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3-Anilino-4-arylmaleimides: Potent and Selective Inhibitors of Glycogen Synthase Kinase-3 (GSK-3)

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Abstract—Potent 3-anilino-4-arylmaleimide glycogen synthase kinase-3 (GSK-3) inhibitors have been prepared using automated array methodology. A number of these are highly selective, having little inhibitory potency against more than 20 other protein kinases.

Glycogen synthase kinase-3 (GSK-3), so called because it was one of originally five kinases known to phosphorylate glycogen synthase (GS),[1] is a serine/threonine kinase which exists as two isoforms (α and β) with molecular weights of 51 and 46 kDa, respectively.[2] The catalytic domains have ~90% identity but GSK-3α has an additional ~60 amino acid residues N-terminal to the kinase domain which is glycine rich. (Recently a third isoform of MW 53 kDa has been discovered which may be a GSK-3α variant formed by post-translational processing.[3]) Differences in function between the isoforms have yet to be established. GSK-3 plays a key role in a number of diverse cellular processes. For example, insulin-stimulated glycogen synthesis in skeletal muscle is mediated by GS which is considered to be the rate-determining enzyme of this process.[4] Phosphorylation of GS by GSK-3 leads to its deactivation.[5] In type 2 diabetics the rate of glycogen synthesis is impaired,[6] the activity of GS is reduced[7] and GSK-3 expression and activity are elevated and inversely correlated with insulin-stimulated glucose disposal,[3] thus implicating elevated GSK-3 in the disease process. GSK-3 is also known to phosphorylate the microtubule associated protein tau in mammalian cells,[8] hyperphosphorylation of which is an early event in neurodegenerative conditions such as Alzheimers disease. GSK-3 inhibition is also implicated in neuronal cell survival.[9]

Bipolar disorder (manic depression) is commonly treated with lithium and the ability of this cation to inhibit GSK-3β (albeit at low millimolar concentration) has been proposed as a possible underlying therapeutic mechanism of action.[10] In support of this, valproate, another drug commonly used for the treatment of bipolar disorder, is also an inhibitor of GSK-3 (both isoforms) at concentrations which have clinical efficacy.[11] Lithium has been shown to protect cerebellar granule neurons from death[12] and chronic lithium treatment has demonstrable efficacy in the middle cerebral artery occlusion model of stroke.[13] Lithium has also been found to mimic some of the actions of insulin by stimulating glycogen synthesis.[14] Lithium exerts other effects apart from weak inhibition of GSK-3, and Jope[15] has argued that it is the integration of these multiple actions of lithium which contribute to its beneficial effects.

More potent and selective inhibitors of GSK-3 than lithium would thus not only help to delineate the relevance of this kinase in a variety of disease states, but may also serve to ameliorate the condition should a suitable therapeutic agent be obtained. Effort towards obtaining such compounds is reported herein.

High throughput screening of the SmithKline Beecham compound collection against rabbit GSK-3α[16] identified a number of maleimide-containing structures as potent inhibitors. Bisindole maleimides of generic structure **1** were originally synthesised as selective protein kinase C (PKC) inhibitors[17] but, since the time of the original publications, inhibition of other kinases has been reported.[18] The ability of this type of compound to inhibit GSK-3 has recently been established[19] but concerns about a possible general selectivity issue with this

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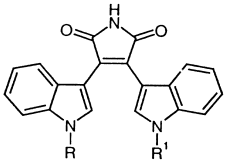
class of compound (see Davies et al.[19c]) precluded development as a template of interest for our studies. By contrast, the lead anilinomaleimide **2** (IC_{50} 910 nM, rabbit GSK-3α) had no published precedent for kinase inhibition and hence was considered a template worthy of further exploration. (Recently, however, 3-anilino-4-indole-maleimides have been disclosed as inhibitors of PKCβ.[20])



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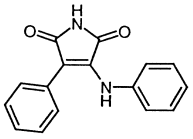

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Chemistry

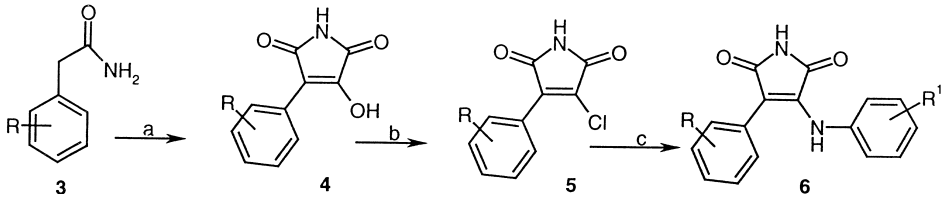
The synthetic route to anilinomaleimides of general structure **6** is shown in Scheme 1. Base-induced condensation of an appropriate arylacetamide **3** with dimethyl oxalate gave the 3-hydroxy-4-arylmaleimides **4**[21] in 53–100% yield which, after treatment with phosphorus oxychloride in *N*,*N*-dimethylaniline[22] or with oxalyl chloride,[23] provided the chlorinated derivatives **5** (15–66%). Treatment of these with anilines furnished the required anilinomaleimides **6**.[22]

Ready access to arylacetamides led to the generation of a number of chloromaleimides which could then be combined array fashion with a diverse range of commercially available anilines and amines. It quickly became clear that the final step of this scheme was ideally suited to robotic synthesis, and conditions were devised for the displacement such that compounds were routinely prepared in one- or two-dimensional, 48-membered arrays (or subsets thereof) using a Bohdan RAM Automatic Synthesiser (chloromaleimide (0.5 mmol), aniline (2.5 equiv), methanol (2 mL), 65 °C, 72 h followed by addition of ethyl acetate (6 mL) and washing with HCl (2 M) and brine). Parallel chromatography (0–10% ether or 0–5% methanol in dichoromethane on silica cartridges) using Sympur (Anachem) or Quad3 (Biotage) instrumentation provided material of generally >95% purity.[24] However, the amine displacement reaction was unsatisfactory under these conditions in those cases where R and R^1 were both *ortho* substituted or where R^1 was a strong *ortho* or *para* electron withdrawing group. In these cases higher yields and/or cleaner reactions were obtained using 4-aryl-3-chloromaleimide and aniline in *N*-methylpyrrolidinone at 150–200 °C for 1–2 h. Whilst the conditions for robotic synthesis were generally satisfactory in the case of anilines, it was found that a large number of products derived from primary amines hydrolysed to the hydroxy maleimide **4** during the acidic work up, presumably as a consequence of the greater basicity of the amine and thus its more facile protonation and better leaving group ability. This was easily overcome by utilising conventional manual work up procedures with concomitant shorter reaction times.

Enzymology and SAR

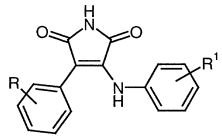
Selected compounds from this series were competitive with ATP[25] when evaluated against recombinant human GSK-3α (hGSK-3α).[26] Compounds were routinely evaluated against both hGSK-3α and β to establish IC_{50}s.[27] In no case was selectivity demonstrated for either enzyme, reflecting the homology of the ATP binding site in these isoforms (53 residues immediately surrounding the ATP binding site of hGSK-3 are identical apart from a Glu^{195}(α) to Asp^{132}(β) modification). The medicinal chemistry strategy was to react an initial set of chloromaleimides (**5**, R = H, 4-Cl, and 4-OMe) with a diverse set of anilines in a 3 (chloromaleimide) ×16 (aniline) two-dimensional array. This gave a number of compounds with submicromolar potency which were then elaborated in a variety of array formats via a combination of rational design, additivity of substituents, and further exploitation of diversity to optimise potency. Aryl substituents that were found to confer sub 100 nM potency were subsequently investigated by one-dimensional arrays to define the limits of the activity boundary. Similarly aniline substituents that were routinely found to confer good potency were used in arrays with newly synthesised chloromaleimide intermediates. Use of these iterative processes rapidly led to identification of compounds with $IC_{50} < 50$ nM.

Those substituents in the aryl and anilino rings that frequently conferred potency are shown as a small subset of data obtained (Table 1). Parent compounds (i.e., either R = H or R^1 = H) are shown, where prepared, to establish baseline data. Interestingly, both electron-withdrawing and electron-donating groups in the non-



Scheme 1. Preparation of 3-anilino-4-arylmaleimides. Reagents: (a) $(MeO_2C)_2$, KOBu^t, DMF; (b) $(COCl)_2$, DCM or $POCl_3$, $PhNMe_2$; (c) R^1-$PhNH_2$, MeOH or *N*-methylpyrrolidinone.

Table 1. IC_{50} values for 3-anilino-4-arylmaleimide inhibitors of hGSK-3α[a]



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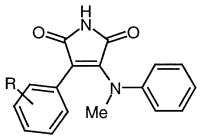

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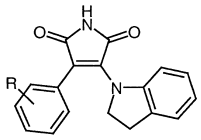

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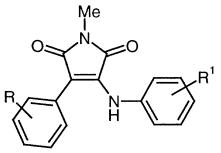

9a R = R^1 = H
9b R = 4-Cl, R^1 = 4-Cl-3-CO_2H
9c R = 4-Cl, R^1 = 3,5-di-Cl-4-OH

	6 R^1									7	8
R	H	3-Cl	3-OH	4-OH	3-Cl-4-OH	3,5-di-Cl-4-OH	3-CO_2H	4-Cl-3-CO_2H	4-SMe		
H	529±59	301±46	704±72			149±8	291±14	143±9	404±74	2613±418	
2-Cl	216±13	195±11	374±36		152±9	93±9	136±11	74±5	161±11		337±8
2-OMe	216±20	114±6	259±7		139±5 (28)	82±7			110±10		187±6
2-NO_2		104±5	251±13		104±10	52±5		28±3			131±10
3-Cl			1478±216		94±6	58±2	134±13	76±8	532±24		460±26
3-OMe		257±8	472±20			142±8	195±13	85±5	203±14	>4500	
3-NO_2	141±11	70±6	236±14	123±9	59±3	20±2	79±6	26±2	152±3	1398±74	161±7
4-Cl	514±22	447±11	407±22	317±42	173±16	91±11	186±27	109±7	529±22	2285±103	1412±70
4-OMe	390±38	156±7	481±76			83±2	214±20		243±17		694±63
4-NO_2						71±10			392±10		

[a]IC_{50} (nM) versus hGSK-3α, see ref 27 for assay details. Results are a mean of two determinations run in duplicate ($n=4$) and are given as mean±SEM. The compound where R = 2-OMe and R^1 = 3-Cl-4-OH was used as an internal standard across all assay plates and is a mean of 28 determinations.

anilino aryl ring resulted in highly potent compounds suggesting that either there is not an overriding electronic component to the activity conferred by this moiety or, that if there is, then the binding mode of those particular compounds is likely to be different. Where comparisons allow, potency is always enhanced over parent by the presence of a 3-carboxyl substituent in the anilino moiety, suggesting a favourable interaction of carboxylate with the protein. Potency is always augmented by the presence of an additional 4-chloro substituent. A similar increase of potency is observed by the addition of 3-chloro or 3,5-dichloro substituents to those compounds containing a 4-hydroxyaniline moiety, suggesting that the greater acidity of the phenol, or the above acid, may result in the generation of a stronger H-bond interaction, or that potency is gained from the additional lipophilicity. Combination of the favoured aryl substituents with those aniline moieties which consistently gave good potency resulted in some of the most potent compounds to be obtained from this series (Table 1, highlighted).

Comparison of *N*-methylanilinomaleimides **7** with the anilino analogues **6** shows a marked reduction in potency of the former compounds. In the absence of information to the contrary, this might suggest that the aniline proton is critical for binding. However, the indolines **8** show potency which, in some cases, is similar to that of the parent aniline, demonstrating that for these compounds at least, an N–H interaction with the protein appears not to be critical. Increased conformational restraint of the indoline may allow a binding mode which is unavailable to the *N*-methylaniline derived compounds.

Potency of the three methylated compounds **9a–c** (**5** + MeI/K_2CO_3 prior to coupling with aniline, or use of the *N*-methylated analogue of **3** in the reaction sequence) is substantially reduced (**9a**, **9b** IC_{50} >5 μM; **9c** IC_{50} >3 μM) relative to the unsubstituted maleimide, implying that the maleimide NH is essential for binding. By analogy with published X-ray structures of indolocarbazole kinase inhibitors such as staurosporine[28] it is likely that the anilinomaleimide adopts a binding mode where the maleimide proton and the adjacent carbonyl oxygen form a donor–acceptor pair with the C=O (*n*) and NH (*n*+2) of three amino acid residues in the hinge region of the ATP site of the kinase. Molecular modelling studies of compounds from this series using an homology model of GSK-3 derived from cyclin dependent kinase-2 (CDK-2) will be discussed in future publications.

Selectivity

Selectivity of kinase inhibition is critical for pathway analysis in cellular systems and in vivo. For this reason some of the above compounds were profiled against a panel of more than 20 kinases (including GSK-3β), Table 2.[29] From the table it is clear that representative examples from the anilinomaleimide series are selective inhibitors of GSK-3 (see footnote to Table 2 for compound descriptors). For the compounds shown, inhibition of the majority of these kinases was <50% at 10 μM in contrast to their potency as inhibitors of GSK-3β. Significantly, since a number of recent publications[30] describe potent inhibitors of CDK-2 which also inhibit GSK-3, two of the most potent GSK-3 inhibitors

Table 2. Selectivity of 3-anilino-4-arylmaleimide inhibitors for GSK-3β[a]

Cmpd. No.*	AMPK	Chk1	CKII	JNK	LCK	MAPK	RSK-2	MAPKAP-K2	MEK1	MSK1	p70S6K	PDK1	PHOS. K	PKA	PKBα	PKCα	PRAK	ROKa	SAPK2a	SAPK2b	SAPK3	SAPK4	SGK	CDK2/Cyclin A	GSK-3β
6a	46	25	18	0	19	3	62	4	12	19	8	8	0	7	5	30	0	0	5	0	0	0	3	ND	**83**
6b	14	22	6	0	24	1	44	0	5	0	1	19	2	1	7	8	11	-7	11	5	0	0	0	ND	**81**
6c	6	20	3	7	0	ND	27	8	0	22	0	5	9	6	0	0	7	11	0	1	0	0	11	11	**100**
6d	7	14	0	0	0	ND	27	6	0	16	0	3	9	1	0	0	0	0	10	0	0	0	0	14	**87**
8a	15	0	0	6	38	5	27	0	6	9	0	15	30	1	3	28	15	0	5	0	0	11	4	ND	**90**

[a]Values are %I @ 10 μM using 100 μM ATP (see ref 19c for kinases used and assay details). ND% I at 10 μM versus this kinase not determined.
* **6a** R = 2-NO_2, R^1 = 3-Cl-4-OH; **6b** R = 4-OMe, R^1 = 3-Cl; **6c** R = 3-NO_2, R^1 = 4-Cl-3-CO_2H; **6d** R = 2-NO_2, R^1 = 4-Cl-3-CO_2H; **8a** R = 2-OMe.

described here (**6c** and **6d**, IC_{50} versus GSK-3α 26 and 28 nM, respectively) are not inhibitors of CDK-2.

Conclusion

An efficient, automated synthetic approach to derivatives of a novel kinase inhibitor template is described and compounds presented here represent some of the most potent and selective GSK-3 inhibitors reported to date. The high potency and good selectivity of compounds from Table 2 should allow their use as tool compounds to aid resolution of the often complex nature of those signalling pathways where GSK-3 is believed to be implicated. The cellular activity of one such compound (**6a**, SB-415286) has already been described.[25,31]

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26. hGSK-3 isoforms with a HexaHis tag were expressed using the baculovirus expression system and protein was recovered from cells by homogenisation and purification using NiNTA Superflow (Qiagen).
27. Using microtitre plates, GSK-3 was assayed in 50 mM MOPS buffer, pH 7.0 containing 5% glycerol, 0.01% Tween-20, 7.5 mM 2-mercaptoethanol, 10 mM magnesium acetate, 8 μM substrate peptide (Biotin-KYRRAAVPPSPSLSRHSS-PHQ(SP)EDEEE, where (SP) is a pre-phosphorylated serine) and 10 μM [γ-^{33}P]-ATP. After incubation for 1 h at room temperature, the reaction was stopped by addition of 50 mM EDTA solution containing Streptavidin coated SPA beads (Amersham) to give a final 0.2 mg of beads per assay well in a 384 microtitre plate format. Following centrifugation, the microtitre plates were counted in a Trilux 1450 microbeta liquid scintillation counter (Wallac). IC_{50} values were generated for each compound by fitting to a four parameter model.
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