

## Supplemental Data

## GSK-3-Selective Inhibitors Derived from Tyrian Purple Indirubins

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### Supplemental Experimental Procedures

#### Natural products extraction and chemistry

##### *Spectral data of isolated indirubins*

###### **6'-Bromoindirubin (2)**

<sup>1</sup>H NMR (DMSO, 400 MHz,  $\delta$  ppm, J in Hz) 11.00 (1H, s, N'-H), 10.90 (1H, s, N-H), 8.75 (1H, d,  $J = 7.7$  Hz, H-4), 7.64 (1H, s, H-7'), 7.59 (1H, d,  $J = 8.1$  Hz, H-4'), 7.27 (1H, t,  $J = 7.7$  Hz, H-6), 7.19 (1H, d,  $J = 8.1$  Hz, H-5'), 7.03 (1H, t,  $J = 7.7$  Hz, H-5), 6.92 (1H, d,  $J = 7.7$  Hz, H-7). <sup>13</sup>C NMR (DMSO, 200 MHz,  $\delta$  ppm) 187.17 (C-3'), 170.33 (C-2), 152.76 (C-7a'), 140.71 (C-7a), 137.58 (C-2'), 130.34 (C-6'), 129.28 (C-6), 125.45 (C-4'), 124.46 (C-4), 123.65 (C-5'), 120.93 (C-5,3a), 117.77 (C-3a'), 115.82 (C-7'), 109.24 (C-7), 107.26 (C-3); CI-MS  $m/z$  341, 343 (M+H)<sup>+</sup>

###### **6-Bromoindirubin (3)**

<sup>1</sup>H NMR (DMSO, 400 MHz,  $\delta$  ppm, J in Hz) 11.10 (1H, s, N'-H), 11.00 (1H, s, N-H), 8.67 (1H, d,  $J = 8.1$  Hz, H-4), 7.65 (1H, d,  $J = 7.5$  Hz, H-4'), 7.58 (1H, t,  $J = 7.5$  Hz, H-6'), 7.42 (1H, d,  $J = 7.5$  Hz, H-7'), 7.22 (1H, dd,  $J = 8.1, 1.7$  Hz, H-5), 7.04 (1H, d,  $J = 1.7$  Hz, H-7), 7.03 (1H, t,  $J = 7.5$  Hz, H-5'). <sup>13</sup>C NMR (DMSO, 200 MHz,  $\delta$  ppm) 188.85 (C-3'), 170.98 (C-2), 152.58 (C-7a'), 142.59 (C-7a), 138.86 (C-2'), 137.31 (C-6'), 125.99 (C-4), 124.52 (C-4'), 123.78 (C-5), 121.58 (C-3,5'), 120.86 (C-3a), 119.06 (C-3a'), 113.64 (C-7'), 112.39 (C-7), 105.42 (C-6); CI-MS  $m/z$  341, 343 (M+H)<sup>+</sup>

###### **6,6'-Dibromoindirubin (4)**

$^1\text{H}$  NMR (DMSO, 400 MHz,  $\delta$  ppm,  $J$  in Hz) 11.20 (1H, s, N'-H), 11.10 (1H, s, N-H), 8.67 (1H, d,  $J = 8.4$  Hz, H-4), 7.68 (1H, d,  $J = 1.7$  Hz H-7'), 7.62 (1H, d,  $J = 8.1$  Hz, H-4'), 7.22 (1H, dd,  $J = 8.1, 1.7$  Hz, H-5'), 7.22 (1H, dd,  $J = 8.4, 1.6$  Hz, H-5), 7.05 (1H, d,  $J = 1.6$  Hz, H-7).  $^{13}\text{C}$  NMR (DMSO, 200 MHz,  $\delta$  ppm) 187.60 (C-3'), 170.69 (C-2), 153.04 (C-7a'), 142.49 (C-7a), 130.99 (C-6'), 129.55 (C-2'), 126.25 (C-4), 126.06 (C-4'), 124.45 (C-5'), 124.08 (C-5), 121.95 (C-3), 121.04 (C-3a), 118.31 (C-3a'), 116.47 (C-7'), 112.50 (C-7), 106.01 (C-6); CI-MS  $m/z$  419, 421, 423 (M+H)<sup>+</sup>

## **Kinase assays**

### ***Buffers***

*Homogenization Buffer* - 60 mM  $\beta$ -glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM  $\text{MgCl}_2$ , 1 mM DTT, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10  $\mu\text{g}$  leupeptin/ml, 10  $\mu\text{g}$  aprotinin/ml, 10  $\mu\text{g}$  soybean trypsin inhibitor/ml and 100  $\mu\text{M}$  benzamidine.

*Buffer A* - 10 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM DTT, 25 mM Tris-HCl pH 7.5, 50  $\mu\text{g}$  heparin/ml.

*Buffer C* - homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors.

### ***Kinase preparations and assays***

Kinase activities were assayed in Buffer A or C (unless otherwise stated), at 30°C, at a final ATP concentration of 15  $\mu\text{M}$ . Blank values were subtracted and activities calculated as pmoles of phosphate incorporated during a 10 min. incubation. The activities are usually expressed in % of the maximal activity, i.e. in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide. In a few cases phosphorylation of the substrate was assessed by autoradiography after SDS-PAGE.

*GSK-3 $\alpha/\beta$*  was purified from porcine brain by affinity chromatography on immobilized axin [S1]. It was assayed, following a 1/100 dilution in 1 mg BSA/ml 10 mM DTT, with 5  $\mu\text{l}$  40  $\mu\text{M}$  GS-1 peptide, a specific GSK-3 substrate, (YRRAAVPPSPSLSRHSSPHQSpEDEEE, synthesized by the Peptide Synthesis Unit, Institute of Biomolecular Sciences, University of Southampton, U.K.), in buffer A, in the presence of 15  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (3,000 Ci/mmol; 1 mCi/ml) in a final volume of 30  $\mu\text{l}$ . After 30 min. incubation at 30°C, 25  $\mu\text{l}$  aliquots of supernatant were spotted onto 2.5 x 3 cm pieces of Whatman P81 phosphocellulose paper, and, 20 sec. later, the filters were washed five times (for at least 5 min. each time) in a

solution of 10 ml phosphoric acid/liter of water. The wet filters were counted in the presence of 1 ml ACS (Amersham) scintillation fluid.

*CDK1/cyclin B* was extracted in homogenisation buffer from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on p9<sup>CKShs1</sup>-sepharose beads, from which it was eluted by free p9<sup>CKShs1</sup> as previously described [S2]. The kinase activity was assayed in buffer C, with 1 mg histone H1 /ml, in the presence of 15  $\mu$ M [ $\gamma$ -<sup>32</sup>P] ATP (3,000 Ci/mmol; 1 mCi/ml) in a final volume of 30  $\mu$ l. After 10 min. incubation at 30°C, 25  $\mu$ l aliquots of supernatant were treated as described above.

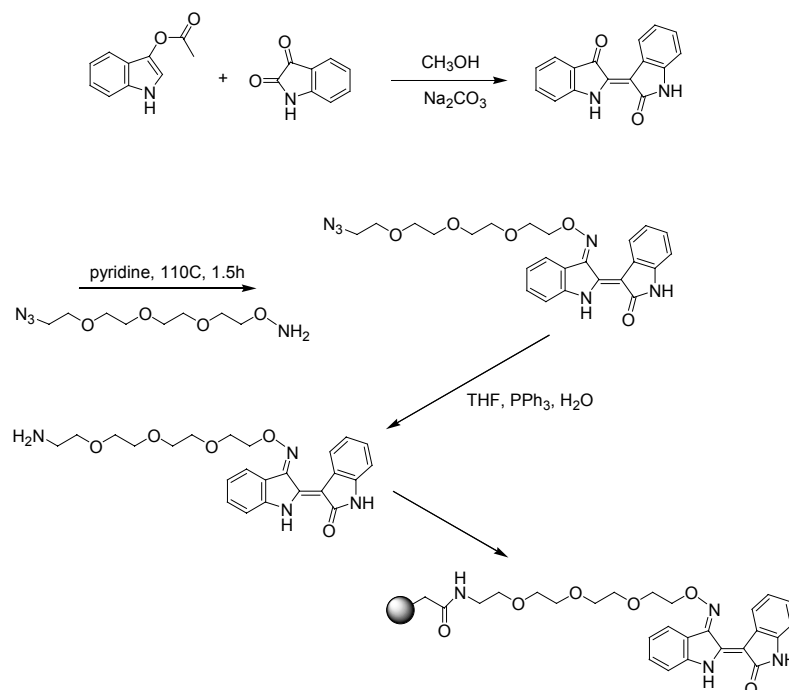
*CDK5/p25* was reconstituted by mixing equal amounts of recombinant mammalian CDK5 and p25 expressed in *E. coli* as GST (Glutathione-S-transferase) fusion proteins and purified by affinity chromatography on glutathione-agarose (vectors kindly provided by Dr. J.H. Wang) (p25 is a truncated version of p35, the 35 kDa CDK5 activator). Its activity was assayed in buffer C as described for CDK1/cyclin B.

*Other kinases* were expressed, purified and assayed as described previously [S3].

## **Affinity chromatography on immobilized indirubin**

### ***Immobilization of indirubin on a matrix***

Indirubin was synthesized by the condensation of 3-acetoxyindol with isatin (all chemicals were from Aldrich) under basic conditions. The indirubin affinity resin compound was then prepared by the formation of an oxime between indirubin and NH<sub>2</sub>-O-(PEG)<sub>3</sub>-N<sub>3</sub> with pyridine at 110°C. The azide group was further reduced to amine, and the resulting compound was captured by active ester agarose (Affi-Gel 15, BioRad). The completion of the resin capture was monitored by the disappearance of the peak of the indirubin derivative in LC/MS.



Scheme 1. Synthesis of immobilized indirubin.

## Crystallization of kinases with indirubins

### *GSK-3 $\beta$*

Human GSK-3 $\beta$  was cloned, and expressed, in the Bac-to-Bac baculovirus expression system (Life Technologies), as previously described [38]. Frozen cells from a 5 liter culture were lysed by thawing, and hand homogenizing on ice in buffer A (50 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 50 mM NaF, 1 mM Na orthovanadate, supplemented with protease inhibitors). The cell extract was centrifuged (48,000 g for 60 min at 4°C) and the clarified supernatant was mixed with 10 ml Talon metal affinity resin (Clontech) for 2 hr at 4°C. The resin was pelleted by centrifugation at 700 g for 3 min at 4°C, packed into an XK 16/20 column (Amersham Biosciences), washed with 20 column volumes of buffer A, and 20 column volumes of buffer A + 5 mM imidazole. The protein was eluted with 50 mM HEPES-NaOH pH 7.0, 300 mM NaCl, 200 mM imidazole, 50 mM NaF, 1 mM Na orthovanadate. 2 mM EDTA and 2 mM DTT were added to the eluted protein, which was then incubated overnight at 4°C with approximately 3 mg (or 20,000 units) of rTEV protease, to remove the histidine tag. The protein was concentrated to 15 ml using Vivaspin 20 ml centrifugal concentrator (Vivascience), and desalted (HiPrep 26/10 desalting column, Amersham Biosciences) in 50 mM HEPES-NaOH pH 7.0, 300 mM NaCl. The protein was mixed with 10 ml Talon for 2 hr at 4°C to separate cleaved GSK-3 $\beta$  from non-cleaved protein, rTEV protease, and other

contaminants. 1 mM EDTA and 1 mM DTT were added to the protein, which was concentrated and diluted in ion exchange buffer A (25 mM HEPES-NaOH pH 7.0, 1 mM DTT) to obtain a NaCl concentration of 50 mM. The protein was applied to an 8 ml Source 15S column, HR 10/10 (Amersham Biosciences). The resin was washed with buffer A and the protein was eluted with a 0-500 mM NaCl gradient over 50 column volumes, in 25 mM HEPES-NaOH pH7.0, 1 mM DTT. The protein was concentrated to ~ 10 mg/ml using a 2ml centricon centrifugal concentrator (Amicon), and the purified GSK-3 $\beta$  was stored at – 80 °C.

Samples of unphosphorylated and tyrosine phosphorylated GSK-3 $\beta$  (130  $\mu$ M) were incubated on ice for 1 hr with 200  $\mu$ M **BIO**. Crystallization trials were conducted using MDL Structure Screen 1, in 2  $\mu$ l hanging drop experiments. Unphosphorylated GSK-3 $\beta$  - **BIO** complex yielded small crystals in several conditions and large crystals with precipitant containing 2.0 M ammonium dihydrogen phosphate, 0.1 M Tris HCl pH 8.5. Crystals large enough for data collection were obtained, by mixing 1  $\mu$ l of phosphorylated GSK-3 $\beta$  - **BIO** complex, in 25 mM HEPES-NaOH pH 7.0, 250 mM NaCl, 1 mM DTT, with 1  $\mu$ l precipitant containing 2.0 M ammonium dihydrogen phosphate, 0.1 M Tris HCl pH 8.5. The crystals were stepped through cryo-buffer drops containing 0, 10, 20 and 30% glycerol in under a minute and flash frozen. Data were collected on a single crystal on beamline ID29 at the ESRF, using a wavelength of 0.92Å, chosen to highlight the bromine position. Data statistics are summarized in Table S1. Data processing was carried out using MOSFLM and SCALA [S4]. The structure was solved by molecular replacement methods [S5] using the GSK-3 $\beta$  structure as search model. Refinement was carried out in CNS [S6] using torsion angle molecular dynamics. A model for the bromoindirubin was built and refinement parameters generated from the HIC-UP server [S7]. In the final stages of refinement 125 water molecules were added.

### ***CDK5/p25***

A dominant-negative version of the CDK5 kinase containing a point mutation of Asp144 to asparagine was used in the co-crystallization experiments as this mutation substantially improved the expression yields [40]. The CDK5/p25 complex was expressed, purified and crystallized as described previously [40]. After CDK5/p25 crystals had formed, small amounts of indirubin-3'-oxime powder were added to the crystallization drops using a cat's whisker, and soaking was protracted for 2 h at 20°C. During this time, binding of the inhibitor to the crystals could be detected by the coloring of the crystals, which assumed a bright purple

color. The crystals were dialyzed overnight in cryo-buffer (mother liquor containing 20% glycerol) as described [40] and flash-frozen. Data were collected from a single crystal at beamline ID14-1 at ESRF, using a wavelength of 0.93 Å, as summarized in Table S1. Data processing was carried out using programs DENZO and SCALEPACK [S8]. The structure was solved by molecular replacement with the program AMoRe [S5] using the CDK5/p25 model as a search model. Following rigid body refinement with CNS [S6],  $(F_o - F_c)_{\text{calc}}$  map showed clear electron density for the bound inhibitor. After several runs of torsion angle molecular dynamics, a model for indirubin-3'-oxime was retrieved from the Cambridge Structural Database and built into the electron density map. All atoms of indirubin-3'-oxime were restrained to lie on a single plane. The model was subjected to a few final rounds of positional and B factor refinement. Towards the end of refinement, 210 water molecules were added. Data collection, processing and refinement statistics are given in Table S1.

**Table S1. Structure determination of the GSK-3 $\beta$  - BIO and CDK5/p25-IO complexes.**

Statistics of the dataset used and of the refined structure.

<b>GSK-3<math>\beta</math> - BIO</b>			
Data Statistics		Refinement Statistics	
Space group	P4 <sub>3</sub> 2 <sub>1</sub> 2	Maximal resolution (Å)	2.8
Resolution (Å)	2.8	Protein atoms	2760
Observations/unique reflections	220805/50596	Reflections (working set/test set)	24570 / 1204
Completeness (last shell) (%)	99.7 (97)	R <sub>cryst</sub> <sup>**</sup> (%)	19.25
R <sub>sym</sub> <sup>*</sup> (last shell) (%)	7.0 (28.9)	R <sub>free</sub> <sup>***</sup> (%) (% of data)	22.50 (5)
I/ $\sigma$ I (last shell)	18.4 (3.7)		
Unit cell dimensions	a = 98.3 Å c = 198.0 Å		

<b>CDK5/p25 – IO</b>			
Data Statistics		Refinement Statistics	
Space group	C2	Maximal resolution (Å)	2.3
Resolution (Å)	30 – 2.3	Protein atoms	6982
Observations/unique reflections	220805/50596	Reflections (working set/test set)	48066/2530
Completeness (last shell) (%)	98.8 (99.9)	R <sub>cryst</sub> <sup>**</sup> (%)	24
R <sub>sym</sub> <sup>*</sup> (last shell) (%)	7.5 (39.6)	R <sub>free</sub> <sup>***</sup> (%) (% of data)	26.5 (5)
I/ $\sigma$ I (last shell)	20.4 (4.5)		
Unit cell dimensions	a = 149.5 Å b = 90.1 Å c = 83.2 Å $\beta$ = 93.3°		

$$* R_{sym} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \overline{I(hkl)}|}{\sum_{hkl} \sum_i I_i(hkl)}$$

$$** R_{cryst} = \frac{\sum_{hkl} |F_{obs} - k|F_{calc}|}{\sum_{hkl} |F_{obs}|}$$

\*\*\* R<sub>free</sub> is equivalent to R<sub>cryst</sub> but is calculated using a disjoint set of reflections excluded from refinement.

## Effects of indirubins on *Xenopus* embryos

### *In vitro* transcription and RT-PCR

The RNA expression vectors were pCS2+DN Xtcf-3, encoding amino acids 88-553 of Xtcf-3 [S9], MTPA2 [50] for axin. RT-PCR was performed as previously described [S10], with the following oligonucleotides: for *ODC* sense 5' CAA CGT GTG ATG GGC TGG AT 3' and antisense 5' CAT AAT AAA GGG TTG GTC TCT GA 3'; for *nrp1* sense 5' GGG TTT CTT GGA ACA AGC 3' and antisense 5' ACT GTG CAG GAA CAC AAG 3'; for *XAG1* sense 5' GAG TTG CTT CTC TGG CA 3' and antisense 5' CTG ACT GTC CGA TCA GAC 3'; for *en2* sense 5' CGG AAT TCA TCA GGT CCG AGA TC 3' and antisense 5' GCG GAT CCT TTG AAG TGG TCG CG 3'; for *Xhoxb9* sense 5' TAC TTA CGG CGT TGG CTG GA 3' and antisense 5' AGC GTG TAA CCA GTT GGC TG 3'; for *chordin* sense 5' CAG TCA GAT GGA GCA GGA TC 3' and antisense 5' AGT CCC ATT GCC CGA GTT GC 3'.

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