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Chemistry & Drug Discovery

Supporting Information

Identification of a Glycogen Synthase Kinase-3β Inhibitor that Attenuates Hyperactivity in CLOCK Mutant Mice

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Kinase selectivity for compound **3a**

1 compound tested against 15 kinases

Compounds were tested in 10-dose IC50 mode with 3-fold serial dilution starting at 100 µM
Control Compound was tested in 10-dose IC50 mode with 3-fold serial dilution starting at 20 µM
Reactions were carried out at 10 µM ATP

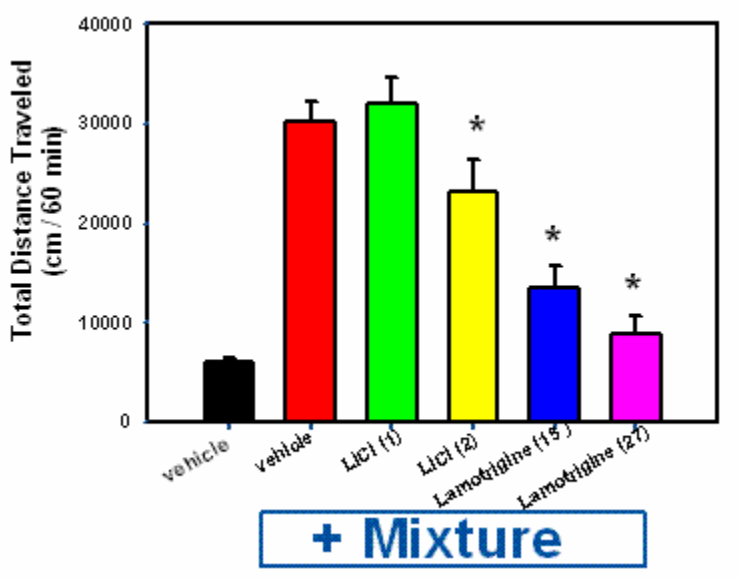
Data pages include raw data, % Enzyme activity (relative to DMSO controls) , and curve fits.

IC50 Summary:

	Compound IC50* (nM):			
Kinase:	**gsp6-79**	**Staurosporine**	**Alternate Control cpd.**	**Alternate compound ID**
AKT1	**3739.00**	**<1.0**		
CDK1/cyclin B	**8617.00**	**<1.0**		
CDK2/cyclin A	**4710.00**	**<1.0**		
CDK5/p25	**31420.00**	**2.76**		
CHK1	**384.80**	**<1.0**		
ERK1		**10240.00**		
FGFR3	**8046.00**	**4.63**		
GSK3a	**38.91**	**3.36**		
JNK1	**83360.00**	**3503.00**		
KDR/VEGFR2	**6414.00**	**2.95**		
MAPKAPK2	**>100000**	**236.80**		
P38a/MAPK14	**>100000**	ND	**41.77**	**SB202190**
PDK1/PDPK1	**15590.00**	**13.38**		
PKA	**305.70**	**<1.0**		
PKCb2	**29.05**	**1.35**		

*** Empty cells indicate no inhibition or compound activity that could not be fit to an IC50 curve**
ND **Indicates compound not tested against enzyme**

Lithium control for the Amph+CDP hyperactivity assay



(http://www.psychogenics.com/CDP.shtml)

X-ray structure determination of the GSK-3β in complex with compound 2

Human GSK-3β was expressed and purified from *Spodoptera frugiperda* insect cells similar to published methods [1]. Purified GSK-3β was co-crystallized with **2** by vapor diffusion using the hanging-drop vapor diffusion method. Crystals of the GSK-3β-**2** complex formed from 25% PEG 3350, 0.1M Na Acetate, 0.1M HEPES, pH 7.3. Crystals were briefly transferred to mother-liquor supplemented with ~7.5% glycerol and ~7.4% PEG400 and then immediately flash-frozen in liquid nitrogen. Crystals were stored in liquid nitrogen until synchrotron time was available. Crystals were transferred from liquid nitrogen into a stream of dry nitrogen gas at 100 ^{o}K for X-ray data collection.

X-ray data were collected at the Advanced Photon Source, Argonne National Laboratory, on beamline 21 ID-G at the Life Sciences-Collaborative Access Team (LS-CAT). X-ray data were recorded on a Rayonix-300 CCD detector at a crystal to detector distance of 300 mm. Exposure times were 3 s, and 200 images with a $\Delta\Phi$ angle of 0.5^{o} were collected for a total of 100^{o} of data coverage. X-ray data were processed and scaled using HKL2000 [2]. The GSK3-β-**2** complex crystallized in space group $P2_12_12_1$ with unit cell dimensions of a=83.0 Å, b=86.7Å, c=177.4 Å with two GSK-3β monomers in the asymmetric unit and a Matthews coefficient [3] of 3.94 $Å^3$ and a solvent content of 68.8%.

Intensities were converted to structure-factor amplitudes by the method of French and Wilson [1] using the program TRUNCATE in the CCP4 program suite [4]. The initial phases for the model were determined by the method of molecular replacement using the program MOLREP [5] in the CCP4 program suite. The search model used for molecular replacement consisted of the monomer of wild-type GSK-3β (PDB:1Q4I) [1] with all side chains intact and all waters and ligands removed. The final and optimal molecular replacement solution contained a single dimer in the asymmetric unit.

An initial round of combined positional and B-factor refinement was performed with the program REFMAC [6] in the CCP4 program suite using a maximum-likelihood target function and no sigma cutoff on structure factor amplitudes. Initial difference Fourier maps were calculated and visualized using the program COOT [7]. The initial Fo-Fc difference maps revealed strong (+4σ), residual electron density peaks in the active site

for the inhibitor **2**. A molecular model for the inhibitor was built using the Monomer Library Sketcher program in the CCP4 program suite, and a monomer library description was created for refinement. Residual electron density was also observed for the n-terminal amino acid residues 25 to 34. The inhibitor and residues 25 to 35 were built into density manually using COOT, and the structure was refined using REFMAC. Iterative rounds of refinement were performed, and water molecules were added manually into strong (+4σ) difference density peaks in the initial refinement stages, and into peaks of (+ 3 σ) in the final stages of refinement. During these iterative refinements, residual electron density consistent with a HEPES molecule from the buffer solution was identified, and this molecule was built into residual density and included in all subsequent refinements. A HEPES molecule is also observed in the structure of GSK-3β (PDB:1H8F) [8].

Iterative refinement using REFMAC was continued until the R_{cryst} and R_{free} values plateaued at their lowest values which were 19.2% and 27.6%, respectively. At this point, the coordinates for the resulting model were submitted to the TLS (translation/libration/screw) server [9] to generate a multi-group TLS model. The resulting TLS groups were visualized using the molecular viewer on the TLS website [10], and 15 TLS groups were chosen. Two rounds of TLS and restrained refinement [11] were performed in REFMAC with the weighting term set at 0.1. The resulting R_{cryst} and R_{free} values were 17.7% and 24.8%, respectively, justifying the inclusion of TLS groups in the standard refinement protocol [11]. The final model was validated using Procheck [12], MolProbity [13], and WhatCheck [14]. The final model coordinates have been

deposited in the PDB under accession code PDB:#### [15]. A summary of the X-ray data collection and refinement statistics is given in Table 1.

Results

The X-ray structure of GSK-3β in complex with **2** clearly shows that the inhibitor is bound within the active site. Difference Fourier electron density maps contoured at +10σ allowed for the unambiguous placement of the bromine atom, and maps contoured at +4σ allowed for the unambiguous placement of the indole ring, ethyl benzofuran ring (Figure 2). The maleimide portion of **2** makes key hydrogen bond interactions with the backbone carbonyl of Asp133 and the backbone amide of Val135 (Figure 2), similar to interactions with other maleimide containing inhibitors and staurosporine [1, 16].

Table S1.Data Collection and Refinement Statistics for GSK-3β in Complex with Inhibitor 2

Data Collection Parameters	
Crystal Conditions	Flash-Cooled at 100 ^{o}K
X-ray Source and Detector	LS-CAT 21-ID-G, Rayonix-300
Wavelength (Å)	0.979
Resolution Limit (Å)	2.3
Space Group	$P2_12_12_1$
Unit Cell Dimensions (Å) a, b, c	83.0,86.7,177.4
Data Processing Statistics	**Overall (Last Shell)**
Data Resolution Range (Å)	50.0 – 2.3 Å (2.38 – 2.30]
Reflections	
Total Recorded (n)	227,532
Averaged (n)	56,569
Completeness (%)[a]	98.1(85.7)
Average Redundancy	4.0
R_{merge} (%)	8.7 (57.0)
Average I / □ I	16.0 (1.7)

Refinement Statistics	**Overall (Last Shell)**
Data Resolution Range (Å)	50.0 – 2.3 Å (2.36 – 2.30]
Reflections in Working Set (n)	53,634 (3,251)
Reflections in Test Set (n)	2,870 (187)
R_{cryst} (%)[b]	21.3(27.1)
R_{free} (%)[c]	27.0(38.4)
Figure of merit (FOM)[d]	0.8
RMS Deviations Bond length (Å)	0.017
RMS Deviation Bond angles (^{o})	1.99
Estimated overall coordinate error based on Maximum Liklihood (Å)	0.157
Molecules in Final Model (#)	**Average B-factor (Chain A, Chain B) (Å^2)**
Protein Chains (2)	36.9
Inhibitor **2** (2)	(67.6, 79.6)
HEPES (2)	(78.3, 82.2)
Solvent H_2O (467)	< 90.0[e]

[a] Completeness for $I/\sigma(I) > 1.0$.
[b] $R_{cryst} = \sum ||F_o|-|F_c|| / \sum |F_o|$
[c] R_{free} was calculated against 5% of the reflections removed at random.
[d] Figure of Merit = ($|\sum P(\alpha)e^{i\alpha} / \sum P(\alpha)|$), where α is the phase and $P(\alpha)$ is the phase probability distribution.
[e] Cutoff criteria on solvent B-factors.

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