Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription

Matthew P Coghlan [1], Ainsley A Culbert [2], Darren AE Cross [2], Stacey L Corcoran [1], John W Yates [1], Nigel J Pearce [1], Oliver L Rausch [2], Gregory J Murphy [1], Paul S Carter [3], Lynne Roxbee Cox [3], David Mills [4], Murray J Brown [4], David Haigh [5], Robert W Ward [5], David G Smith [5], Kenneth J Murray [4], Alastair D Reith [2] and Julie C Holder [1]

Background: Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase, the activity of which is inhibited by a variety of extracellular stimuli including insulin, growth factors, cell specification factors and cell adhesion. Consequently, inhibition of GSK-3 activity has been proposed to play a role in the regulation of numerous signalling pathways that elicit pleiotropic cellular responses. This report describes the identification and characterisation of potent and selective small molecule inhibitors of GSK-3.

Results: SB-216763 and SB-415286 are structurally distinct maleimides that inhibit GSK-3α in vitro, with K_is of 9 nM and 31 nM respectively, in an ATP competitive manner. These compounds inhibited GSK-3β with similar potency. However, neither compound significantly inhibited any member of a panel of 24 other protein kinases. Furthermore, treatment of cells with either compound stimulated responses characteristic of extracellular stimuli that are known to inhibit GSK-3 activity. Thus, SB-216763 and SB-415286 stimulated glycogen synthesis in human liver cells and induced expression of a β-catenin-LEF/TCF regulated reporter gene in HEK293 cells. In both cases, compound treatment was demonstrated to inhibit cellular GSK-3 activity as assessed by activation of glycogen synthase, which is a direct target of this kinase.

Conclusions: SB-216763 and SB-415286 are novel, potent and selective cell permeable inhibitors of GSK-3. Therefore, these compounds represent valuable pharmacological tools with which the role of GSK-3 in cellular signalling can be further elucidated. Furthermore, development of similar compounds may be of use therapeutically in disease states associated with elevated GSK-3 activity such as non-insulin dependent diabetes mellitus and neurodegenerative disease.

[1]Department of Vascular Biology, SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AD, UK
[2]Department of Neuroscience Research, SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AD, UK
[3]Department of Biotechnology and Genetics, SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AD, UK
[4]Department of Molecular Screening Technologies, SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AD, UK
[5]Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AD, UK

Correspondence: Matthew P Coghlan
E-mail: matthew_coghlan-1@sbphrd.com

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Introduction

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase encoded by two highly homologous and ubiquitously expressed genes. The catalytic domains of mammalian GSK-3α and GSK-3β are 95% identical at the amino acid level, whereas the amino- and carboxy-termini are less conserved [1]. GSK-3 was originally identified by virtue of its ability to phosphorylate and inactivate glycogen synthase, the rate limiting enzyme in glycogen synthesis [2]. However, it is now apparent that GSK-3 has many putative targets, including IRS-1 [3], the translation initiation factor eIF2B [4], transcription factors c-jun [5], CREB [6], NFAT [7], β-catenin [8], C/EBPα [9] and the neuronal microtubule associated proteins MAP-1B [10,11] and Tau [12–15] (Figure 1).

A variety of extracellular stimuli inhibit cellular GSK-3 activity, including insulin [16], growth factors [17–23], Wnt cell specification proteins [24] and cell adhesion [25]. Since these stimuli elicit a diverse range of responses in a number of different cell types, inhibition of GSK-3 activity is potentially pivotal in mediating pleiotropic cellular responses to external stimuli (Figure 1). However, the potential role of GSK-3 inhibition in any given response is

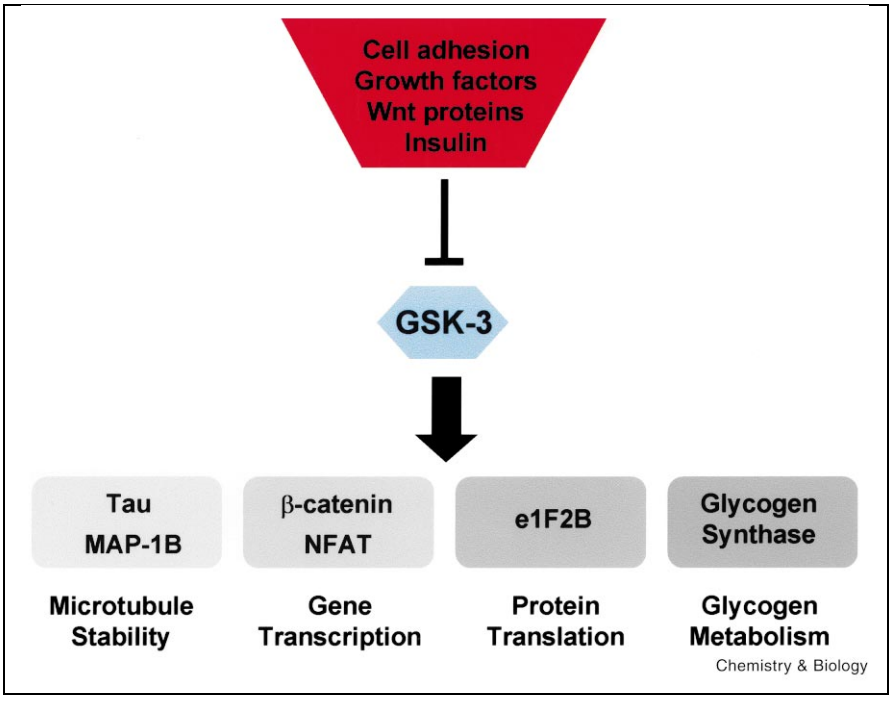

Figure 1. Inhibition of GSK-3 activity mediates pleiotropic cellular responses to external stimuli. GSK-3 phosphorylates and modulates the activity of a number of key regulatory proteins (arrow in the figure) in the cell including: microtubule associated proteins (Tau, MAP-1B); transcription factors (β-catenin, NFAT); a translation initiation factor (eIF2B) and glycogen synthase. Consequently, external stimuli that inhibit GSK-3 activity (hammerhead in the figure) have the potential to regulate a broad range of cellular processes including microtubule stability, gene transcription, protein translation and glycogen metabolism.

complicated by the fact that stimuli often initiate additional signalling pathways to the one that affects GSK-3 activity. Therefore, in order to more definitively implicate GSK-3 inhibition in a response it is necessary to selectively inhibit this kinase and assess whether this alone is sufficient to induce the response.

Antisense targeting of GSK-3 and the overexpression of a dominant negative form of the kinase have both been used to inhibit cellular GSK-3 activity [26,27]. However, these techniques may have spurious effects on cell physiology that are independent of GSK-3 inhibition. This is because full or partial sequences targeted by antisense oligonucleotides are predicted to be present in multiple mRNAs, not just the one encoding the intended target (for review see [28]). Sequestration of kinase activating factors or co-factors by dominant negative kinases may also inhibit kinases in addition to the target kinase. These genetic techniques also have the disadvantage of being invasive, chronic interventions that again raise concerns about non-specific effects on cell physiology.

LiCl is an ATP noncompetitive inhibitor of GSK-3β activity (K_i 2 mM) that has been used extensively in studies investigating the functional role of GSK-3 [10,12,29–31]. It has the advantage over genetic approaches of effecting an acute inhibition of GSK-3 in cells. However, LiCl is not a selective inhibitor of GSK-3 and has a number of additional activities that complicate interpretation of data generated from its use. Recently, LiCl has been reported as a low mM inhibitor of casein kinase-2, p38 regulated/activated kinase and MAPK activated protein kinase-2 [32]. Additionally, LiCl is a μM inhibitor of polyphosphate 1-phosphatase and inositol monophosphatase, two enzymes required for de novo synthesis of inositol and thus to support phosphoinositide signalling (for review see [33]). Paradoxically, LiCl has also been reported to acutely elevate phosphatidylinositol 3-phosphate levels in rat cerebellar granule cells, thereby activating PKB [34]. Activated PKB phosphorylates and inhibits GSK-3, suggesting that LiCl has the potential to inhibit this kinase both directly and indirectly in cells.

Recent drug discovery programmes have demonstrated the feasibility of identifying highly selective, cell permeable, small molecule inhibitors of protein kinases (for reviews see [35,36]). This report is the first to describe the directed identification and characterisation of small molecule inhibitors of GSK-3. These compounds potently and selectively inhibited GSK-3 in vitro. Furthermore, the compounds elicited cellular responses that have been previously attributed to inhibition of GSK-3. Therefore, these compounds will be valuable pharmacological tools with which to further elucidate the role of GSK-3 inhibition in mediating cellular responses to extracellular stimuli. Moreover, these compounds and their derivatives may be of therapeutic use in treating conditions associated with elevated GSK-3 activity such as non-insulin dependent

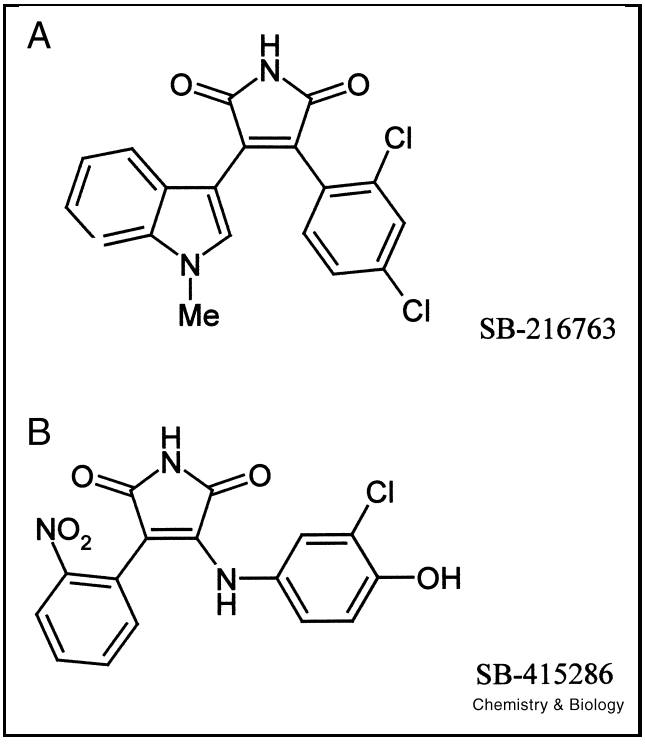

Figure 2. The chemical structures of SB-216763 and SB-415286. **(A)** SB-216763 is 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione. **(B)** SB-415286 is 3-(3-chloro-4-hydroxyphenylamino)-4-(2-nitrophenyl)-1*H*-pyrrole-2,5-dione.

diabetes mellitus [37,38] and neurodegenerative disease [26,39].

Results

Identification of SB-216763 and SB-415286 as inhibitors of GSK-3

Maleimide derivatives were identified as leads from a high throughput screen of the SmithKline Beecham compound bank against rabbit GSK-3α. The arylindolemaleimide SB-216763 and anilinomaleimide SB-415286 [46] were subsequently identified as potent inhibitors of human GSK-3α using a peptide-based protein kinase assay as described in Materials and methods (structures in Figure 2). SB-216763 and SB-415286 inhibited human GSK-3α with IC_{50}s of 34 nM and 78 nM respectively, when assayed in the presence of 0.01 mM ATP (Figure 3A,B). These compounds inhibited GSK-3α in an ATP competitive manner (Figure 4A,B). SB-216763 and SB-415286 inhibited GSK-3α with K_is of 9 nM and 31 nM respectively (Figure 4A,B). SB-216763 and SB-415286 were equally effective at inhibiting human GSK-3α and GSK-3β (data not shown).

Selectivity of SB-216763 and SB-415286 as GSK-3 inhibitors

The selectivity of SB-216763 and SB-415286 was investigated by testing the ability of these compounds to inhibit a panel of 25 different serine/threonine and tyrosine protein kinases. In the presence of 0.1 mM ATP, 10 μM SB-216763 and SB-415286 inhibited GSK-3β kinase activity by 96% and 83% respectively (Table 1). In contrast, these compounds exhibited little or no inhibition of the other 24 members of the kinase selectivity panel.

SB-216763 and SB-415286 stimulate glycogen synthesis in a human liver cell line

There is substantial evidence that insulin-induced inhibition of GSK-3 activity contributes to the ability of this hormone to stimulate glycogen synthesis in muscle, liver and fat cells [31,40–42]. Consistent with these reports, both

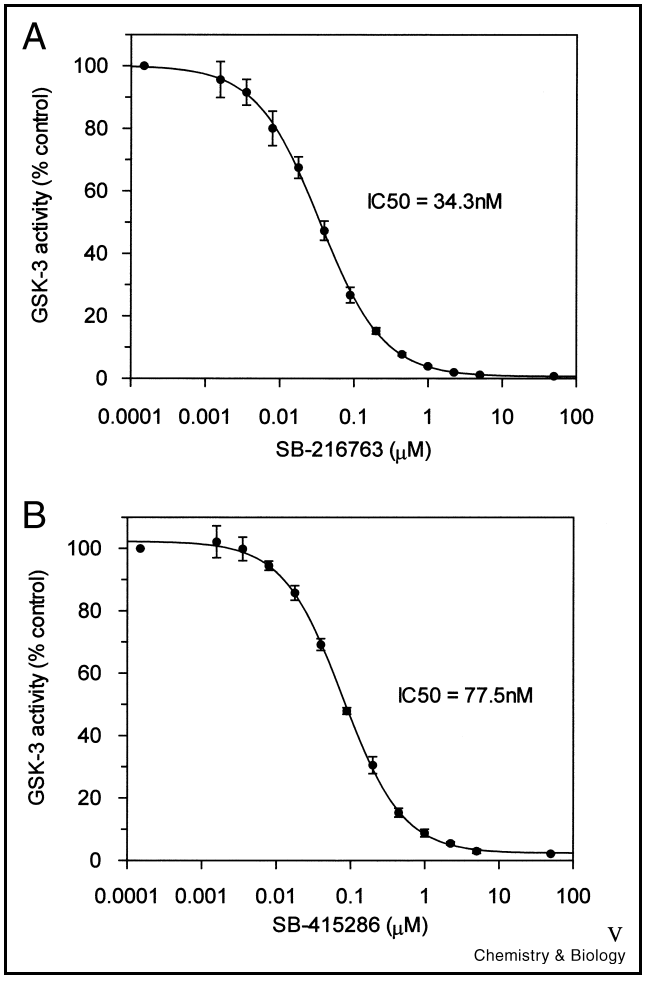

Figure 3. SB-216763 and SB-415286 inhibit GSK-3 activity in vitro. The ability of GSK-3α to phosphorylate a peptide substrate was assessed in the presence of the indicated concentrations of SB-216763 **(A)** or SB-415286 **(B)**. Results are presented as the percentage of GSK-3α activity in control incubations in which compound was omitted. Data are mean ± S.E.M. from at least four independent experiments, where each point was assayed in quadruplicate.

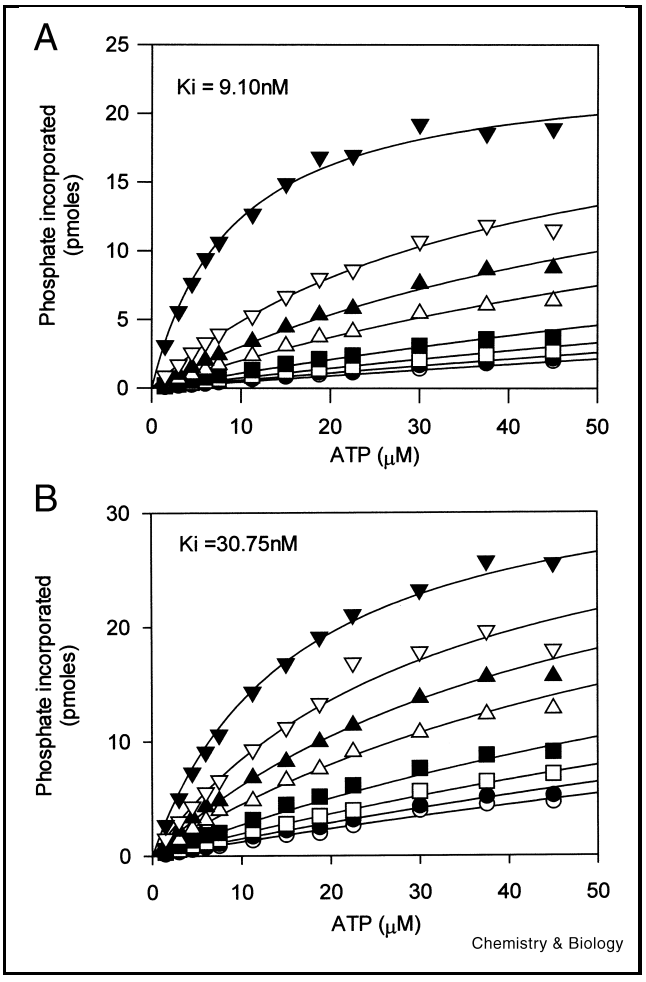

Figure 4. SB-216763 and SB415286 inhibit GSK-3 activity in an ATP competitive manner. The ability of SB-216763 **(A)** and SB-415286 **(B)** to inhibit GSK-3α activity in the presence of the indicated concentrations of ATP was studied. Control incubations (▼) and incubations carried out in the presence of SB-216763 or SB-415286 at 30 nM (▽), 60 nM (▲), 100 nM (△), 200 nM (■), 300 nM (□), 400 nM (●) and 500 nM (○). Results are presented as pmol of phosphate incorporated into peptide substrate/10 μl of sample/30 min. Data are means of duplicate assay wells.

SB-216763 and SB-415286 stimulated glycogen synthesis in the Chang human liver cell line with EC_{50}s of 3.6 μM and 2.9 μM respectively (Figure 5A). The maximum effective concentrations of SB-216763 and SB-415286 were 30 μM and 10 μM respectively. These concentrations of the compounds stimulated glycogen synthesis to a greater degree than a maximum stimulatory concentration of insulin (Figure 5B). The non-selective GSK-3 inhibitor, LiCl, at its maximum effective concentration (for LiCl dose response see Figure 5C) stimulated glycogen synthesis similarly to the compounds (Figure 5B). Maximum effective concentrations of LiCl plus either SB-216763 or SB-415286 were not additive in their stimulation of glycogen synthesis (Figure 5B). In contrast, insulin was additive with either compound or LiCl in the stimulation of glycogen synthesis (Figure 5B).

SB-216763 and SB-415286 activate glycogen synthase in Chang human liver cells and in HEK293 cells

To determine whether the ability of SB-216763 and SB-415286 to stimulate glycogen synthesis in liver cells resulted from inhibition of GSK-3 activity in these cells, the activity of glycogen synthase was assessed in compound-treated cells. This enzyme represents a marker of cellular GSK-3 activity as its activity is directly inhibited by GSK-3 mediated phosphorylation. SB-216763, SB-415286,

Table 1
Effect of SB-216763 and SB-415286 on the activities of protein kinases in vitro.

Protein kinase	Activity (% control)	
	SB-216763	SB-415286
AMPK	81	54
Chk1	90	75
CKII	95	82
GSK-3β	4	17
IRTK	84	81
JNK	99	101
Lck	101	81
MAPK	88	97
MAPKAPK-1β	96	38
MAPKAPK-2	94	96
MKK1	94	88
MSK1	95	81
p70 S6K	94	92
PDK1	100	92
Phos.K	117	100
PKA	102	93
PKBα	96	95
PKCα	77	70
PRAK	80	101
ROKα	92	109
SAPK2α	81	95
SAPK2β	95	106
SAPK3	104	111
SAPK4	82	92
SGK	98	97

Protein kinases were assayed ±10 μM SB-216763 or SB-415286 in the presence of 0.1 mM ATP. Activities are given as the mean of duplicate determinations relative to control incubations in which the inhibitors were substituted with DMSO vehicle. Similar results were obtained in a second independent screen of the same panel of protein kinases. AMPK, AMP activated protein kinase; Chk, checkpoint kinase; CKII, casein kinase-2; GSK-3, glycogen synthase kinase-3; IRTK, insulin receptor tyrosine kinase; JNK, c-Jun N-terminal kinase; Lck, lymphocyte c-src kinase; MAPK, mitogen activated protein kinase; MAPKAPK, MAPK activated protein kinase; MKK, MAPK kinase; MSK1, mitogen and stress activated protein kinase-1; p70 S6K, p70 ribosomal protein S6 kinase; PDK1, 3-phosphoinositide dependent protein kinase-1; Phos.K, phosphorylase kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PRAK, p38 regulated/activated kinase; ROK, Rho dependent protein kinase; SAPK, stress activated protein kinase; SGK, serum and glucocorticoid induced kinase.

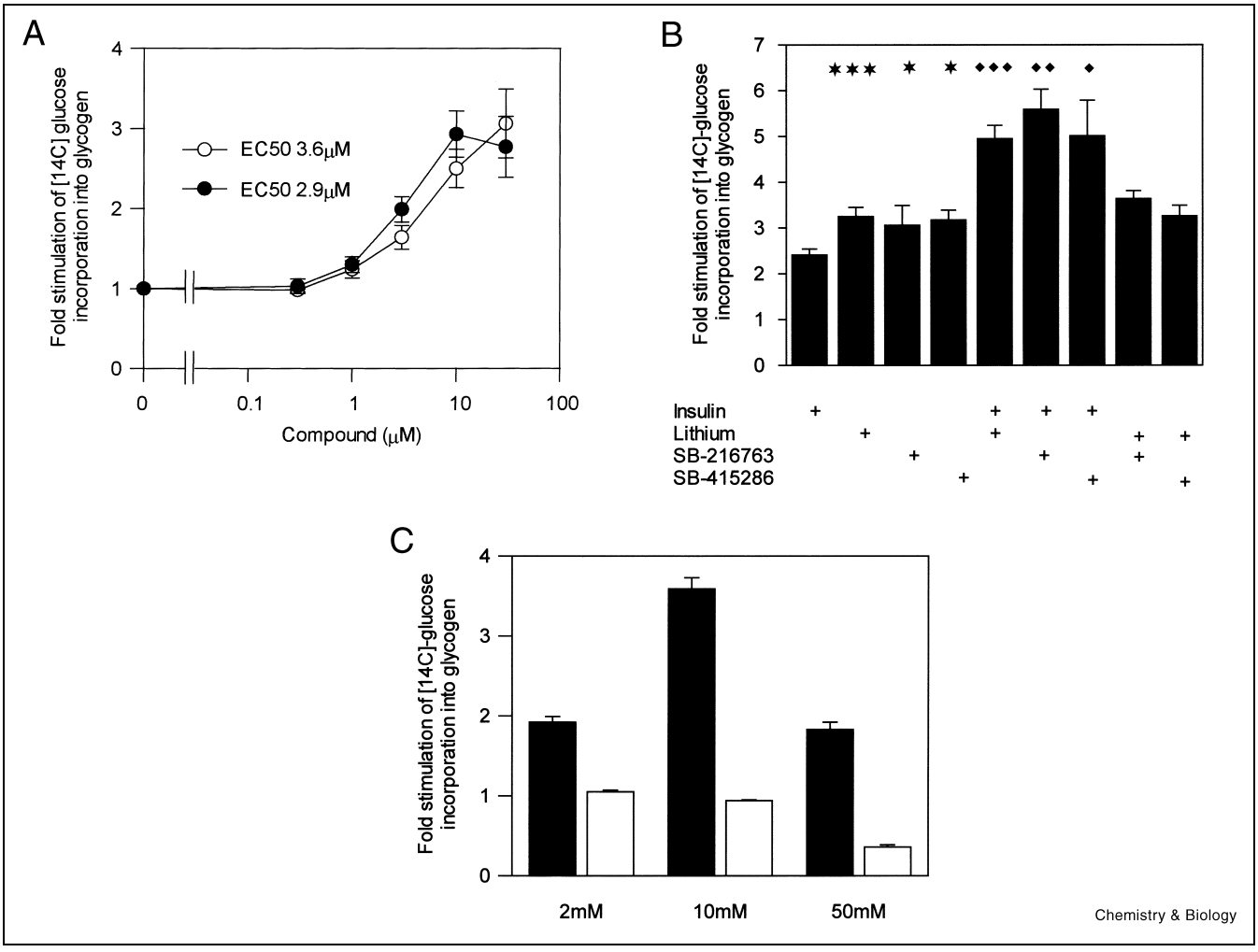

Figure 5. SB-216763 and SB-415286 stimulate glycogen synthesis in Chang human liver cells. Chang cells were incubated in serum-free medium for 16 h. Serum starved Chang cells were treated for 60 min with the indicated concentration of **(A)** SB-216763 (○) or SB-415286 (●), **(B)** 30 μM SB-216763, 10 μM SB-415286, 10 mM LiCl or 1 μM insulin and **(C)** 2, 10 and 50 mM LiCl (filled bars) or NaCl (open bars). Cells not exposed to compound were exposed to DMSO vehicle. After 60 min of treatment, [^{14}C]glucose was added and the cells were incubated for a further 75 min. Cells were then extracted into 20% (w/v) KOH and total cell glycogen was isolated by ethanol precipitation. Glycogen pellets were resuspended in H_2O and [^{14}C]glucose incorporation was assessed by scintillation counting. Results are expressed as fold stimulation of glycogen synthesis observed in control cells treated with DMSO vehicle. Data are mean ± S.E.M. for four independent experiments where each point represents quadruplicate cell treatments. $^{*}P < 0.05$, $^{***}P < 0.001$ vs insulin alone and $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$ and $^{\blacklozenge\blacklozenge\blacklozenge}P < 0.001$ vs LiCl or compound alone.

LiCl and insulin each stimulated glycogen synthase activity in Chang human liver cells (Figure 6A). The compounds and LiCl each activated glycogen synthase to a greater degree than insulin. Treatment of cells with either compound plus LiCl did not elicit an additive activation of glycogen synthase. However, the activation of glycogen synthase by insulin was additive to that effected by compounds or LiCl (Figure 6A). SB-216763, SB-415286 and LiCl were also each capable of inhibiting GSK-3 activity in the HEK293 human embryonic kidney cell line as demonstrated by their ability to activate glycogen synthase in these cells (Figure 6B).

SB-216763 and SB-415286 induce transcription of a β-catenin-LEF/TCF regulated reporter gene in HEK293 cells

GSK-3 phosphorylation of β-catenin targets this protein for ubiquitin mediated proteasomal degradation [8]. Consequently, in quiescent cells in which GSK-3 activity is high, cytosolic and nuclear levels of β-catenin remain low. Inhibition of GSK-3 activity by the cell specification factor Wnt [24] promotes the stabilisation and accumulation of cytosolic β-catenin. Accumulated cytosolic β-catenin binds to members of the lymphoid enhancer factor/T cell factor (LEF/TCF) transcription factor family. The resulting complex is shuttled to the nucleus where it regulates

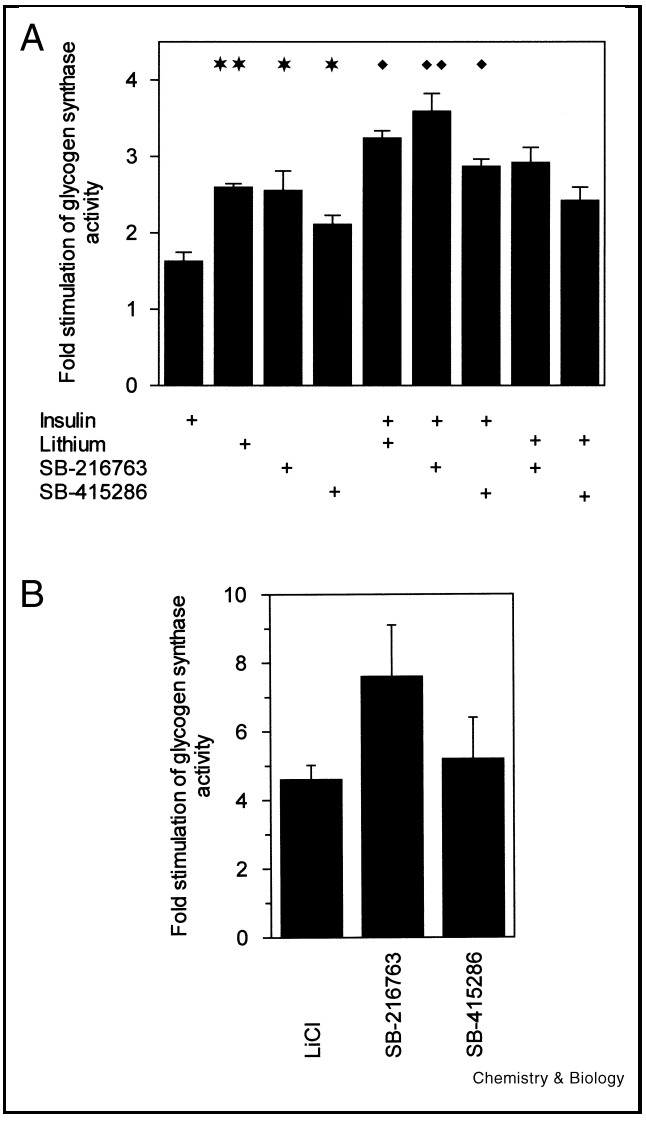

Figure 6. SB-216763 and SB-415286 activate glycogen synthase in Chang human liver cells and HEK293 cells. Serum starved Chang cells were treated for 60 min with **(A)** 30 μM SB-216763, 10 μM SB-415286, 10 mM LiCl or 1 μM insulin. Serum starved HEK293 cells **(B)** were treated for 90 min with 5 μM SB-216763, 40 μM SB-415286 or 20 mM LiCl. Cells not exposed to compound were exposed to DMSO vehicle. Cells were harvested and cell lysates were prepared. Lysate supernatants were assayed for glycogen synthase activity ± glucose 6-phosphate. Results are expressed as fold stimulation of the glycogen synthase activity ratio observed in control cells treated with DMSO vehicle. Data are mean ± S.E.M. for three independent experiments where each point represents duplicate cell treatments. $^{*}P<0.05$, $^{**}P<0.01$ vs insulin alone and $^{\blacklozenge}P<0.05$, $^{\blacklozenge\blacklozenge}P<0.01$ vs LiCl or compound alone.

gene expression [24,43]. Using a HEK293 transient transfection β-catenin-LEF/TCF luciferase reporter assay, SB-216763 and SB-415286 were observed to dose-dependently induce transcription of the β-catenin dependent gene (Figure 7A,B). The maximum effective concentrations of SB-216763 and SB-415286 in this assay were 5 μM and 30 μM respectively. Neither compound induced the expression of a control reporter gene which is insensitive to β-catenin owing to mutation of the LEF/TCF binding sites (Figure 7A,B). Similar to the compounds, LiCl induced a dose dependent activation of the reporter gene (Figure 7C). In contrast to SB-216763 and SB-415286, LiCl also stimulated induction of the control, β-catenin independent reporter (Figure 7C). NaCl had no effect on transcription of either reporter gene (Figure 7D).

Discussion

This report describes the identification of the arylindolemaleimide SB-216763 and the anilinomaleimide SB-415286 as potent inhibitors of GSK-3. In common with most other protein kinase inhibitors these compounds inhibit their target protein kinase in an ATP competitive manner [35,36]. Therefore, these compounds inhibit GSK-3 in vitro at 0.01 mM ATP with IC_{50}s less than 100 nM but approximately 100-fold higher concentrations are required in order to observe compound effects in cells where ATP is present at 1–2 mM [35].

The utility of protein kinase inhibitors as research tools or potential therapeutic agents is dependent upon their ability to selectively inhibit the target protein kinase. SB-216763 and SB-415286 are highly selective inhibitors of GSK-3. Therefore, under conditions in which SB-216763 and SB-415286 completely abolished GSK-3 activity, these two compounds did not affect the activity of 24 different serine/threonine and tyrosine protein kinases that were tested. Moreover, the finding that neither SB-216763 nor SB-415286 inhibited the activity of those kinases required for insulin signalling and cell survival (IRTK, p70 S6K, PDK1, phosphorylase kinase, PKB) suggests that these compounds may be of use therapeutically in disease states associated with elevated GSK-3 activity such as diabetes and neurodegenerative disorders.

The value of these compounds as tool GSK-3 inhibitors was evidenced by their ability to induce cellular responses that had previously been attributed to inhibition of GSK-3 activity. SB-216763 and SB-415286 both stimulated glycogen synthesis in human liver cells and this effect was additive with the stimulation elicited by insulin. This suggests that in these cells, insulin and GSK-3 inhibitors effect their responses through distinct molecular pathways (Figure 8). It is likely that in this case insulin acts through stimulating the MAPK/MAPKAPK-1β/$PP1_G$ pathway, rather than through inhibition of GSK-3, to stimulate glycogen synthase and hence glycogen synthesis [40,44]. The non-selective GSK-3 inhibitor, LiCl, also stimulated glycogen synthesis in these cells and this effect was also additive with that of insulin. However, co-treatment of cells with either SB-216763 or SB-415286 in combina-

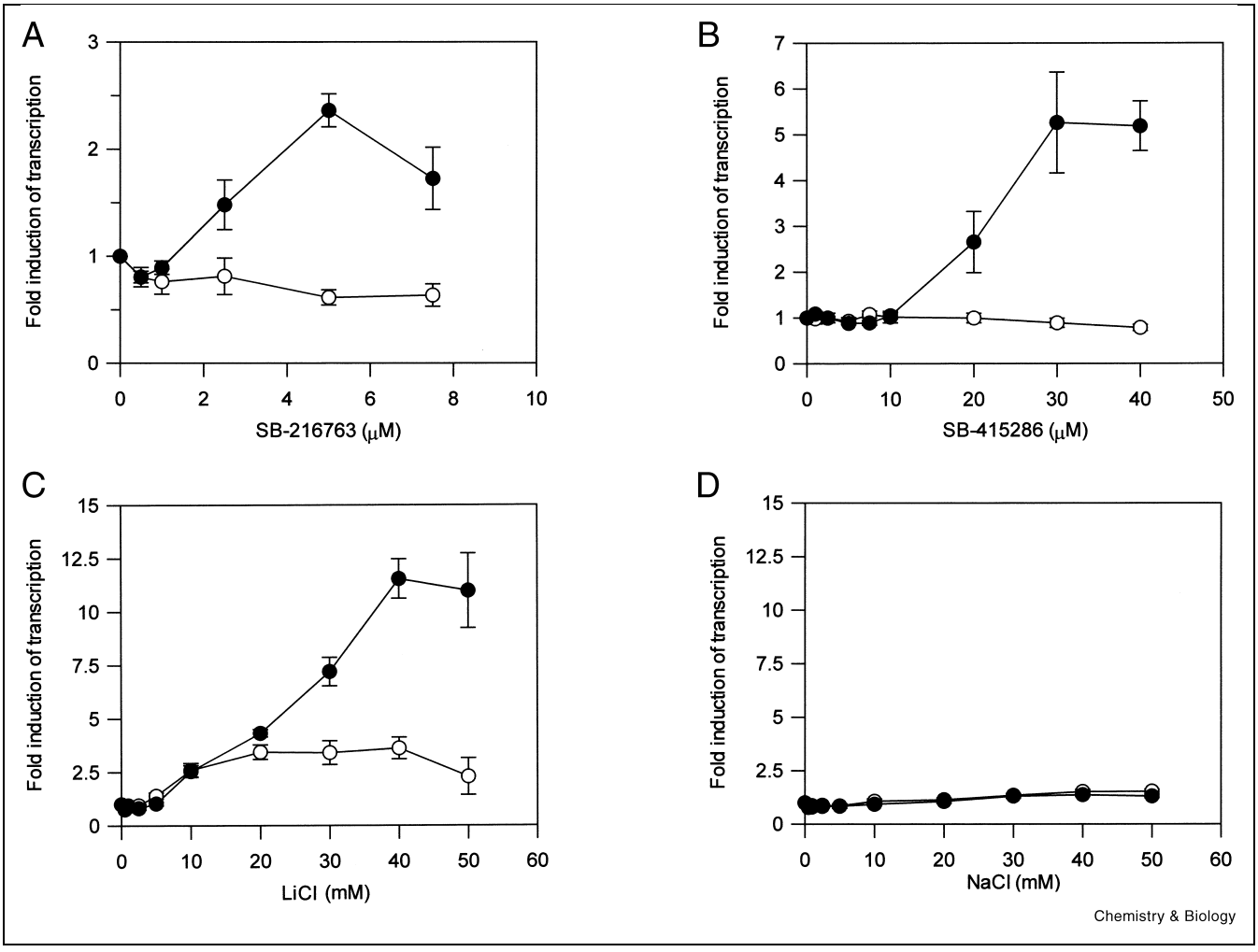

Figure 7. SB-216763 and SB-415286 induce transcription of a β-catenin-LEF/TCF regulated reporter gene. HEK293 cells were transiently transfected with β-catenin-LEF/TCF sensitive (●) or β-catenin-LEF/TCF insensitive (○) luciferase reporter genes. Serum starved cells were then treated for 16 h with the indicated concentration of SB-216763 **(A)**, SB-415286 **(B)**, LiCl **(C)** or NaCl **(D)**. Cell extracts were prepared and assayed for luciferase activity. Results are expressed as fold induction of reporter gene expression over that observed in control cells treated with DMSO vehicle **(A,B)** or serum-free medium alone **(C,D)**. Data are mean ± S.E.M. for four independent experiments where each point represents triplicate cell treatments.

tion with LiCl did not produce an additive stimulation of glycogen synthesis. This is consistent with the effects of the compounds being mediated by inhibition of GSK-3 activity.

The best established functional consequence of GSK-3 inhibition is the activation of glycogen synthase, the rate limiting enzyme in glycogen synthesis. As expected, from their ability to induce glycogen synthesis, SB-216763 and SB-415286 each activated glycogen synthase in human liver cells. Furthermore, the additivity or otherwise of compound effects with those of insulin or LiCl at the level of glycogen synthase paralleled those effects observed at the level of glycogen synthesis. Thus, compound or LiCl plus insulin exhibited an additive activation of glycogen synthase whereas compound plus LiCl did not. The additive stimulation of glycogen synthase and glycogen synthesis by maximum effective concentrations of LiCl plus insulin has been reported previously in liver and fat cells [31,45].

Further evidence for the utility of SB-216763 and SB-415286 as tool GSK-3 inhibitors was provided by the ability of these compounds to induce expression of a β-catenin regulated reporter gene in HEK293 cells. In this assay, SB-216763 produced a maximum 2.5-fold induction of the reporter gene at 5 μM whereas SB-415286 produced a

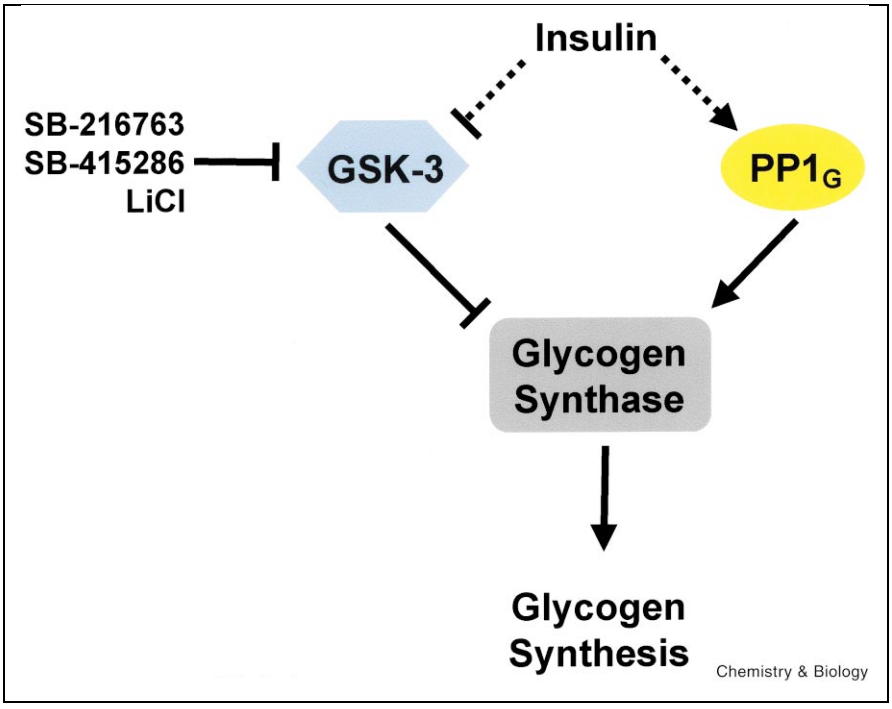

Figure 8. Proposed mechanism by which small molecule GSK-3 inhibitors in combination with insulin induce an additive stimulation of glycogen synthase/synthesis in Chang liver cells. Insulin can stimulate glycogen synthase/synthesis through both GSK-3 dependent and GSK-3 independent pathways. The latter pathway involves the activation of a protein serine/threonine phosphatase ($PP1_G$ glycogen targeted protein phosphatase-1) that dephosphorylates and activates glycogen synthase. The relative role of these two pathways in mediating insulin's stimulation of glycogen synthase/synthesis is cell type dependent (see [40–42,44]). The additive stimulation of glycogen synthase/synthesis in Chang cells elicited by SB-216763, SB-415286 or LiCl in combination with insulin suggests that in this cell line insulin exerts its effects through the activation of $PP1_G$ rather than through the inhibition of GSK-3. Hammerheads and arrows denote inhibition and activation respectively. Solid lines represent direct effects. Broken lines indicate a multi-component pathway.

maximum 5-fold induction at 30 μM. It seems unlikely that the lower effect of SB-216763 compared to SB-415286 results from its poorer cell penetration in 293 cells as both compounds induced a similar activation of glycogen synthase in these cells. However, the cells were only exposed to compounds for 90 min prior to preparation of cell extracts for glycogen synthase assay, whereas the reporter assay involved compound treatment of cells for 16 h. This is important as SB-216763 was observed to precipitate out of solution in a time dependent manner. SB-216763 containing medium that had been incubated for 16 h, under conditions identical to those used in the reporter assay, was microscopically examined. At concentrations above 5 μM, SB-216763 was clearly observed to precipitate out of solution in this culture medium (unpublished observations). No such precipitation of SB-216763 was apparent after shorter incubation of this compound under conditions identical to those used in glycogen synthase or glycogen synthesis assays. Therefore, the reduced ability of SB-216763 compared with SB-415286, to induce β-catenin regulated reporter gene transcription can be attributed to the low solubility of this compound upon prolonged incubation in aqueous media.

The observation that LiCl dose dependently induced the expression of a β-catenin insensitive, control reporter gene highlights the concerns associated with the use of this non-selective agent as a tool GSK-3 inhibitor. However, the use of LiCl in combination with SB-216763 or SB-415286 can be used to implicate GSK-3 inhibition in a given response as demonstrated by their non-additivity in stimulating glycogen synthesis/glycogen synthase in liver cells.

Significance

Elucidation of the functional consequences of inhibiting GSK-3 activity in a given cell type requires a means by which the activity of this kinase can be selectively inhibited. Genetic approaches to reducing cellular GSK-3 activity such as the use of antisense oligonucleotides or the overexpression of dominant negative GSK-3 are chronic, invasive and labour intensive techniques that are likely to have additional non-specific effects on cell physiology. Whilst LiCl is capable of acutely inhibiting cellular GSK-3 activity, it also inhibits a number of other protein kinase and non-kinase targets. This report describes the identification of the arylindolemaleimide SB-216763 and the anilinomaleimide SB-415286, as highly selective, potent and cell permeable inhibitors of GSK-3. These compounds acutely reduce cellular GSK-3 activity as assessed by activation of glycogen synthase. Consistent with this, SB-216763 and SB-415286 are each capable of eliciting responses that had been previously attributed as resulting from the inhibition of GSK-3 activity. Therefore, SB-216763 and SB-415286 represent valuable pharmacological tool compounds that will be of use in further elucidating the role of GSK-3 in cell signalling pathways. Furthermore, development of similar compounds may be of use therapeutically in disease states associated with elevated GSK-3 activity such as non-insulin dependent diabetes mellitus and neurodegenerative disease.

Materials and methods

Materials

SB-216763 and SB-415286 were synthesised according to procedures previously described [46,47]. 100 mM stock solutions of compounds were prepared in dimethylsulphoxide (DMSO) and the final DMSO concentration in cellular assays was 0.1% (v/v). Cell culture reagents were purchased from Life Technologies. Cell lines were from the American Type Culture Collection. Radioisotopes were purchased from Amersham Pharmacia. Human insulin was ActRapid from Novo Nordisk. Unless otherwise indicated all other reagents were purchased from Sigma Chemical Company.

Cell culture

Chang human liver cells were cultured in minimal essential medium supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) non-essential amino acids. E1A-transformed human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat inactivated FCS and 2 mM glutamine. Chang cells were grown to confluence and serum starved for 16 h prior to experimental use. Incubation of cells with compounds, LiCl or insulin was performed at 37°C, 5% (v/v) CO_2.

Expression and purification of recombinant human GSK-3α

Full length GSK-3α was amplified by polymerase chain reaction from a human adult liver cDNA library. Following sequence confirmation, the GSK-3α clone was subcloned into a baculovirus transfer vector containing a HexaHis tag. Recombinant baculovirus encoding GSK-3α was produced and used to infect Sf9 insect cells. GSK-3α was purified from lysates prepared from these cells using a four step strategy as follows: NiNTA (Qiagen) chromatography (elution with 20 mM imidazole followed by 300 mM imidazole); Source15S (Amersham Pharmacia) cation exchange chromatography (NaCl gradient elution); NiNTA chromatography (20–300 mM imidazole gradient elution) and Source15S cation exchange chromatography (NaCl gradient elution). The peak fractions from the final stage were 94% pure by reverse phase HPLC. The protein was stored in a 50% glycerol buffer at −80°C and retained activity for greater than 6 months.

GSK-3 activity assay

GSK-3 kinase activity was measured, in the presence or absence of SB-216763 or SB-415286, in a reaction mixture containing final concentrations of: 1 nM human GSK-3α or rabbit GSK3α (Upstate Biotechnology Inc.); 50 mM MOPS pH 7.0; 0.2 mM EDTA; 10 mM Mg-acetate; 7.5 mM β-mercaptoethanol; 5% (w/v) glycerol; 0.01% (w/v) Tween-20; 10% (v/v) DMSO; 28 μM GS-2 peptide substrate (Upstate Biotechnology Inc.). The GS-2 peptide sequence corresponds to a region of glycogen synthase that is phosphorylated by GSK-3 as previously described [18]. The assay was initiated by the addition of 0.34 μCi [^{33}P]γ-ATP (IC_{50} determinations) or 2.7 μCi [^{33}P]γ-ATP (K_i determinations). The total ATP concentration was 10 μM (IC_{50} determinations) or ranged from 0 to 45 μM (K_i determinations). Following 30 min incubation at room temperature the assay was stopped by the addition of one third assay volume of 2.5% (v/v) H_3PO_4 containing 21 mM ATP. Samples were spotted onto P30 phosphocellulose mats and these were washed six times in 0.5% (v/v) H_3PO_4. The filter mats were sealed into sample bags containing Wallac betaplate scintillation fluid. ^{33}P incorporation into the substrate peptide was determined by counting the mats in a Wallac microbeta scintillation counter.

Selectivity panel protein kinase assays

Human insulin receptor was partially purified from a cell line overexpressing this gene as described previously [48]. Insulin receptor tyrosine kinase was activated by pre-incubation with 100 nM insulin. Compound effects on insulin receptor tyrosine kinase activity and that of the other members of the kinase selectivity panel were assessed as described previously [32]. Briefly, activated protein kinases were assayed for their ability to phosphorylate the appropriate peptide/protein substrate in the presence of 10 μM SB-216763 or SB-415286 at 0.1 mM ATP. All protein kinase activities were linear with respect to time over the course of the assay incubations.

Glycogen synthesis assay

Chang cells in 48 well plates were pre-incubated with insulin, compound or DMSO vehicle. After 60 min, 2 μCi [^{14}C]glucose was added to each well and the plates incubated for a further 75 min. Wells were then aspirated and washed with 1×400 μl phosphate buffered saline (PBS) before cell extraction into 150 μl 20% (w/v) KOH for 1 h at 37°C. 90 μl of 1 mg/ml carrier glycogen was added to each well. Following the addition of 540 μl absolute ethanol, total glycogen was precipitated overnight at −20°C. Glycogen precipitates were recovered by centrifugation at 1100×*g* for 20 min at room temperature. Precipitates were resuspended in 75 μl hot tap water and transferred to Packard 96 well OptiPlates. 150 μl of Microscint-40 (Packard) was added to each well and [^{14}C]glucose incorporation into glycogen was assessed using a Packard TopCount scintillation counter.

Glycogen synthase assay

Cells on 10 cm diameter dishes were treated with compound, DMSO vehicle, LiCl or insulin for 60 (Chang cells) or 90 min (HEK293 cells). Incubation medium was removed and cells washed with 1×5 ml ice-cold PBS prior to lysis, on ice, in 450 μl 20 mM Tris–HCl pH 7.4, 50 mM NaF, 5 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 1% (w/v) Triton X-100. Cell lysates were centrifuged at 15 000×*g* for 5 min at 4°C. Lysate supernatants were snap frozen on liquid nitrogen and stored at −80°C prior to assay. Lysates were assayed for glycogen synthase activity in buffer (67 mM Tris–HCl pH 7.5, 5 mM DTT, 6.7 mM EDTA, 13 mg/ml glycogen, 8.9 mM [^{14}C]UDP-glucose) in the presence or absence of 20 mM glucose 6-phosphate as described previously [41]. Data are expressed as fold increases in glycogen synthase activity ratios over those of control. Activity ratios of glycogen synthase in control cells were approximately 0.15 and 0.05 in Chang and HEK293 cells respectively.

β-Catenin-LEF/TCF regulated reporter gene assay

Two 10 cm diameter dishes were seeded with 3×10^6 HEK293 cells. The following day, the cells were transfected with 4 μg of either a β-catenin-LEF/TCF sensitive or a β-catenin-LEF/TCF insensitive reporter vector (Upstate Biotechnology Inc.) using Lipofectamine Plus reagent (GibcoBRL) according to the manufacturer's instructions. The next day, cells were trypsinised, washed into serum-free medium, counted and seeded at 40 000 cells per well in a 96 well plate. Subsequent to cell attachment, LiCl or GSK-3 inhibitor compounds were added to the medium to the required concentrations. Control cells were DMSO vehicle treated. 16 h after inhibitor addition, cells were analysed for luciferase activity using the Steady-Glo Luciferase assay system (Promega) according to the manufacturer's instructions.

Statistical analysis

Results are presented as mean± S.E.M. unless stated. Statistical analysis was performed by a Dunnett test for unpaired data using Statistica for Windows (Release 5.1, 1997 edition).

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