silico modeling, was supported by the observed SAR. Within the series the Ar_1 group was shown to be highly sensitive to substitution, with the *meta*-methoxyphenyl and 4-pyridyl groups optimal for potency. As predicted by the model, the Ar_2 group is much more tolerant of substitution, with a large variety of groups allowed. Incorporation of small alkyl groups at the 3-position of the pyrazole ring was also allowed. The ability of these compounds to effect glycogen synthesis and their in vivo antidiabetic properties will be described in subsequent publications.

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9. 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, 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. The standard GSK-3 inhibitor SB-415286 gave similar IC_{50} values in this assay as compared to those reported in the literature, see: Smith, D. G.; Buffet, M.; Fenwick, A. E.; Haigh, D.; Ife, R. J.; Saunders, M.; Slingsby, B. P.; Stacey, R.; Ward, R. W. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 635.
10. The molecular modeling was performed with the Maestro software package from Schrodinger. The ligand was manually docked into the active site and then the complex was energy minimized using the Merck molecular force field. Structural co-ordinates for the GSK-3 structure was taken from: Bax, B.; Carter, P. S.; Lewis, C.; Guy, A. R.; Bridges, A.; Tanner, R.; Pettman, G.; Mannix, C.; Culbert, A. A.; Brown, M. J. B.; Smith, D. G.; Reith, A. D. *Structure* **2001**, *9*, 1143.
11. All compounds were determined to be >95% pure by both ^{1}H NMR and LC/MS spectroscopy; the spectral data was consistent with the reported structure. Aryl-hydrazines and aryl-aldehydes were either commercially available or were prepared by reported methods.
12. Compound treatment effects on glycogen accumulation in differentiated L6 cells were determined by an adaptation of the method of Thomas, J. A.; Schlender, K. K.; Larner *J. Anal. Biochem.* **1968**, *25*, 486–499.
13. Compounds **58**–**63** were prepared in a similar manner as shown in Scheme 1. Ethoxymethylenemalonitrile was replaced with the required alkyl derivative, and for compounds **62** and **63** 4-acetylpyridine was used in the final condensation step. NMR and LC/MS spectroscopy were consistent with the reported structure.