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An ESI-MS/MS Method for Screening of Small-Molecule Mixtures against Glycogen Synthase Kinase-3β (GSK-3β)

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Glycogen synthase kinase-3β (GSK-3β) is involved in the hyperphosphorylation of previously phosphorylated (primed) substrates, and is currently assayed using an approach based on the incorporation of γ-^{32}P-radiolabelled isotopes into substrate peptides. The requirement to detect hyperphosphorylation of a primed substrate poses a particular challenge for development of a high-throughput screening assay, as many current kinase assays are designed to produce a signal in the presence of any phosphorylation site, and thus are only suitable for β-unphosphorylated substrates. Herein, we have developed an electrospray-ionization tandem mass spectrometry (ESI-MS/MS) assay to allow for direct detection of a hyperphosphorylated product which is formed in a solution reaction involving a primed peptide substrate (GSM peptide) and GSK-3β. Optimum reaction conditions (level of Mg^{2+}, buffer type, ionic strength, pH, enzyme concentration, and reaction time) were established to both maintain the activity of GSK-3β and allow for substrate and product quantification through ESI/MS/MS. We show that the MS-based assay allows for rapid determination of GSK-3β activity from reaction volumes of ~40 μL and that it can be used to assess IC_{50} values and the site of action of known inhibitors. It also can be used for automated screening of small-molecules mixtures to identify inhibitors of GSK-3β.

Introduction

Phosphorylation is a key post-translational protein modfication that controls a variety of regulatory processes in mammalian cells.[1] Indeed, kinases have been implicated in a wide range of disease states, and as such are highly touted as potential targets for drug development.[2,3] A particularly important member of this family is the serine/threonine kinase, glycogen synthase kinase 3 (GSK-3), which plays an intricate role within the framework of mammalian cellular metabolism, and is involved in many metabolic regulatory pathways. In addition to its role in the regulation of glycogen metabolism,[4] it is now known that GSK-3 is involved in cytoskeletal regulation,[5] cell cycle progression,[6,7] apoptosis,[8] cell fate and speciation,[9] and transcriptional/translational initiation.[10,11] Altered GSK-3 activity, normally involving hyperphosphorylation of its targets, can contribute to a number of pathological processes including bipolar mood disorder,[12–14] schizophrenia,[15] heart disease,[16,17] neurodegeneration,[18] Alzheimer's disease,[11,19] and diabetes mellitus.[20] Its involvement in Alzheimer's disease is thought to stem from its role (in conjunction with cyclin-dependent kinase 6 (CDK6) and microtubule affinity-regulating kinase (MARK) in the hyperphosphorylation of tau. The role of GSK-3β as the key isoform responsible for hyperphosphorylation of tau has been unequivocally established by Takashima et al. through GSK-3β antisense treatment and phosphatidylinositol-(3,4,5)triphosphate (PtdIns(3,4,5)P_3) inhibition studies.[21] As a consequence, there is a clear need for the development of potent and selective inhibitors of GSK-3β.

A complication in screening for inhibitors of GSK-3β is that the enzyme has an unusual preference for target proteins that have undergone a previous phosphopriming event; the enzyme generally recognizes substrates with a Ser-X-X-X-Ser(P) motif.[22,23] An ideal inhibitor will target the primed substrate binding site rather than the ATP binding site so as to increase specificity toward particular GSK-3β pathways and avoid dysregulation of other pathways regulated by GSK-3β, such as the Wnt signaling pathway.[24] Thus, a suitable diagnostic assay must be able to follow the formation of hyperphosphorylated products and assess the site of action of potential inhibitors.

At present, there are a wide variety of kinase assays that can be used to measure inhibition. These follow loss of reactants (ATP), production of products (ADP or phosphorylated substrates), or competition between test compounds and suitably labeled ligands that can bind to the active site of the kinase.[24] Specifically these include: 1) cell-based assays;[25] 2) ATP-dependent assays (colorimetric, fluorescent and bioluminescent readouts);[24] 3) antibody-based, time-resolved fluorescence resonance energy transfer (FRET) readout assays;[26] 4) radioassay methods; 5) ligand displacement assays (fluorescence polarization or FRET);[27,28] and 6) phosphospecific staining methods (AlphaScreen™,[29] iron quenching,[30] immobilized metal ion affinity based polarization (IMAP),[31] Pro-Q™ Diamond stain[32]).

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Unfortunately, most of these assays are either unable to discriminate primed substrates from hyperphosphorylated product (i.e., FRET or phosphostaining methods), require secondary assays to reduce false positives (i.e., coupled enzyme assays for ATP), or require significant manipulation of the intracellular machinery (i.e., knock-out or knock-in experiments) to be able to provide specific information regarding the inhibition of GSK-3β. Radioassay methods can be used to assess hyperphosphorylation of primed substrates by GSK-3β,[33] but such assays are not scalable to high-throughput screening of large compound collections and have significant issues related to the use and disposal of radioactive materials. Hence, there is a need for new screening technologies to allow for the rapid discovery of new inhibitors of GSK-3β.

In recent years, there have been several reports on the use of mass spectrometry-based assays for both evaluation of enzyme activity and screening of enzyme inhibitors.[34–38] Several groups have described studies in which enzyme reactions were carried out in wells or other vessels containing the free enzyme, followed by off-line MS analysis of substrates, products and/or inhibitors to evaluate enzyme activity and ligand binding.[39–44] Other approaches have used immobilized ligands to screen enzymatic activity, with MALDI/MS providing the ability to detect conversion of the ligands.[45] Still other methods have utilized flow-through reactors wherein both the enzyme and substrate/inhibitor flow through a reaction loop followed by infusion of all components into an ESI/MS system to monitor enzyme activity,[46] or immobilized enzyme reactors interfaced off-line to MALDI/MS[47] or on-line to ESI-MS/MS[48] for evaluation of enzyme activity and inhibition. Several affinity-based screening formats have also been coupled with MS detection,[49] using methods such as gel permeation chromatography,[50] size-exclusion chromatography,[51] pulsed ultrafiltration,[52] competitive displacement,[53] affinity capillary electrophoresis[54] or frontal affinity chromatography (FAC-MS).[55] However, such methods do not provide functional information on enzyme activity and require appropriate competitive ligands to assess the site of action.

Two recent papers have highlighted the potential of MS for following the reactions of kinases. Irth and coworkers reported on an ESI-MS assay for protein kinase A and showed that appropriate conditions could be obtained to allow infusion of the kinase reaction components into the MS for quantitative determination of enzyme activity and inhibition.[56] O'Gorman and coworkers reported on the use of surface-enhanced laser desorption ionization (SELDI) MS for assessment of GSK-3 activity from cell lysates and showed the potential of the MS method to discriminate primed substrates and hyperphosphorylated products.[57] However, neither of these studies explored the use of MS for assessing the site of action of inhibitors or for semi-automated screening of compound mixtures.

This study focuses on the development of an ESI-MS/MS method for screening GSK-3β activity and inhibition. This kinase assay utilizes a peptide substrate derived from glycogen synthase (GSM peptide) that has a serine residue at a position equivalent to the GSK-3 phosphorylation site on GS (position *n*) and phosphoserine at the $n+4$ position. The assay involves performing the kinase reaction in an optimized MS-compatible buffer that is simply quenched with an acidic methanol solution and immediately subjected to ESI/MS/MS analysis with no further sample treatment. The assay allows for rapid, quantitative assessment of GSK-3β activity, determination of inhibition constants (IC_{50} or K_i), and can be used to determine the site of action of inhibitors. We also show that interfacing to an autosampler provides a platform for semi-automated screening of small-molecule mixtures to identify inhibitors of GSK-3β.

Results and Discussion

Optimization of buffer conditions for MS-based analysis of GSK-3β activity

An important issue with MS-based enzyme assays is the need to find suitable solution conditions that allow high enzyme activity and a high MS signal. However, these two requirements are often at odds with each other, since enzyme activity is frequently dependent upon concentrations of cofactors that tend to suppress ionization of other compounds and consequently decrease the MS signal. A particular problem for GSK-3β assays is the requirement for Mg^{2+} as a cofactor, as this salt ion readily suppresses signals in ESI-MS. The presence of high concentrations of Mg^{2+} also generates significant salt adducts by complexing with the compounds present in the ESI-MS, substantially reducing the $[M+H]^+$ signal and lowering the signal-to-noise (S/N) level in Q1 scans.

To determine the effect of Mg^{2+} concentration on the activity of GSK-3β, a ^{32}P-ATP radio assay was performed, with 62.5 μM GSM, 125 μM ATP and 2.0 U enzyme incubated in 2 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) with 0–2.5 mM $Mg(OAc)_2$. The data (Figure 1 A) showed that the minimal concentration of Mg^{2+} needed for robust enzymatic function was 0.8 mM, as indicated by a sharp drop in GSK-3β activity below this concentration of $Mg(OAc)_2$.

The buffer type and concentration were also optimized to obtain good enzyme activity and MS compatibility. In general, MS assays require the use of volatile buffers, thus the MOPS buffer utilized in the previous radioassay was exchanged for the more volatile and MS-compatible NH_4OAc buffer. The reaction mixtures containing radiolabelled ATP were then incubated with 1–21 mM NH_4OAc in 0.8 mM $Mg(OAc)_2$ for one hour, and activity was assessed by scintillation counting. Enzyme activity was significantly affected at low NH_4OAc concentrations, with the product signal reaching a plateau around 6 mM NH_4OAc and remaining constant beyond this point (Figure 1 B). 6 mM NH_4OAc was therefore chosen as the optimal reaction buffer concentration to use in GSK-3β assays, as any lower concentrations would result in less kinase activity, while higher concentrations could potentially contribute to ion suppression, decreasing GSM/pGSM signal intensity.

Once suitable buffer conditions were determined, GSK-3β activity in solution was assessed as a function of time using ^{32}P-ATP radio assays in order to quantify enzyme activity. 62.5 μM GSM, 2.0 U GSK-3β and 125 μM ATP were incubated with the 6 mM NH_4OAc/0.8 mM $Mg(OAc)_2$, pH 7.4 buffer. The

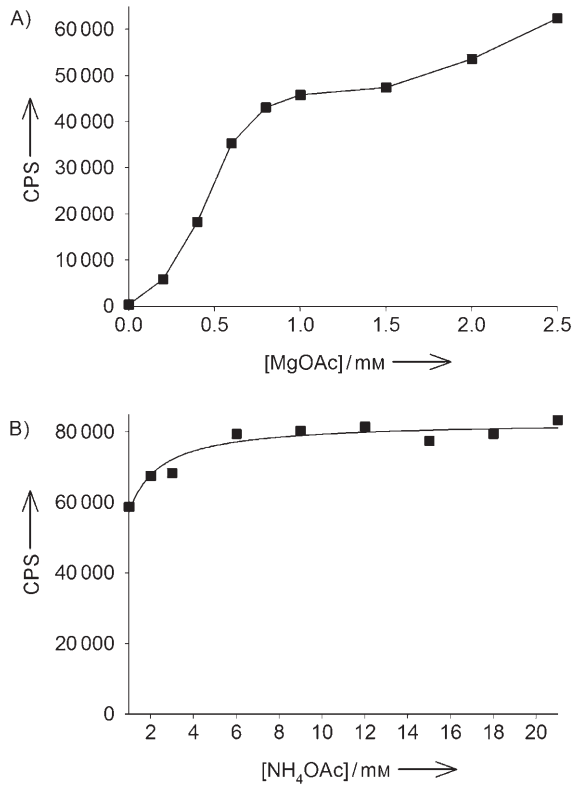

Figure 1. ^{32}P-ATP radio assay of GSK-3β activity in solution as a function of Mg^{2+} (A) and buffer concentrations (B). A) 0–2.5 mM $Mg(OAc)_2$ concentration assay (in 2 mM MOPS); and B) 1–21 mM NH_4OAc concentration assay (with 0.8 mM $Mg(OAc)_2$). CPS = counts per second.

results of the assay, measured in product concentration, revealed a linear relationship between enzyme activity and time for the +GSK-3β reaction and essentially no signal for the negative control (-GSK-3β) reaction (see the Supporting Information). For the positive control, the quantity of pGSM reached 7 μM (~11% conversion from 62.5 μM substrate) after 2 h, indicative of a relatively slow turnover rate for GSK-3β. This is in fact favorable for monitoring enzymatic activity, as the substrate-to-product conversion should ideally remain at 10% or less in order to retain the initial reaction rate proportional to product concentration. Based on this data, MS assays were typically run for one hour, as this provided sufficient signal in MS based assays while not unduly increasing the assay time (see below).

Detection of substrate and product and optimization of MS assay conditions

Since GSK-3β ESI-MS-based screening assays are dependent on monitoring product/substrate ratios by MS/MS, obtaining distinct and unambiguous GSM and pGSM MS precursor and product ion signals was one of the key goals in the early stages of this study. Based on the data provided above, the analysis of GSM was carried out first with 62.5 μM GSM dissolved in 6 mM NH_4OAc/1.6 mM $Mg(OAc)_2$, pH 7.4, injected into the ESI-MS/MS at a flowrate of 5 μL min^{-1}. The resulting GSM Q1 spectrum (see the Supporting Information), showed a prominent peak at *m/z* 527.7, corresponding to the expected *m/z* of the substrate peptide in the +5 charge state (peptide mass is 2637 Da). Additional peaks at *m/z* values of 531.9 and 536.0 were also present, corresponding to sodium ion adducts. Analysis of the *m/z* 527.7 ion in MS2 mode revealed a prominent daughter ion peak at *m/z* 507.7, corresponding to the loss of a PO_4 group, which provided a useful multiple reaction monitoring (MRM) transition to monitor the presence of the GSM peptide with ESI-MS/MS. Note that there were also usable peaks observed for the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ charge envelopes; however, the $[M+5H]^{5+}$ charge envelope showed the highest signal-to-noise levels under the conditions employed in our assay, and provided sufficient sensitivity to produce a robust assay (see below).

To obtain a sufficient pGSM signal for MS analysis with a reasonable reaction time, reaction mixtures containing 2.0 U GSK-3β were incubated for 120 min. MS scans of these mixtures are shown in the Supporting Information. A Q1 scan of the reaction containing 2.0 U GSK-3β revealed the presence of a product signal at *m/z* 543.7, which corresponds to a mass increase of ~80 Da (16 unit shift in *m/z*, +5 charge state), as expected for the addition of a PO_3 group. Several additional peaks also appeared at *m/z* values of 551.9, 573.9 and 595.9, but these peaks did not provide high quality MS/MS spectra (data not shown). The MS/MS spectrum of the *m/z* 543 peak showed the presence of only the *m/z* 527 and 507 peaks, with the *m/z* 543→*m/z* 527 transition providing the highest intensity.

We note that while the signal intensity of pGSM in the GSK-3β containing solutions is much higher than in the enzyme-free control solution, a distinct peak at *m/z* 543.7 is still present in the control despite the absence of enzyme to generate pGSM. The *m/z* 543.7 ion in the enzyme-free sample displays an identical daughter ion spectrum to pGSM, providing strong evidence for the presence of small amounts of pGSM prior to the introduction of GSK-3β. Although the origin of this pGSM is unknown, the presence of this contaminant did not adversely affect the ability to detect product formation or GSK-3β inhibition, as described below.

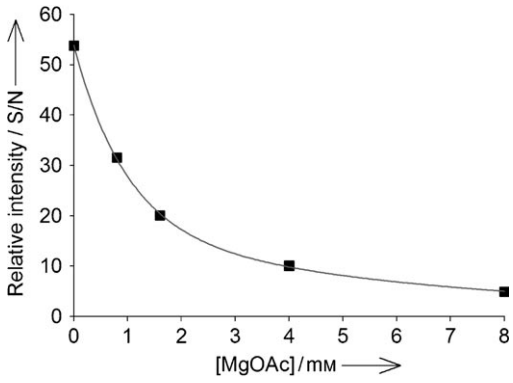

Figure 2. The effect of Mg^{2+} ions on GSM Q1 signal. 62.5 μM GSM in 6 mM NH_4OAc with 0, 0.8, 1.6, 4, and 8 mM $Mg(OAc)_2$ were infused into the ESI-MS at 5 μL min^{-1} with a syringe pump.

Once suitable MRM transitions were obtained, the amount of signal was assessed as a function of Mg^{2+}, with 6 mM NH_4OAc as the buffer. As shown in Figure 2, detection of the GSM peptide in the absence of $Mg(OAc)_2$ produced a S/N ratio of approximately 54:1, while the inclusion of 8 mM $Mg(OAc)_2$ produced only a 5:1 S/N ratio. Thus, the presence of high levels of $Mg(OAc)_2$ decreased the GSM signal by >90% compared to the control values. Increases in Mg^{2+} did not lead to an observable increase in $[M+Mg]^{2+}$ adducts, providing evidence for overall signal suppression by Mg^{2+} rather than adduct formation. Concentrations of Mg^{2+}, in the range of 0.8 to 1.6 mM, produced S/N values of 32 and 20. Based on the activity and S/N ratio data, a final buffer composition of 6 mM NH_4OAc with 0.8 mM $Mg(OAc)_2$ at pH 7.4 was used with a one hour incubation time. Note that in some assays which used low ATP or GSM levels, the $Mg(OAc)_2$ level was increased to 1.6 mM to provide somewhat higher product concentrations (see below).

Validation of GSK-3β activity using a solution-based ESI-MS method

The application of the solution-based ESI-MS/MS technology for monitoring GSK-3β activity possesses many potential benefits, such as the capacity for the rapid detection of previously unknown inhibitors. To validate that the MS-based assay was accurately measuring GSK-3β activity, an enzyme concentration assay was performed. The reaction mixture containing 62.5 μM GSM, 125 μM ATP in 6 mM NH_4OAc/0.8 mM $Mg(OAc)_2$, pH 7.4 was incubated with varying levels of GSK-3β for one hour and then assessed for pGSM concentration. The results demonstrated a linear increase of pGSM signal with kinase concentration, as expected (data not shown) and suggested an optimal level of 2.0 U for GSK-3β concentration.

In order to ensure the reliability and reproducibility of the results in GSK-3β inhibitor screening experiments, statistical validation of the solution-based MS assay was required. Application of the Z′ test allowed for the determination of the statistical average and standard deviation in enzyme/no enzyme controls. Reactions were run with and without GSK-3β using 1.6 mM $Mg(OAc)_2$, which provided a significant increase in kinase activity and in Z′ values. The assays were run sequentially with both direct injection and semi-automated delivery of the methanol-quenched reaction solutions and the resulting product-to-substrate (P/S) signal ratio was recorded for each reaction. We note here that variations in ionization efficiency between pGSM and GSM result in the signal ratio being somewhat different from the actual concentration ratio. However, the use of a signal ratio reduces errors owing to short and long-term drift in instrument sensitivity, since such fluctuations are corrected by taking the ratio. The mean P/S signal ratio and standard deviation for the direct injection method were 0.78 ± 0.06 for the GSK-3β high control and 0.14 ± 0.01 for the low control (Figure 3A). For semi-automated delivery, the GSK-3β P/S ratio for the high control was 0.99 ± 0.06 and for the low control 0.27 ± 0.03 (Figure 3B). The corresponding Z′ factors were calculated to be 0.68 for direct injection and 0.60 for the autosampler assays, each resulting in "an excellent assay" ranking as interpreted by Zhang et al.[58] The successful validation of the assay method thus allowed high confidence that the enzyme performed reproducibly over multiple reactions. This indicated that the assay could be applied to semi-automated MS-based screening of small molecules against GSK-3β.

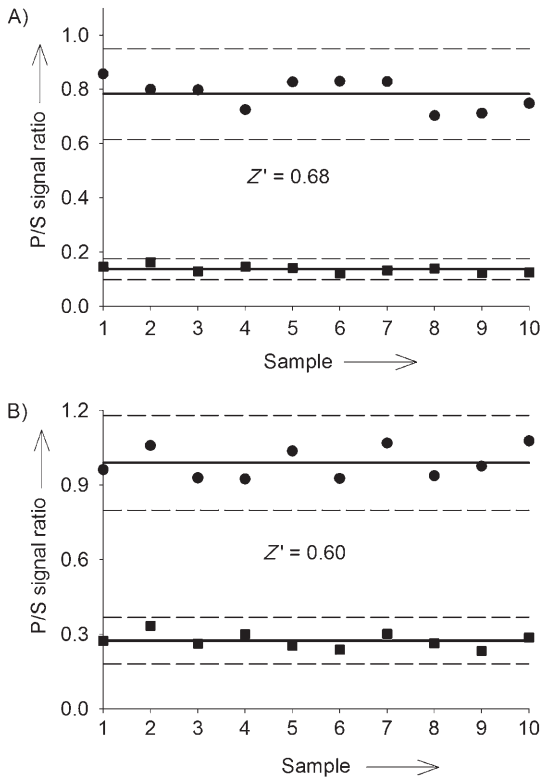

Figure 3. Z′ analysis of the GSK-3β solution-based MS assay with A) direct injection and B) automated injection. +GSK-3β positive control reactions (●); -GSK-3β negative control reactions (■); solid lines denote mean P/S signal ratios, broken lines represent three standard deviations on the mean P/S signal ratios.

Determination of IC_{50} values and site of action

To further validate the assay IC_{50} values were obtained for known ATP-competitive and non-ATP-competitive inhibitors to demonstrate the capacity of ESI-MS to accurately determine inhibition constants and monitor the site of inhibition. Initial experiments were conducted with 6 mM NH_4OAc/1.6 mM $Mg(OAc)_2$, pH 7.4 buffer, 62.5 μM GSM, 125 μM ATP and 2.0 U GSK-3β with 0.001-100 μM of the ATP competitive inhibitor SB-415286 added to the reaction mixtures. Following a one hour incubation period the solutions were quenched with acidic MeOH and analyzed by ESI-MS/MS. As shown in Figure 4A, the P/S signal ratio decreased with increasing inhibitor concentration, producing an IC_{50} of 0.81 ± 0.10 μM (see Table 1 for all IC_{50} values). Dropping the ATP level fivefold reduced the IC_{50} by exactly fivefold to 0.18 ± 0.03 μM, confirming the ATP binding site to be the site of action and competitive inhibition to be the mode of action. Analysis of GSK-3β Inhibitor I, a known allosteric inhibitor, over a concentration range of 0.1–200 μM,

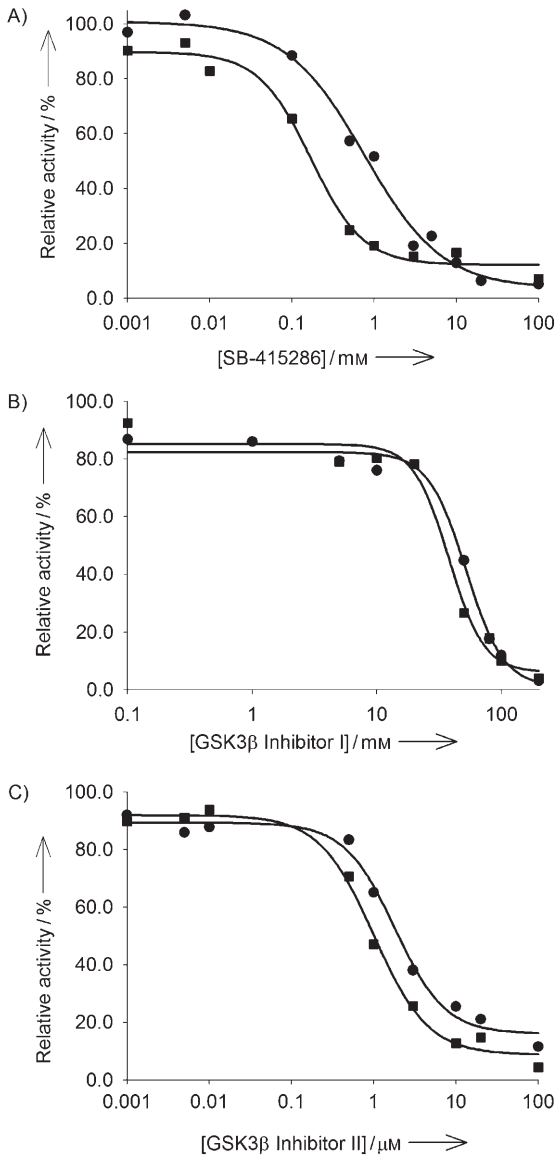

Figure 4. IC_{50} curves for GSK-3β inhibitors for A) SB-415 286; B) GSK-3β inhibitor I; and C) GSK-3β Inhibitor II. Assays utilized 25 μM ATP (●) or 125 μM ATP (■) with 62.5 μM GSM and 2.0 U GSK-3β incubated in 6 mM NH_4OAc/1.6 mM $Mg(OAc)_2$, pH 7.4 buffer for 1 h. Data is plotted as normalized P/S signal ratios and fit to the Hill equation.

resulted in an IC_{50} value of 45 ± 5 μM for the higher and 35 ± 5 μM for the lower ATP concentration (Figure 4B), as would be expected for a non-ATP-competitive compound. To further evaluate this method, the IC_{50} values were determined for a compound with an uncharacterized mode of action, GSK-3β inhibitor II. IC_{50} values of 2.1 ± 0.3 and 1.0 ± 0.2 μM were obtained at high and low ATP concentrations, respectively (Figure 4C). These results strongly suggest that this inhibitor is not ATP-competitive, and is consistent with either allosteric or possibly mixed inhibition.

Further evaluation of the three inhibitors was done by using high and low levels of the GSM substrate (twofold concentration difference). These studies yielded no substantial change in the IC_{50} values for any of the compounds tested (Table 1). This finding confirms not only that SB-415286 is an ATP-competitive inhibitor and that GSK-3β inhibitor I is an allosteric inhibitor, but also suggests that the previously uncharacterized GSK-3β Inhibitor II is likely an allosteric inhibitor as well, as its mode of action was demonstrated to be largely noncompetitive with respect to both ATP and GSM. These results highlight the potential of the ESI-MS/MS method to provide detailed information not only on inhibition constants but also on site and mode of action.

Semi-automated ESI-MS compound mixture screening

Following the establishment of IC_{50} values for GSK-3β inhibitors, the application of the solution-based ESI-MS/MS approach towards mixture screening was assessed. The introduction of an autosampler unit operating in tandem with the MS greatly increased the efficiency of the assays and allowed for the automated injection of samples for screening of multiple compounds against the enzyme. However, in the absence of conventional liquid handling technology, the reactions themselves were all prepared and quenched manually, subsequently resulting in the "semi-automated" state of the assay. The need for a relatively long incubation step is the result of the extremely low turnover of the enzyme (see the Supporting Information). This step of the assay, including mixing and quenching steps, could also be readily automated with a conventional liquid handler or could be integrated into the assay by incubating reactions in the autosampler with on-line quenching,[59] however this was not done for this study.

Table 1. Summary of IC_{50} experiments performed with inhibitors SB-415 286, GSK-3β inhibitor I and GSK-3β inhibitor II. Low [ATP]: 25 μM ATP, high [ATP]: 125 μM ATP, low [GSM]: 62.5 μM GSM and high [GSM]: 125 μM GSM.

Inhibitor	IC_{50}		Mode of action	
	Low [ATP] High [ATP]	(lit.)	Low [GSM] High [GSM]	(exper.)
SB-415286	0.18 ± 0.03 μM 0.81 ± 0.10 μM	ATP-competitive	0.81 ± 0.10 μM 0.74 ± 0.9 μM	ATP-competitive
GSK-3β inhibitor I	35 ± 5 μM 45 ± 5 μM	allosteric	45 ± 5 μM 31 ± 4 μM	allosteric
GSK-3β inhibitor II	1.0 ± 0.2 μM 2.1 ± 0.3 μM	unknown	2.1 ± 0.3 μM 3.0 ± 0.3 μM	allosteric

As a proof-of-concept for the autosampler-based assay, we performed a small screen of ten mixtures containing ten compounds each. This study was designed to demonstrate the potential of the MS-based assay for mixture screening. The 100 compounds selected for this screen were all drug-like (according to Lipinski's rules[60]) and were either from commercial sources or were synthesized and purified to high purity. Each compound was dissolved from an initial 1 mM stock in DMSO to a concentration of 1 μM in assay buffer to minimize the amount of DMSO present—this compound has previously been shown to have a deleterious effect on the electrospray process and must be minimized in ESI/MS-based assays.[48] Final DMSO concentrations in test mixtures were typically ~0.1% (*v/v*). A compound concentration of 1 μM was chosen for screening as higher concentrations could lead to ion suppression, while lower concentrations may lead to some of the weaker inhibitors being missed in the assay.

The results of the automated mixture screening assay are shown in Figure 5. Panel A shows the raw data (P/S signal ratio) obtained during the assay, while Panel B shows a bar graph (on the same time axis) that provides the average P/S signal ratio from Panel A. Also shown on both panels is the signal obtained by monitoring the SB-415286 daughter ion (*m/z* 360.0→*m/z* 140.0; CE = 15 V), which was spiked into Mixture **2**. P/S signal ratios obtained for low activity controls (LC; 10 μM SB-415 286 in a sample containing no other potential inhibitors) and a high activity control (HC; no inhibitor) are also shown. The results show that both the low activity control and Mixture **2** resulted in a significant drop in the P/S ratio to similar levels, indicating the presence of an inhibitor, while all other samples remained at high P/S ratios similar to the high activity control. Panels C and D show the raw and processed data obtained for automated injection of each individual compound in Mixture **2**, and demonstrate that it is SB-415286 (compound **20**) that is responsible for the inhibitory action observed in Mixture **2**. This clearly demonstrates the ability of the MS-based assay to detect inhibitors within compound mixtures as a means for primary screening, and to subsequently deconvolute these mixtures to identify the active compound. Although this screen involved only 100 compounds in 10 mixtures, it is possible to screen and deconvolute a considerably larger quantity of compounds per sample to increase throughput.[51] Indeed, even in the present study it was possible to screen 100 compounds by using only 20 assays (not including controls); an even better ratio of compounds per assay is possible with more complex mixtures.

One issue with the current assay is the relatively slow speed (ca. 20 min per sample). The long assay time is a result of the slow flowrate (which is typical of nanoflow LC pumps) and the large injection volume. The larger volumes were used to provide sufficient time to allow for a stable MS signal. Based on the data in Figure 5, a shorter time of perhaps 1–2 min per

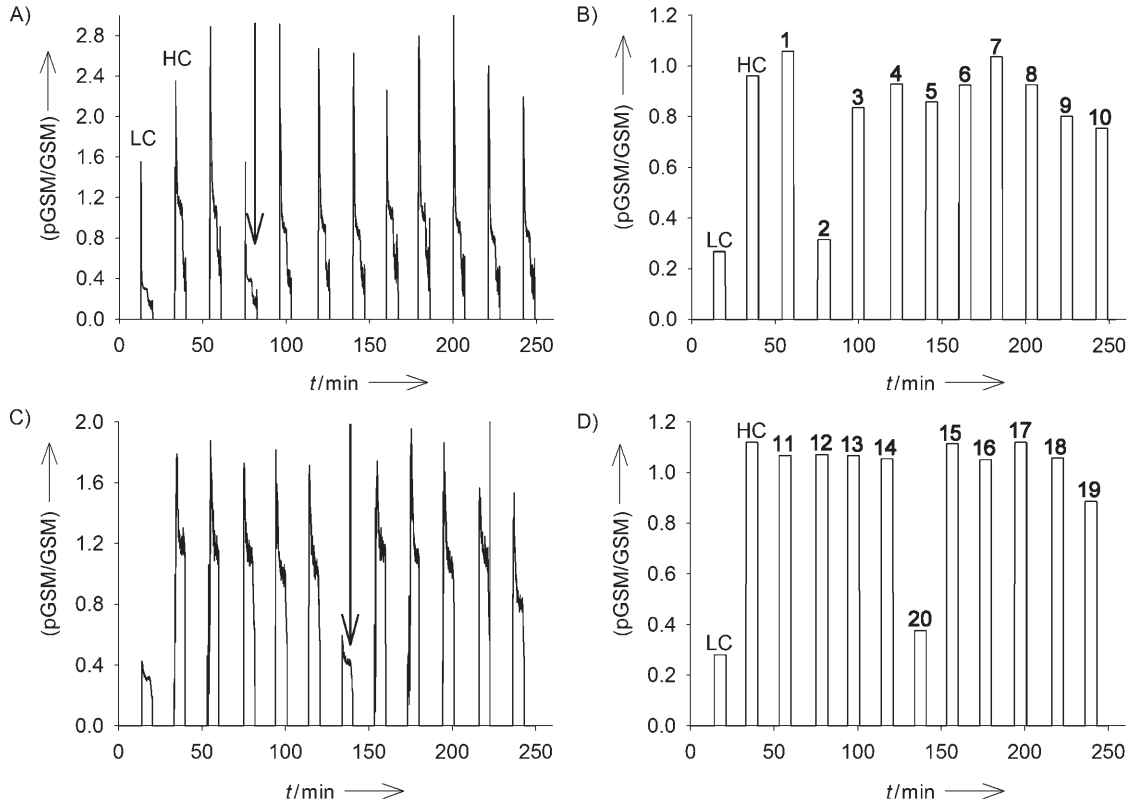

Figure 5. Preliminary solution-based ESI-MS 100-compound inhibitor-mixture screen with GSK-3β showing the raw pGSM/GSM signal ratio (panels A and C) and the P/S signal ratios averaged over their respective 6 min injection periods (panels B and D). Low control (LC), high control (HC) and mixtures **1**–**10** are shown in panels A and B. Low control, high control, and individual compounds **11**–**20** from Mixture **2** are shown in panels C and D (See Table S1 in the Supporting Information for compound data). The arrows denote cases in which the inhibitor SB415 286 was present.

point would be possible by using a lower injection volume. Use of a higher flow rate pump could also provide significant improvements in assay time, but at this time we are limited to a maximum flow rate of 20 μL min^{-1}. With higher flow rates and lower injection volumes it might be possible to increase assay speed to the range of ~30 s per sample, which is on part with results from Biotrove Inc.[61] Thus, with further optimization in terms of both mixture complexity (ca. 100 compounds per mixture) and assay speed (one mixture every 30 s) it should be possible to screen up to 200 compounds per min, or ~300 000 compounds per day per instrument (not including time for deconvolution of hits). Clearly, any mixture that contains a hit will need to be further assessed, but again this can be done by using ten mixtures of ten compounds and finally by testing the active small mixture one compound at a time for the hit. Alternatively, bioactive mixtures could be tested by using more conventional affinity-based methods such as gel permeation chromatography,[50] size-exclusion chromatography[51] or pulsed ultrafiltration,[52] interfaced to MS detection, with hit confirmation done using the function-based assay described herein. Progress toward this goal is ongoing in our lab and the results of these studies will be presented in due course.

Other recently reported MS-based kinase assays involve either ESI-MS to study protein kinase A (PKA) activity,[56] or a SELDI-TOF MS method to assess GSK-3β activity.[57] The SELDI method utilized enzyme reactions run in conventional buffers followed by a desalting step (using a C-18 Ziptip®) and deposition on a SELDI chip. While the method was amenable to the assaying of GSK-3β activity, the difficulty associated with quantifying product/substrate ratios by using MALDI-MS[36] makes the determination of IC_{50} values complicated. The method described herein is most similar to that described by de Boer et al.,[56] which also used ESI-MS to follow kinase-catalyzed phosphorylation reactions. As was the case for de Boer et al., significant optimization of buffer conditions and the use of volatile buffers with low Mg^{2+} levels were required to achieve a stable electrospray, and modifiers needed to be added to enzyme solutions after the enzymatic reaction to obtain maximum signal levels. However, in our assay the use of product/substrate signal ratios avoided the need for addition of internal standards, while use of multiple reaction monitoring instead of selected ion monitoring provided a significant improvement in signal-to-noise levels. We also demonstrate the ability to screen mixtures, which could improve throughput when the method is applied to high-throughput screening. However, there is still a need to perform the enzyme assay in MS compatible buffers, which can limit the activity of some enzymes, and further improvements are needed in assay speed and throughput. Furthermore, the slow turnover of the GSK-3β enzyme may preclude flow-through reactions, which typically require enzyme–substrate contact times of a minute or less.

A significant advantage to the use of a MS-based assay for GSK-3β is the ability to use a wide variety of substrates which are either native unphosphorylated or primed. This is particularly relevant in the case of GSK-3β since this enzyme has a wide array of potential substrates and thus there is a need to selectively inhibit hyperphosphorylation of specific substrates, such as tau, while retaining the ability to phosphorylate other substrates such as glycogen synthase or β-catenin. Since the MS assay described herein simply measures shifts in *m/z* as a result of phosphorylation, it should be possible to apply the assay to study the selectivity of GSK-3β inhibitors and ultimately their mode of action.

Conclusions

Through the optimization of GSK-3β-specific reaction parameters, such as buffer/Mg^{2+} concentrations and GSK-3β substrate/product MS signal parameters, the development of a new, robust solution-based ESI-MS screening technology capable of monitoring changes in GSK-3β activity was possible. Unlike the majority of commonly used kinase assays, this method presents a distinct advantage in the field of chemical biology and drug screening, as it can readily distinguish between the monophosphorylated substrate and the diphosphosporylated product of GSK-3β. IC_{50} values for specific GSK-3β inhibitors provided insights into both site and mode of action, showing the utility of the method for identifying novel leads. Operation in a semi-automated mode allowed for efficient compound screening as compared to the traditional ^{32}P radioassay technique currently used to assess GSK-3β activity, and could be extended to screening of mixtures, which may allow assaying of complex libraries or natural product extracts. The application of automated liquid handling can further improve the throughput of the solution-based assay. Further improvements in throughput can be realized by increasing mixture complexity and flow rates and reducing injection volumes. In this manner, thousands of compounds originating from synthetic libraries and natural product extracts could be rapidly screened, resulting in low protein consumption per compound screened. The speed and accuracy of this system should prove to be an invaluable tool in the future development and discovery of GSK-3β and other kinase inhibitors.

Experimental Section

Materials: Histidine-tagged recombinant rabbit glycogen synthase kinase-3β (GSK-3β; Cat. No. G1663, >90% pure, 15 units per μg protein of which one unit will transfer 1 pmol of phosphate from ATP to phosphatase inhibitor 2 per min at pH 7.5 at 30 °C) and SB-415 286 were purchased from Sigma–Aldrich (Oakville, Ontario, Canada). Upon reconstitution with water (100 μL), the GSK-3β solution contains 200 U of enzyme in Tris (20 mM, pH 7.5), EDTA (2 mM), EGTA (2 mM), DTT (1 mM), sucrose (5%), Brij® 35 (0.1%), leupeptin (1 μg mL^{-1}), and 4-(2-aminoethyl)benzene sulfonyl fluoride hydrochloride (AEBSF; 0.1 mM). This is further diluted upon addition of the enzyme stock solution (1 μL) to an assay solution (40 μL) containing NH_4OAc (6 mM) and $Mg(OAc)_2$ (0.8 or 1.6 mM). GSK-3β Inhibitor I and GSK-3β inhibitor II were purchased at Calbiochem (San Diego, California, USA). HPLC grade water was purchased from Caledon Laboratory Chemicals (Georgetown, Ontario, Canada). Synthetic muscle glycogen synthase 1 peptide (GSM; structure: Arg-Arg-Arg-Pro-Ala-Ser-Val-Pro-Pro-Ser-Pro-Ser-Leu-Ser-Arg-His-Ser-pSer-His-Gln-Arg-Arg; 2673 Da; pSer = phosphoserine) was purchased from Upstate USA (Charlottesville, VA, USA). [γ-^{32}P]

ATP and ACS™ aqueous scintillation cocktail were obtained from GE Healthcare Bio-Sciences (Baie d'Urfe, Québec, Canada). C96-microwell plates were purchased from VWR International (Mississauga, Ontario, Canada). All reagents were used as received. Other compounds listed in Table S1 in the Supporting Information were from commercial or synthetic sources.

GSK-3β reaction optimization: Kinase activity was measured by including GSM substrate (62.5 μM) and [γ-^{32}P] ATP (0.5 μCi μL^{-1}) with recombinant GSK-3β (2.0 U) in a reaction mixture containing NH_4OAc (1–21 mM), $Mg(OAc)_2$ (0–2.5 mM) and ATP (125 μM) in a total volume of 40 μL. After 60 min, the samples were spotted onto Whatman P81 phosphocellulose paper and washed three times with 0.75% *o*-phosphoric acid and once with acetone. ^{32}P incorporation onto the substrate was then determined by scintillation counting, as previously described.[62,63]

LC/MS settings: Mobile-phase delivery for substrate/product-signal optimization studies was performed with a Hamilton syringe (1.0 mL, 4.6 mm i.d.) using an AB/Sciex Q-Trap Mass Spectrometer controlled by Analyst v.1.4 software. Parent/daughter ion signals were followed using Q1 and MS2 modes of analysis in positive-ion mode under the following conditions: Curtain Gas = 45.0, Collision gas = low, Ion Spray Voltage = 5000 V, Temperature = 175 °C, Ion Source Gas 1 = 40.0, Ion Source Gas 2 = 40.0. GSM was monitored using the *m/z* 527.7→*m/z* 507.8 transition (CE 15 V) while pGSM was monitored using the *m/z* 543.7→*m/z* 527.7 transition (CE 15 V). All scans used a declustering potential of 30 V, an entrance potential of 7 V and an exit cone potential of 6 V. The total scan time was 5 s per point.

ESI-MS signal optimization: GSM (62.5 μM) was added to $Mg(OAc)_2$ (0, 0.8, 1.6, 4 and 8 mM) in NH_4OAc (6 mM, pH 7.4) in a total volume of 40 μL. The GSM Q1 MS signal was detected through direct injection at a flowrate of 5 μL min^{-1} after diluting the samples to 120 μL with acidic MeOH. The ratio of the GSM signal over the average of the noise was then calculated for all samples.

GSK-3β solution-based ESI-MS reproducibility assays: Kinase activity was measured by including recombinant GSK-3β (2.0 U) in a reaction mixture containing GSM substrate (62.5 μM), NH_4OAc (6 mM), $Mg(OAc)_2$ (0.8 or 1.6 mM) and ATP (125 μM) at pH 7.4 in a total volume of 40 μL. After a set incubation time of 1 h, the samples were diluted to 120 μL (syringe) or 240 μL (autosampler) with acidic MeOH and injected directly or delivered with a LC pump/autosampler into the ESI-MS at a flowrate or 5 μL min^{-1}, where the substrate and product signals were monitored in MS2 and multiple reaction monitoring (MRM) modes, respectively. 10 samples for low control (-GSK-3β) and high control (+GSK-3β) were included in both the manual and semi-automated Z′ assays. The Z′ factor was calculated for each assay using the formula (1):

$$Z' = 1 - \frac{3\sigma_C + 3\sigma_S}{|\mu_S - \mu_C|} \quad (1)$$

where σ_c is the standard deviation of the low control, σ_s is the standard deviation of the high control, μ_s is the average signal of the high control and μ_c is the average signal of the low control.

GSK-3β IC_{50} assays: Kinase activity was measured in the reaction mixture above with $Mg(OAc)_2$ (1.6 mM) and either variable ATP concentrations (25 or 125 μM ATP with 62.5 μM GSM) or variable GSM concentrations (62.5 or 125 μM GSM with 125 μM ATP) and SB-415 286 or GSK-3β Inhibitor II (0.001–100 μM) or GSK-3β Inhibitor I (0.1–200 μM) in a total volume of 40 μL. After a 1 h incubation time, the samples were diluted to 120 μL with acidic MeOH and injected directly into the ESI-MS with the syringe pump at 5 μL min^{-1}, where substrate and product signals were detected in MS2 mode. The ratio of P/S signals for each concentration was then obtained by utilizing the daughter-ion peak intensities for GSM and pGSM. In all cases the binding isotherms were fit to the Hill equation (2):[64]

$$B = B_0\left(\frac{B_{max}[I]^n}{IC_{50} + [I]^n}\right) \quad (2)$$

where B is P/S signal ratio at a given concentration of inhibitor [I], B_0 and B_{max} are the minimum and maximum P/S ratios, respectively, IC_{50} is the inflection point of the isotherm and n is the Hill number which influences the pitch of the inflection and can be used to determine the number of binding sites per enzyme (assumed to be 1).

Mixture screen: Reaction solutions contained NH_4OAc (6 mM), $Mg(OAc)_2$ (1.6 mM), ATP (125 μM), GSM (62.5 μM), GSK-3β (2.0 U), and 10 compounds in a mixture (1 μM each, 10 mixtures; except in low control (LC) solutions which contained no enzyme or inhibitors (10 μM SB-415 286)and in high control (HC) solutions which contained GSK-3β (2.0 U) and no inhibitors) at pH 7.4 in a total volume of 40 μL. SB-415 286 (1 μM) was added to Mixture **2** as a control to demonstrate GSK-3β inhibition. After a 1 h incubation time, the samples were diluted to 240 μL with pure acidic MeOH and placed into 0.35 mL sample vials sealed with septum caps. Samples were injected into the ESI-MS using an Eksigent nanoLC system interfaced to an AS-1 Autosampler. Channels A and B of the NanoLC pump (each 5 mL internal volume) were filled with a buffer consisting of NH_4OAc (6 mM) and $Mg(OAc)_2$ (1.6 mM) at pH 7.4 and used as the mobile phase for all assays with a flowrate of 5 μL min^{-1}. In a typical assay the sample in the vial was aspirated into a PEEK sample loop (250 μm i.d.) with an uptake volume of 120 μL and the mobile phase was then passed through the sample loop to introduce the sample to the MS/MS system. The GSM, pGSM and SB-415 286 signals were then monitored in MRM mode. PEEK tubing (75 μm i.d.) was used elsewhere in the LC system to minimize non-specific binding of compounds, which was observed in some cases when fused silica tubing was used. Blank solutions containing only mobile phase were injected by the autosampler in between every sample vial in order to rinse the system between assays.

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