

Soluble 3',6-Substituted Indirubins with Enhanced Selectivity toward Glycogen Synthase Kinase -3 Alter Circadian Period

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Glycogen synthase kinase -3 (GSK-3) is a key enzyme involved in numerous physiological events and in major diseases, such as Alzheimer's disease, diabetes, and cardiac hypertrophy. Indirubins are bis-indoles that can be generated from various natural sources or chemically synthesized. While rather potent and selective as GSK-3 inhibitors, most indirubins exhibit low water solubility. To address the issue of solubility, we have designed novel analogues of 6-bromo-indirubin-3'-oxime with increased hydrophilicity based on the GSK-3/indirubins cocrystal structures. The new derivatives with an extended amino side chain attached at position 3' showed potent GSK-3 inhibitory activity, enhanced selectivity, and dramatically increased water solubility. Furthermore, some of them displayed little or no cytotoxicity. The new indirubins inhibit GSK-3 in a cellular reporter model. They alter the circadian period measured in rhythmically expressing cell cultures, suggesting that they might constitute tools to investigate circadian rhythm regulation.

Introduction

Among the 518 protein kinases that constitute the human kinome, glycogen synthase kinase -3 (GSK-3^a) stands out as a particularly interesting and well-studied family of serine/threonine kinases.^{1–5} There are only two GSK-3 forms (GSK-3 α and GSK-3 β), which share extensive similarity (84% overall identity, 98% within the catalytic domain), the main difference coming from an extra Gly-rich stretch in the N terminal domain of GSK-3 α . GSK-3s are highly conserved protein kinases present from unicellular parasites^{6,7} to yeast⁸ up to mammals. These kinases are involved in numerous critical physiological events such as Wnt and Hedgehog signaling, embryonic development (pattern specification and axial orientation), transcription, insulin action, cell division cycle, cell death, cell survival, differentiation, multiple neuronal functions, circadian rhythm regulation, stem cell differentiation, etc. In addition, GSK-3s are implicated in a large diversity of human diseases, including nervous system disorders such as Alzheimer's disease,^{9–11} schizophrenia,¹² bipolar disorder,¹³ diabetes,¹⁴ heart hypertrophy,^{15,16} renal diseases,¹⁷ shock and inflammation,¹⁸ cancers,¹⁹ etc. There is thus a strong rationale supporting the search for potent and selective GSK-3 inhibitors for their use as pharmacological tools in basic research, as potential drugs

for the treatment of specific diseases, and for the maintenance of pluripotent stem cells in the absence of feeder cells.²⁰ Numerous GSK-3 inhibitory scaffolds have been described.^{21–24} Interestingly, many of these inhibitors also interact with cyclin-dependent kinases (CDKs), another family of well-studied key regulatory enzymes.²⁵

Among GSK-3 inhibitors, derivatives of the bis-indole indirubin (collectively referred to as indirubins) appear as a class of original and promising tools and agents.²⁶ Their moderate selectivity might be an inconvenient when used as a research reagent, but their combined effects on several disease-relevant targets (in particular CDKs and GSK-3) may constitute an advantage for potential therapeutic applications.²⁴ Among many indirubins, 6-bromo-indirubin-3'-oxime (6BIO)^{27–29} has been widely used to investigate the physiological role of GSK-3 in various cellular settings and to alter the fate of embryonic stem cells.²⁰ While highly potent and relatively selective kinase inhibitory indirubins have been developed, they usually exhibit low water solubility. To address the solubility problem of these promising compounds, we have designed novel analogues of 6BIO with increased hydrophilicity. Improvement of the hydrophilic character of a molecule may be approached by several ways. The decrease of the aromatic character of indirubin scaffold by changing the hybridization state of an aromatic carbon atom to sp³ has been proposed as a way to enhance solubility.³⁰ An alternative method is the introduction of hydrophilic groups on the molecule.³¹ Obviously, it is essential that the optimization of hydrophilicity does not negatively impact on either the potency or on the selectivity of the molecule toward the target kinase. The choice of the substitution position is thus highly significant because there are two important areas of the molecule that cannot be altered without dramatic decrease of efficacy on kinases. The first one is the pharmacophore consisting of the lactam nitrogen and carbonyl and the heterocyclic nitrogen of the bis-indole core that form the key hydrogen bonding interaction pattern with the active site of the kinase

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^a Abbreviations: 6BIO, 6-bromoindirubin-3'-oxime; CDK, cyclin-dependent kinase; FCS, fetal calf serum; GSK-3, glycogen synthase kinase-3; IO, indirubin-3'-oxime; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium).

targets.^{27,28,32–36} The second is the bromine substitution at position 6, which is the selectivity determinant of 6BIO toward GSK-3 β .^{27,28,37} A detailed analysis of the crystal structure of GSK-3 β in complex with 6BIO^{27,28} provided critical information suggesting that the 3' position might be ideal for carrying out chemical modifications on the indirubin scaffold. We thus designed and synthesized a series of 6-bromo-indirubins with various substitutions on position 3'. Some of these molecules displayed high potency toward GSK-3, enhanced selectivity, and much increased water solubility. These molecules were evaluated for their GSK-3 inhibitory actions in several cellular systems.

Results and Discussion

Rationale: Binding Cavity Mapping. The biochemical and potential therapeutic importance of 6BIO being a rather potent and selective GSK-3 β ATP-competitive inhibitor is undermined by its poor water solubility (<5 mg/l). Chemical synthesis was chosen to address the question of improving this physicochemical property. Modifications of the 6BIO molecule had to be done with care, as the aim was to introduce substitutions that might improve solubility and simultaneously maintain affinity and selectivity.

Both affinity and selectivity are attributed to particular sites of the 6BIO molecule that, as a consequence, should not be altered. The pharmacophore is an essential part of the molecule that needs to be kept intact. It accommodates the main stabilizing interactions of the ligand, which is anchored at the kinase active site through three hydrogen bonds. Furthermore, substitution at position 6 is crucial for selectivity. A bulky group at that position takes advantage of the difference in the active site “gatekeeper” residue between GSK-3 β (Ile132) and the CDKs (Phe80^{CDK2}). Selectivity results from the steric clash between the substituent at position 6 and the phenylalanine residue of the CDKs. Positions 4 and 7 are not suitable for substitution. An atom larger than hydrogen at position 4 distorts the planarity of the molecule. Substitution on position 7 causes unfavorable contacts with the protein backbone.^{37,38} An introduction of hydrophilic groups at position 5 has already been performed.^{39,40} Nevertheless, an unfavorable interaction with the catalytic residues Lys85 and Asp200 of the active site is possible. Substituents at position 5' and 6' would be oriented toward the side chain of Arg141, forming π – π face-to-edge stacking interactions, but analogues carrying bulky substitutions at these positions have demonstrated a diminished potency.^{25,27,28,40,41}

The study of crystal structures of indirubin 3'-oxime bound to CDK5/p25 (1UNH)³⁴ and 6BIO bound to GSK3 β (1UV5)^{27,28} indicated the oxime moiety as an ideal site for introducing groups that will increase the hydrophilic character of 6BIO. The oxime of the bound ligand lies at the periphery of the binding cleft being partly exposed to the solvent (Figure 1). As a consequence, the introduction of bulky groups or chains at this position seems to be sterically acceptable. Most importantly, the rich polar environment provided by the ribose subsite where the oxime is positioned could facilitate novel favorable interactions. The binding mode of ATP to CDK2 as it appears in crystal structures (1B38, 1HCK) demonstrates the significance of interactions formed at the ribose subsite on stabilizing the inhibitor–receptor complex (Figure 2). The ribose moiety of ATP is anchored by the side chain carboxyl of Asp86 and by the backbone carbonyl of Gln131 through hydrogen bonds (Figure 1). In the structure of ATP bound to CDK2/cyclin A (1FIN), a conformational change is induced by cyclin binding that results in a reorientation of ATP in the cavity. However,

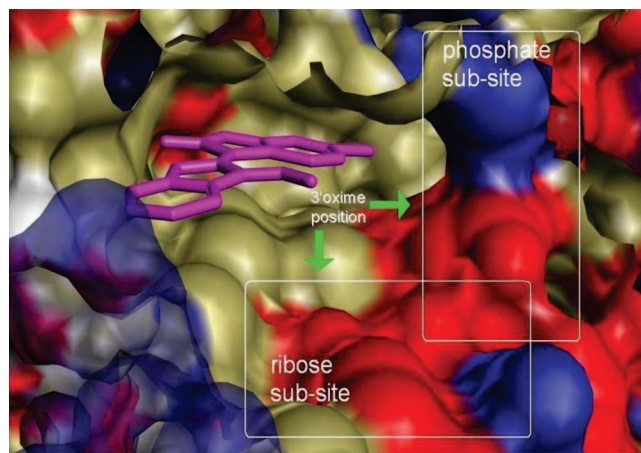


Figure 1. Mapping of the hydrophobic (green), positive (blue), and negative (red) regions of the binding pocket of GSK-3. Three distinct regions are visible: the hydrophobic active site where 6BIO molecule (magenta) is bound, the ribose subsite where the sugar of ATP forms stabilizing interactions with the kinase, and the phosphate subsite where the three phosphate groups interact with catalytic residues of the kinase. The ribose and phosphate subsites provide a polar environment that could accommodate favorable electrostatic or hydrogen bonding interactions with the inhibitor molecule.

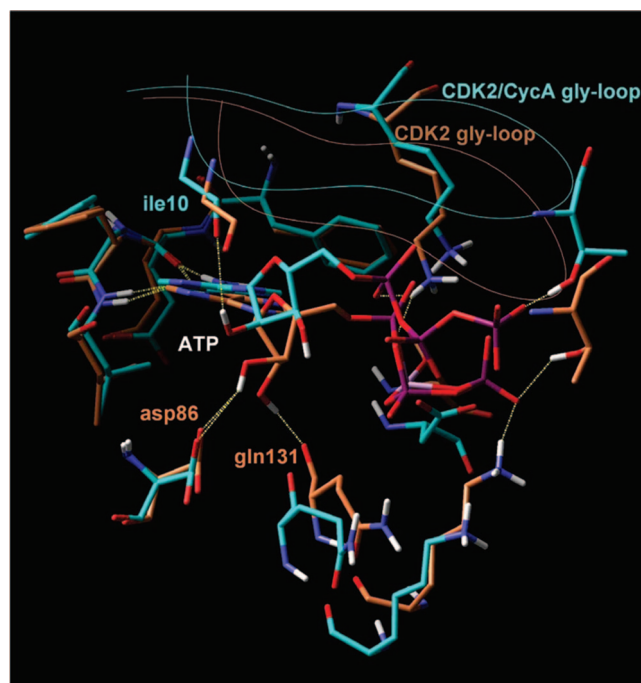
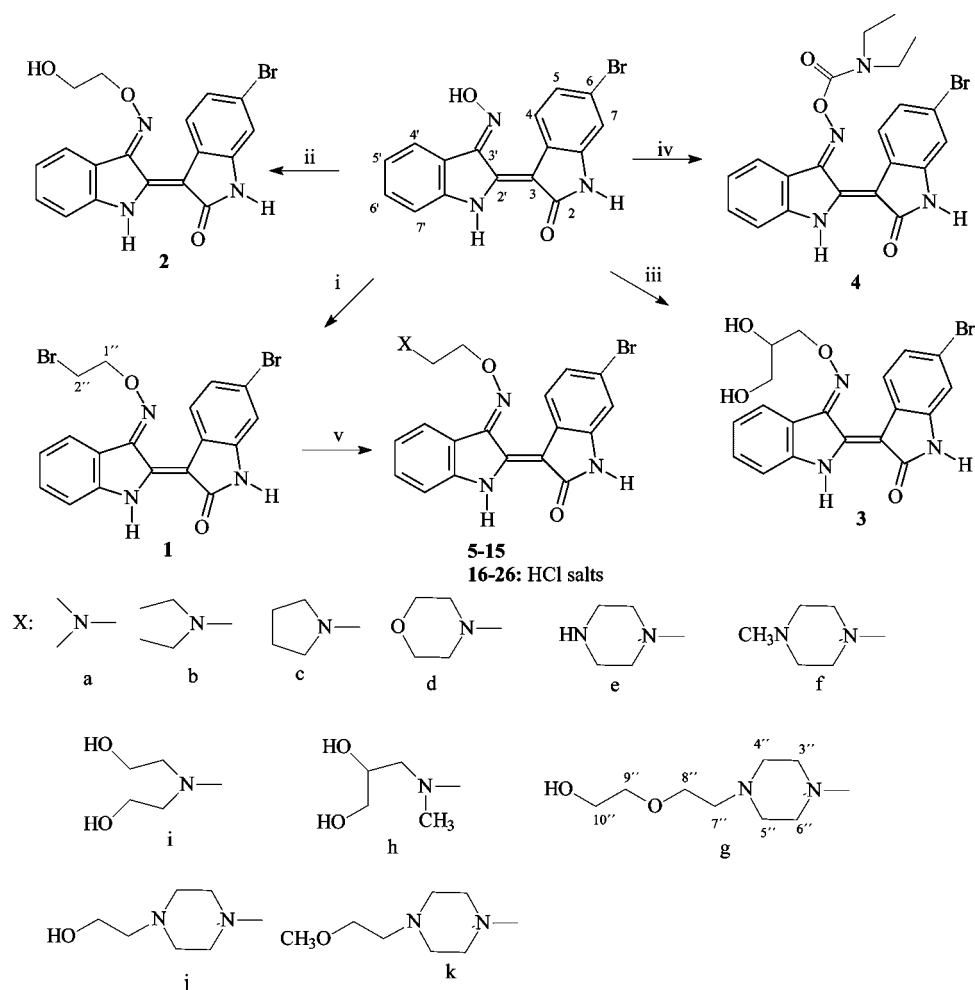


Figure 2. Superposition of crystal structures of ATP bound to monomeric CDK2 (orange) and CDK2/cyclin A (blue). In both cases, two hydrogen bonds formed between the adenine and the receptor (yellow dashed lines) stabilize the complex. An additional set of favorable interactions exists at the ribose subsite of the binding pocket, where the sugar moiety of ATP interacts through hydrogen bonds with residues that constitute the periphery of the binding pocket, namely Asp86 and Gln131 in the monomeric kinase and Ile10 in the CDK2/cyclin A dimer. Visible is also the C α trace of the glycine loop at the top of the binding pocket.

ribose retains a strong interaction with the cavity environment, in this case with the backbone carbonyl of Ile10 of the glycine loop. The ribose subsite of the GSK-3 β binding cavity is similar to that of CDK2. Ile10^{CDK2} corresponds to Ile62^{GSK3 β} , Gln131^{CDK2} to Gln185^{GSK3 β} , and Asp86^{CDK2} to Thr138^{GSK3 β} . The

Scheme 1^a

^a Reagents: (i) dibromoethane, triethylamine, DMF anhyd, 25 °C; (ii) 2-bromoethanol, triethylamine, DMF anhyd, 25 °C; (iii) 3-bromo-1,2-propanediol, triethylamine, DMF anhyd, 25 °C; (iv) *N,N*-diethylcarbonyl chloride, triethylamine, DMF anhyd, 25 °C. (v) DMF anhyd, 25 °C, amine a–k.

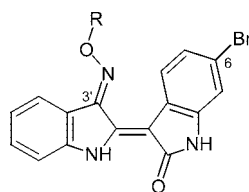
ribose subsite thus provides a rich environment of polar groups that could be targeted as interaction partners of improved 6BIO analogues.

By examining crystal structures of ATP or ATP-competitive inhibitors bound to various kinases, an additional part of the cavity was located where favorable interactions could be facilitated. It is the place where the phosphate groups of ATP interact with the receptor, called the phosphate subsite. Key interactions between the ATP phosphates and the kinase are formed there and more specifically between the α -phosphate and the side chain of lys33, between the β -phosphate and the side chains of lys129 and gln131, and between the γ -phosphate and the side chains of lys129 and thr14. These residues along with asn132 and the catalytic asp145 (the D of the DFG conserved motif) form the phosphate subsite in CDK2. In GSK-3 β , the homologous residues are ser66 (thr14^{CDK2}), lys85 (lys33^{CDK2}), lys183 (lys129^{CDK2}), gln185 (gln131^{CDK2}), asn186 (asn132^{CDK2}), and asp200 (asp145^{CDK2}). A grid-based mapping of the hydrophilic and hydrophobic sites of the binding cavity of GSK-3 β was performed by the Sitemap module of Maestro. Finally, two water molecules (PDB 1UV5, waters 49 and 74) that are proposed to bridge the interaction between thr138 and the oxime were considered as part of the cavity and possible mediators of favorable interactions.²⁸

Rationale: Energetics. On the basis of the GSK-3 β active site inspection, the substitution of the 3' oxime by a hydrophilic

chain containing appropriate chemical groups that could interact with residues of the ribose and the phosphate sites of the receptor cavity was considered as suitable (Figure 1). Such a substitution is expected to resolve the solubility problem of indirubins without affecting the selectivity toward GSK-3 β as the 6Br substitution remains unaltered. Introducing hydrophilic chemical groups on 6BIO could have undesirable consequences on binding affinity, as an increase of the polar character of a molecule results in a larger desolvation energy penalty upon binding. In addition, conformational entropy cost proportional to molecular flexibility could further decrease binding affinity. Energetics of binding imply that the finest way to overcome the impact of introducing flexible hydrophilic groups on the affinity of 6BIO for GSK-3 β is the formation of novel stabilizing interactions between these groups and the kinase. Maximizing the attractive interactions in an otherwise highly flexible moiety could equilibrate the expected entropic loss in binding affinity due to the degrees of freedom inherent to such a system as well as the desolvation energy loss and optimize the affinity of the inhibitors.

Synthesis of 3'-Substituted, 6-Bromo-indirubins. In an effort to prepare derivatives consistent with the above rationale, we synthesized 3'-substituted oximes of 6BIO bearing side chains with amine groups **5–15** and the corresponding salts **16–26** (Scheme 1). The synthesis was based on the reaction of the 3'-[O-(2-bromoethyl)oxime] intermediate **1** with the ap-

Table 1. Effects of Indirubins 1–26 on Three Protein Kinases and on the Survival of Human Neuroblastoma SH-SY5Y Cells^a

Cpd #	R	GSK3	CDK1	CDK5	SH-SY5Y
6BIO	H	0.005	0.320	0.083	9.0
1	—CH ₂ CH ₂ Br	0.14	> 10	> 10	> 100
2	—CH ₂ CH ₂ OH	0.03	> 10	> 10	> 100
3	—CH ₂ CH(OH)CH ₂ OH	0.034	0.110	0.025	0.94
4		0.03	> 10	10	> 100
5		0.033	0.490	0.100	5.4
16		0.029	0.19	0.053	5.8
6		0.035	0.09	0.02	78.0
17		0.027	0.19	0.04	> 100
9		0.040	0.60	0.21	6.6
20		0.041	1.00	0.10	9.0
10		0.067	0.24	0.24	5.8
21		0.023	0.15	0.10	6.0
7		0.026	0.50	0.13	5.5

Cpd #	R	GSK3	CDK1	CDK5	SH-SY5Y
18		0.054	0.45	0.10	4.2
8		0.060	1.10	0.60	72
19		0.110	1.80	0.90	74
11		0.0033	0.3	0.2	13.2
22		0.0013	0.2	0.18	5.9
12		0.0070	0.4	0.4	5.4
23		0.0050	0.3	0.3	5.4
13		0.0050	0.6	0.2	28.0
24		0.0042	0.4	0.2	16.7
14		0.0110	2.8	0.5	> 100
25		0.0200	1.0	0.4	> 100
15		0.014	0.90	0.31	94.4
26		0.033	0.50	0.33	97.6

^a Indirubins were tested at various concentrations on GSK-3 α/β , CDK1/cyclin B, CDK5/p25, as described in Experimental Section. IC₅₀ values, calculated from the dose–response curves, are reported in μ M. The compounds were tested at various concentrations for their effects on SH-SY5Y cells survival after 48 h incubation as estimated using the MTS reduction assay. IC₅₀ values, calculated from the dose–response curves, are reported in μ M.

appropriate amine: pyrrolidine, morpholine, piperazine, *N*-methylpiperazine, hydroxyethylpiperazine, methoxyethylpiperazine, dimethylamine and diethylamine, *N,N*-bis-2-hydroxyethylamine, *N*-2,3-dihydroxypropyl-*N*-methyl amine, and *N*-2-hydroxyethoxyethyl piperazine. The intermediate **1** was prepared by the reaction of 6BIO with 1,2-dibromoethane in DMF and Et₃N at room temperature. In addition, the carbamate **4** was prepared by the reaction of 6BIO with *N,N*-diethylcarbonyl chloride. The alcohols **2** and **3** were prepared by the reaction of 6BIO with the appropriate bromo alcohol in DMF and Et₃N at room temperature. Indirubin and 6BIO were synthesized as previously reported.²⁸

Building Analogues: Kinase Inhibitory Properties. Several chemical groups were introduced on the oxime oxygen of 6BIO and designed analogues along with their IC₅₀ values for three different kinases (GSK-3 α/β , CDK1/cyclin B, CDK5/p25) are summarized in Table 1. As the initial objective was to increase

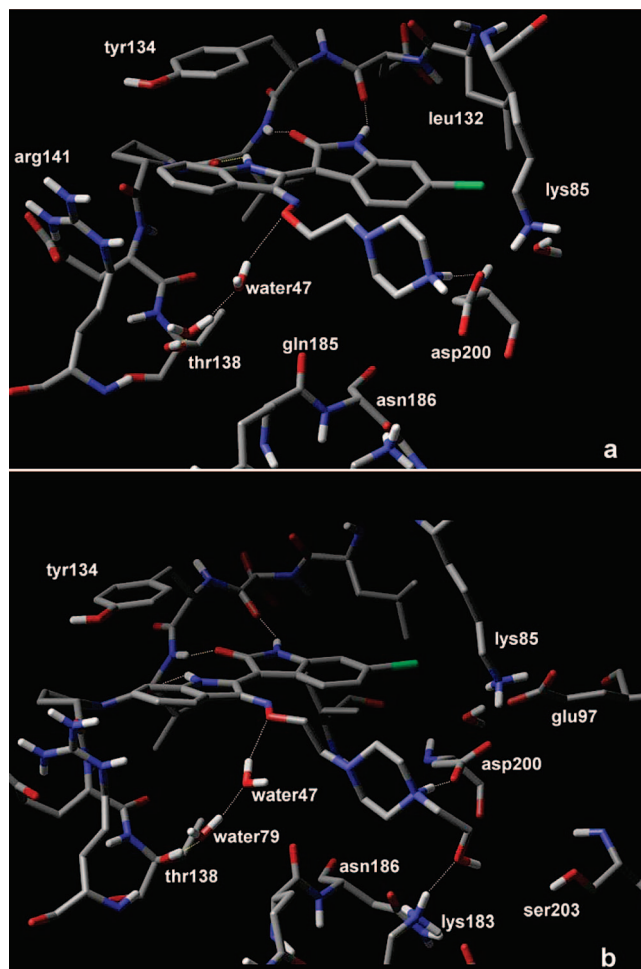
solubility, these groups were chosen according to their polarity and their potential to ionize. The biological results of the new 6BIO analogues have provided interesting information about structure–activity relations concerning GSK-3. In addition, docking calculations have provided insight in the binding mode of the novel analogues. The physicochemical properties of representative compounds such as calculated p*K*_a, clog*D*, and water solubility are summarized in Table 2.

Analogue **3** carries a side chain with two aliphatic hydroxyls at a distance of two carbon atoms from the oxime oxygen, which were expected to mimic the 2' and 3' hydroxyl groups of ATP ribose. It demonstrated a 6-fold decrease of activity compared to 6BIO. This decrease was accompanied by a mediocre improvement of solubility. This result was indicative of the unfavorable influence of chain flexibility on affinity. As flexibility was implicit for groups that would be used to improve solubility, the amplification of their stabilizing interactions was

Table 2. Water Solubility of Indirubin Salts and Calculated Physicochemical Properties pK_a and $\log D$ at pH 7.4 of Corresponding Bases

no.	solubility (g/L)	pK_a	$\log D$
6BIO	<0.005		2.59
16	0.141	8.59	1.69
17	0.192	9.38	1.19
18	0.195	9.18	1.16
21	1.45	7.48	1.79
22	1.61	8.85	-0.87
23	1.50	7.65	1.74
24	1.14	7.65	1.41
25	0.57	7.47	1.90
26	4.253	7.58	1.46

considered as a way to equalize the entropic loss. A series of analogues carrying a tertiary amine two carbons from the oxime was prepared in order to enhance the strength of hydrogen bonding interactions that are expected to form at the ribose subsite. Analogue **5** carries a dimethylamino group two carbon atoms from the oxime oxygen. Analogue **6**, carrying a bulkier diethylamino group, was designed to scan the extent by which the ATP channel could accommodate larger hydrophobic interactions. The ethyl carbons of **6** were fused to a pyrrolidine ring in analogue **7** to reduce conformational entropy. Their activity was only slightly improved. Docking calculations demonstrated that the main interaction formed by those analogues was a charge-assisted hydrogen bond between the protonated secondary amine nitrogen and the side chain of the catalytic asp200. However, the geometry of this bond was not optimal. As a result, alternative binding modes were observed where residues asn64 (glycine loop), asn186 (ribose-site), and water 47 participated with polar interactions. Keeping the tertiary amine unaltered was encouraged by this observed binding mode. As a result, further scanning of the cavity toward the phosphate subsite was performed by the extended chains of **9**, **10** and **8**. The first two carry a short aliphatic extension bearing two hydroxyl groups. The free rotation of the extended chain was restricted in **8**, where the two hydroxyethyl groups were fused to a morpholine ring. Compounds **8** and **9** demonstrated a noteworthy decrease of affinity (8-fold and 12-fold). According to these results and modeling, it was deduced that affinity could not be optimized by introduction of hydroxyl groups. Interactions formed by hydroxyls at the ribose subsite included hydrogen bonds with gln185 and a water-mediated bridge with thr138. Docking calculations suggested that these bonds could not compensate for the hydroxy-carrying group inherent flexibility. The conversion of the heterocyclic oxygen of the rigid morpholine to nitrogen would thus add a tertiary positively charged amine group closer to asp200. Analogue **11/22**, carrying a piperazine ring, demonstrated the highest affinity (3.3 nM/1.3 nM). The proposed binding mode of **11** is depicted in Figure 3a and shows an optimal pattern of interactions between the ligand and the active site of GSK-3 β , accounting for the excellent affinity of this compound. The 4-nitrogen of the piperazine ring is protonated and donates a high-quality charge-assisted hydrogen bond to the carboxylate oxygen of asp200. The piperazine ring adopts the lowest energy chair conformation, which permits an optimal approach and orientation between donor and acceptor atoms (1.8 Å). In addition, the oxime oxygen forms a hydrogen bond with water49 in a similar way to the other compounds mode. However, the high ionizing potential of **11** has a negative impact on its physicochemical profile, which is reflected on the calculated $\log D$ value (-0.87). A conversion of the secondary 4-nitrogen to tertiary was utilized as a way to limit its basicity. This conversion was combined

**Figure 3.** Binding mode of analogues **11** (A) and **13** (B) to the ATP binding pocket of GSK-3 β . The piperazine substitution of both analogues interacts with asp200 and residues located at the phosphate subsite of the binding pocket in addition to the hydrogen bonds (yellow dashed lines) formed between the indirubin scaffold and the kinase backbone.

with a gradual extension of the molecule that assisted on mapping the full extent by which the cavity can accommodate bulky ligands. The piperazine ring was substituted by a methyl, a hydroxyethyl, a methoxyethyl, and a hydroxyethoxyethyl group in analogues **12**, **13**, **14**, and **15**, respectively (and corresponding salts: **23**, **24**, **25**, **26**). Within this last group of analogues, **12** and **13** share the best consensus pharmacological and physicochemical profile. They demonstrate affinity similar to that of 6BIO, improved selectivity of 1 order of magnitude for GSK-3, increased water solubility, and acceptable lipophilicity. The proposed binding mode of **13** is depicted in Figure 3b.

Cytotoxicity of the 6BIO Analogues. All compounds were next tested for their effects on survival of SH-SY5Y neuroblastoma cells using an MTS reduction assay. These assays revealed that increased potency on GSK-3 was not associated with enhanced cell death (Table 1). Analogues **13**, **14**, and **15** (and their corresponding salts, **24**, **25**, **26**) had little cell death inducing activities. The IC_{50} values were respectively 28 μM , > 100 μM , 94 μM (salts: 17 μM , > 100 μM , 98 μM) to compare with the IC_{50} of 6BIO, 9 μM . Therefore, the substituted piperazine ring extension not only favors selectivity and efficacy toward GSK-3, allows better solubility, but also reduces their cytotoxicity. These features are particularly favorable for the use of these compounds in the study of GSK-3 in cellular

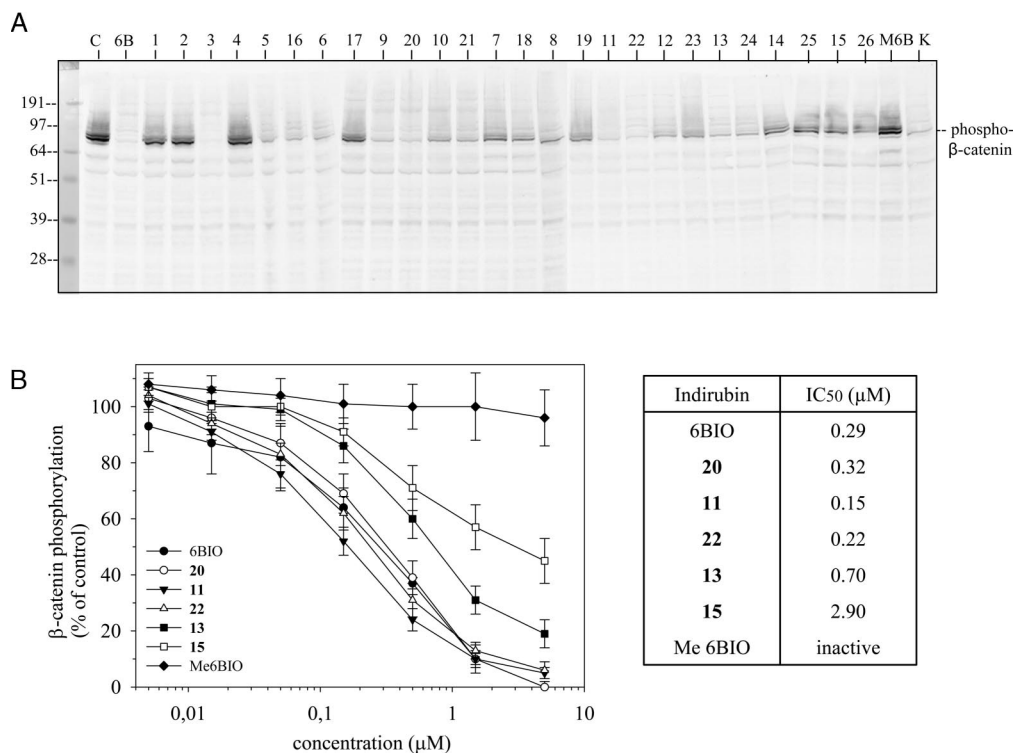


Figure 4. β -Catenin phosphorylation at GSK-3 phosphorylation sites is inhibited by the indirubin derivatives. (A) SH-SY5Y neuroblastoma cells were exposed for 6 h to 10 μ M of each indirubin in the presence of a constant 2 μ M level of the proteasome inhibitor MG132. The level of GSK-3-phosphorylated β -catenin was estimated by Western blotting following SDS-PAGE, using an antibody that specifically cross-reacts with β -catenin phosphorylated by GSK-3. Lack or reduction of the signal indicates that the indirubin has been able to inhibit GSK-3 within the neuroblastoma cells. C, Control (DMSO); 6B, 6BIO; M6B, methyl-6BIO (a control inactive analogue of 6BIO²⁷); K, kenpallone, a structurally unrelated GSK-3 inhibitor.⁴⁹ (B) Dose-response curves for a selection of indirubins were run in an ELISA assay using the same antibodies directed against GSK-3 phosphorylated β -catenin. SH-SY5Y cells were exposed for 6 h to a range of concentrations of each indirubin, in the presence of MG132, and extracts were assessed in the ELISA assay. Activity was expressed as percentage of phosphorylated β -catenin in untreated control cells.

systems and also as potential therapeutic leads in the context of neurodegenerative diseases and diabetes.

Confirmation of Intracellular Inhibition of GSK-3 by Indirubins. To investigate whether the new indirubins were effective at inhibiting GSK-3 in a cellular context, we measured their effects on the phosphorylation of β -catenin at GSK-3 specific sites in SH-SY5Y neuroblastoma cells. Cells were exposed to various concentrations of 10 μ M of each indirubin in the presence of a constant level of MG132 (an inhibitor of the proteasome, which prevented the rapid degradation of β -catenin once phosphorylated by GSK-3). The level of GSK-3-phosphorylated β -catenin was estimated either by Western blotting (with an antibody that specifically cross-reacts with β -catenin when phosphorylated at a GSK-3 site) following SDS-PAGE (Figure 4a) or by an ELISA assay (Figure 4b). Results revealed a dose-dependent inhibition of GSK-3 selective phosphorylation sites on β -catenin, demonstrating that these compounds are actually able to inhibit GSK-3 in cells. The most efficient compounds were 6BIO, 3, 5, 9, 11, 12, and 13 (and their salts when available, i.e., 16, 20, 22, 23, 24). Dose-response curves were obtained with ELISA assay (Figure 4b). The kinase inactive derivative 1-methyl-6BIO was ineffective in the cellular assay.

Effects on Circadian Rhythm in Mammalian Cell Cultures. GSK-3 is a key regulator of the circadian rhythm (aka the daily biological clock).^{44,45} The circadian rhythm can be partially reproduced in a cellular system, which is an excellent model system for circadian clocks in non-neural, peripheral tissues.⁴⁶ We therefore used this system to explore the possibility that GSK-3 inhibition could affect the circadian rhythm. Rat-1 fibroblasts stably transfected with a P_{Per2}::Fluc reporter construct

show a robust circadian rhythm of luminescence as a gauge of clock-controlled *Per2* promoter (P_{Per2}). Cells were cultured and treated first with 10 μ M indirubin 15 as described in the Experimental Section, and their circadian rhythm of *Per* expression dependent luminescence was monitored during 4 days. A gradual shortening of the period length was clearly observed (Figure 5a). Similar experiments were next performed with a small selection of indirubins, and the period length was calculated as in Figure 5a. The most efficient compounds in shortening the period length (Figure 5b) were also the most efficient at inhibiting β -catenin phosphorylation in the cellular assay (Figure 4), supporting the hypothesis that the action of the indirubins in shortening the circadian period is dependent upon GSK-3. Previous studies have suggested a key action of GSK-3 in regulating the circadian rhythm of mammalian cells by using lithium as a pharmacological tool.⁴⁵ Lithium lengthens the period of the circadian rhythm, whereas indirubins shorten the period (Figure 5). However, the concentrations of lithium used in the previous investigations of circadian rhythms and GSK-3 were 10–20 mM,⁴⁶ whereas our results with indirubins were obtained with concentrations 1000 \times lower (10 μ M). These comparisons suggest that the circadian period-lengthening effects of lithium may be due to side effects of lithium. Therefore, indirubins might constitute a useful pharmacological tool to investigate the role of GSK-3 in the regulation of the circadian rhythm.

Conclusions

Through a rationale analysis of the key interactions of indirubins at the ATP-binding site of the disease-relevant glycogen synthase kinase -3 and the synthesis and biological

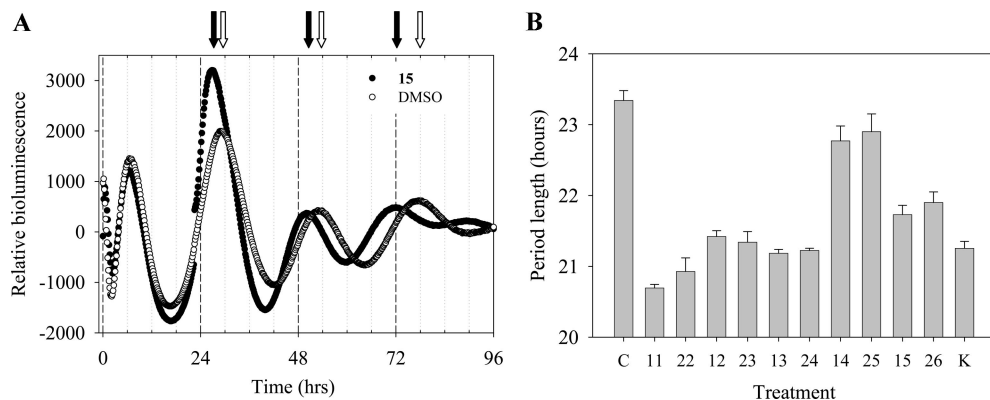


Figure 5. Indirubins alter the circadian period in mammalian fibroblasts. Rat-1 fibroblasts stably transfected with a P_{Per2} ::Fluc reporter construct show a robust circadian rhythm of luminescence as a gauge of clock-controlled *Per2* promoter (P_{Per2}) activity. Cells were cultured up to 100% confluence and treated for 2 h with 0.1 μ M dexamethasone to synchronize the oscillators. The medium was then replaced with assay medium supplemented with 0.1 mM luciferin, and the luminescence rhythm was monitored for 4 days or more. Compounds were added to the culture dishes at 10 μ M and left continuously. DMSO was used as a solvent control. Regression analyses were used to determine period and phase of the luminescence rhythms. (A) Time-course of a typical luminescence rhythm recorded in control cells (○) or cells treated with indirubin **15** (●). White arrows indicate the peaks of luminescence in control cells, black arrows indicate the peaks of luminescence in indirubin **15** treated cells. (B) Period lengths calculated in cells exposed to various compounds. C, control; K, kenpaullone.

evaluation of analogues exploring various modifications at the 3' site, we were able to uncover new interaction sites, offering further stabilization to the inhibitor/GSK-3 complexes. Consequently, extensions at this site provide enhanced activity and selectivity toward GSK-3 and also provided the opportunity to introduce substitutions favoring enhanced water solubility. The novel indirubin analogues were less cytotoxic than the parent 6BIO compound and demonstrated potent GSK-3 inhibition in two cellular models. These results open new directions toward the design of pharmacologically favorable indirubins with potential development against Alzheimer's disease, diabetes, heart hypertrophy, or in the field of embryonic stem cell pluripotency maintenance.

Experimental Section

Chemistry. General Chemistry Experimental Procedures. All chemicals were purchased from Aldrich Chemical Co. NMR spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers (^1H (400 and 200 MHz) and ^{13}C (50 MHz)); chemical shifts are expressed in ppm downfield from TMS. The ^1H - ^1H and the ^1H - ^{13}C NMR experiments were performed using standard Bruker microprograms. Melting points were determined with a Sanyo Gallencamp apparatus. MS spectra were determined on a MSQ Thermofinnigan spectrometer. All UV/vis spectra were recorded on a Shimadzu UV-160A spectrophotometer.

Solubility Measurements. Equilibrium solubilities were determined by adding an excess amount of solid to the medium (water, double distilled), followed by 5 min of sonification and overnight equilibration by stirring at ambient temperature (25 ± 0.1 °C). The samples were centrifuged, and aliquots were removed. Standard solutions were prepared for each compound in order to quantify the aforementioned saturated solutions, and reference curves were plotted for each compound. The absorbance of each saturated and standard solution was measured with a UV/vis spectrophotometer at wavelength that varied between 515 and 518 nm.

General Procedure for the Preparation of the Ethers 1–3. To a solution of 6-bromoindirubin-3'-oxime (1.0 g, 2.83 mmol) in DMF (90 mL) were added triethylamine (0.5 mL) and the appropriate bromide (2 equiv) under Ar, and the mixture was stirred for 17 h at room temperature. Then water (300 mL) was added and the precipitate formed was collected by filtration and washed with water.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-bromoethyl)-oxime] (1). Yield: 95%; mp 252 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ ppm, J in Hz) 11.67 (1H, s, H-1'), 10.93 (1H, s, H-1), 8.49

(1H, d, $J = 8.3$ Hz, H-4), 8.21 (1H, d, $J = 7.8$ Hz, H-4'), 7.45 (2H, brs, H-6', H-7'), 7.16 (1H, dd, $J = 8.3/2.0$ Hz, H-5), 7.07 (2H, m, H-5', H-7), 4.93 (2H, t, $J = 5.6$ Hz, H-1''), 3.97 (2H, t, $J = 5.6$ Hz, H-2''). APCI-MS m/z 462, 464, 466 ($M + \text{H}$) $^+$. Anal. ($\text{C}_{18}\text{H}_{13}\text{N}_3\text{O}_2\text{Br}_2$) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-hydroxyethyl)-oxime] (2). Yield: 96%; mp >300 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ ppm, J in Hz) 11.70 (1H, brs, H-1'), 10.90 (1H, brs, H-1), 8.54 (1H, d, $J = 8.4$ Hz, H-4), 8.18 (1H, d, $J = 7.6$ Hz, H-4'), 7.44 (2H, m, H-7', H-6'), 7.16 (1H, dd, $J = 8.4/1.9$ Hz, H-5), 7.05 (2H, m, H-5', H-7), 5.02 (1H, t, $J = 5.4$ Hz, -OH), 4.62 (2H, t, $J = 4.8$ Hz, H-1''), 3.89 (2H, m, H-2''). CI-MS m/z 400, 402 ($M + \text{H}$) $^+$. Anal. ($\text{C}_{18}\text{H}_{14}\text{N}_3\text{O}_3\text{Br}$) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2,3-dihydroxypropyl)-oxime] (3). Yield: 95%; mp >300 °C. ^1H NMR (400 MHz, DMSO- d_6 , δ ppm, J in Hz) 11.70 (1H, s, H-1'), 10.90 (1H, s, H-1), 8.56 (1H, d, $J = 8.5$ Hz, H-4), 8.17 (1H, d, $J = 7.7$ Hz, H-4'), 7.44 (2H, m, H-6', H-7'), 7.15 (1H, dd, $J = 8.5, 1.9$ Hz, H-5), 7.06 (1H, m, H-5'), 7.03 (1H, d, $J = 1.9$ Hz, H-7), 5.14 (1H, d, $J = 5.0$ Hz, -CHOH), 4.84 (1H, t, $J = 5.6$ Hz, -CH₂OH), 4.65 (1H, dd, $J = 10.9, 3.7$ Hz, H-1''a), 4.50 (1H, dd, $J = 10.9, 6.6$ Hz, H-1''b), 3.99 (1H, m, H-2''), 3.50 (2H, m, H-3''). APCI-MS (+) m/z 430, 432 ($M + \text{H}$) $^+$. Anal. ($\text{C}_{19}\text{H}_{16}\text{N}_3\text{O}_4\text{Br}_2$) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(N,N-diethylcarbamyl)-oxime] (4). To a solution of 6-bromoindirubin-3'-oxime (36 mg, 0.11 mmol) in DMF (10 mL) were added triethylamine (0.5 mL) and 0.3 mL (3.66 mmol) of *N,N*-diethylcarbamylchloride under Ar, and the mixture was stirred for 12 h at room temperature. Then water (40 mL) was added and the precipitate formed was collected by filtration and washed with water to give **4** quantitatively. Yield: 90%; mp 237 °C. ^1H NMR (400 MHz, pyridine- d_5 , δ ppm, J in Hz) 12.36 (1H, s, H-1'), 12.15 (1H, s, H-1), 9.93 (1H, d, $J = 8.6$ Hz, H-4), 8.13 (1H, d, $J = 7.8$ Hz, H-4'), 7.61 (1H, dd, $J = 8.6/1.9$ Hz, H-5), 7.43 (1H, m, H-6'), 7.35 (1H, d, $J = 1.9$ Hz, H-7), 7.14–7.06 (2H, m, H-5', H-7), 3.44 (4H, brs, -N(CH₂CH₃)₂), 1.18 (6H, t, $J = 7.0$ Hz, -N(CH₂CH₃)₂). APCI-MS (+) m/z 455, 457 ($M + \text{H}$) $^+$. Anal. ($\text{C}_{21}\text{H}_{19}\text{N}_4\text{O}_3\text{Br}$) C, H, N.

General Procedure for the Preparation of the Amines 5–15. First, 100 mg of 6-bromoindirubin-3'-[O-(2'-bromoethyl)-oxime] (**1**) was dissolved in 5 mL of anhydrous DMF. An excess of the appropriate amine was added under magnetic stirring and the mixture was then heated at 50 °C. After the completion of the reaction, the mixture was poured into water (30 mL) and the precipitate was filtered and washed with water and cyclohexane. Dimethylamine, diethylamine, pyrrolidine, morpholine, diethanolamine, 3-methylamine-1,2-propanediol, piperazine, 1-methylpiperazine, 1-(2-methoxyethyl) pip-

erazine, 1-(2-hydroxyethyl) piperazine, and 1-[2-(2-hydroxyethoxy)-ethyl] piperazine afforded products (5–15), correspondingly, in qualitative yields.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-dimethylaminoethyl)-oxime] (5). mp 230 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.71 (1H, brs, H-1'), 10.92 (1H, brs, H-1), 8.55 (1H, d, *J* = 8.5 Hz, H-4), 8.14 (1H, d, *J* = 7.5 Hz, H-4'), 7.44 (2H, d, *J* = 4.1 Hz, H-6', H-7'), 7.15 (1H, dd, *J* = 8.5/2.0 Hz, H-5), 7.05 (2H, m, H-5', H-7), 4.69 (2H, t, *J* = 5.8 Hz, H-1''), 2.80 (2H, t, *J* = 5.8 Hz, H-2''), 2.27 (6H, s, -N(CH₃)₂). APCI-MS *m/z* 427, 429 (M + H)⁺. Anal. (C₂₀H₁₉N₄O₂Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-diethylaminoethyl)-oxime] (6). mp 232 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.71 (1H, s, H-1'), 10.92 (1H, s, H-1), 8.55 (1H, d, *J* = 8.2 Hz, H-4), 8.16 (1H, d, *J* = 7.8 Hz, H-4'), 7.44 (2H, d, *J* = 3.4 Hz, H-6', H-7'), 7.13 (1H, dd, *J* = 8.2/2.1 Hz, H-5), 7.09–7.02 (2H, m, H-7, H-5'), 4.65 (2H, t, *J* = 6.0 Hz, H-1''), 2.94 (2H, t, *J* = 6.0 Hz, H-2''), 2.58 (2H, q, *J* = 7.2 Hz, -N(CH₂CH₃)₂), 0.98 (6H, t, *J* = 7.2 Hz, -N(CH₂CH₃)₂). APCI-MS (+) *m/z* 455, 457 (M + H)⁺. Anal. (C₂₂H₂₃N₄O₂Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-pyrrolidin-1-ylethyl)oxime] (7). mp 208 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.70 (1H, s, H-1'), 10.93 (1H, s, H-1), 8.54 (1H, d, *J* = 8.5 Hz, H-4), 8.14 (1H, d, *J* = 7.7 Hz, H-4'), 7.45 (2H, m, H-6', H-7'), 7.14 (1H, d, *J* = 8.5/1.9 Hz, H-5), 7.05 (2H, m, H-5', H-7), 4.70 (2H, t, *J* = 5.8 Hz, H-1''), 2.98 (2H, brt, *J* = 5.8 Hz, H-2''), 2.57 (4H, brs, H-3'', H-6''), 1.69 (4H, m, H-4'', H-5''). APCI-MS (+) *m/z* 453, 455 (M + H)⁺. Anal. (C₂₂H₂₁N₄O₂Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-morpholin-1-ylethyl)oxime] (8). mp 235 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.70 (1H, s, H-1'), 10.90 (1H, s, H-1), 8.52 (1H, d, *J* = 8.5 Hz, H-4), 8.15 (1H, d, *J* = 7.6 Hz, H-4'), 7.43 (2H, m, H-6', H-7'), 7.14 (1H, dd, *J* = 8.5/1.9 Hz, H-5), 7.05 (1H, m, H-5'), 7.02 (1H, d, *J* = 1.9 Hz, H-7), 4.70 (2H, t, *J* = 5.8 Hz, H-1''), 3.57 (4H, t, *J* = 4.5 Hz, H-4'', H-5''), 2.86 (2H, t, *J* = 5.8 Hz, H-2''), 2.50 (4H, m, H-3'', H-6''), overlapped with DMSO. APCI-MS (+) *m/z* 469, 471 (M + H)⁺. Anal. (C₂₂H₂₁N₄O₃Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-(*N,N*-(2-hydroxyethyl)aminoethyl)oxime] (9). mp 201 °C. ¹H NMR (400 MHz, pyridine-*d*₅, δ ppm, *J* in Hz) 12.31 (1H, brs, H-1'), 12.25 (1H, brs, H-1), 8.93 (1H, d, *J* = 8.2 Hz, H-4), 8.42 (1H, d, *J* = 7.8 Hz, H-4'), 7.47 (1H, dd, *J* = 8.2, 1.8 Hz, H-5), 7.39 (1H, d, *J* = 1.8 Hz, H-7), 7.34 (1H, t, *J* = 7.2 Hz, H-6'), 7.04 (2H, m, H-5', H-7'), 5.89 (1H, brs, -OH), 4.86 (2H, t, *J* = 6.3 Hz, H-1''), 4.00 (4H, m, -N(CH₂CH₂OH)₂), 3.38 (2H, t, *J* = 6.3 Hz, H-2''), 3.08 (4H, t, *J* = 5.9 Hz, -N(CH₂CH₂OH)₂). APCI-MS (+) *m/z* 487, 489 (M + H)⁺. Anal. (C₂₂H₂₃N₄O₄Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-[*N*-methyl,*N*-(2,3-dihydroxypropyl)amino]ethyl)oxime] (10). mp 195 °C. ¹H NMR (400 MHz, pyridine-*d*₅, δ ppm, *J* in Hz) 12.27 (2H, m, H-1, H-1'), 8.90 (1H, d, *J* = 8.8 Hz, H-4), 8.41 (1H, d, *J* = 7.5 Hz, H-4'), 7.46 (1H, dd, *J* = 8.8, 1.8 Hz, H-5), 7.38 (1H, d, *J* = 1.8 Hz, H-7), 7.36 (1H, t, *J* = 7.5 Hz, H-6'), 7.05 (2H, m, H-5', H-7), 4.80 (2H, t, *J* = 6.1 Hz, H-1''), 4.29 (1H, m, H-4''), 4.11 (1H, dd, *J* = 11.0, 4.6 Hz, H-5''a), 4.04 (1H, dd, *J* = 11.0, 5.5 Hz, H-5''b), 3.14 (2H, t, *J* = 6.1 Hz, H-2''), 2.93 (2H, m, H-3''), 2.49 (3H, s, -NCH₃). CI-MS *m/z* 487, 489 (M + H)⁺. Anal. (C₂₂H₂₃N₄O₄Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-piperazine-1-ylethyl)oxime] (11). mp 255 °C (dec). ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.69 (1H, s, H-1'), 10.92 (1H, s, H-1), 8.53 (1H, d, *J* = 8.5 Hz, H-4), 8.15 (1H, d, *J* = 7.4 Hz, H-4'), 7.43 (2H, m, H-6', H-7'), 7.14 (1H, d, *J* = 8.5 Hz, H-5), 7.03 (2H, m, H-5', H-7), 4.69 (2H, br t, H-1''), 2.83 (2H, br t, H-2''), 2.71 (4H, brs, H-4'', H-5''), 2.46 (4H, brs, H-3'', H-6''), partially overlapped with DMSO. APCI-MS (+) *m/z* 468, 470 (M + H)⁺. Anal. (C₂₂H₂₂N₅O₂Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-(4-methylpiperazin-1-yl)ethyl)oxime] (12). mp 222 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.68 (1H, s, H-1'), 10.90 (1H, s, H-1), 8.40 (1H, d, *J* = 8.5 Hz, H-4), 8.14 (1H, d, *J* = 7.7 Hz, H-4'), 7.42 (2H, m, H-6', H-7'), 7.13 (1H, dd, *J* = 8.5/1.9 Hz, H-5), 7.04 (1H,

m, H-5'), 7.02 (1H, d, *J* = 1.9 Hz, H-7), 4.68 (2H, t, *J* = 5.9 Hz, H-1''), 2.85 (2H, t, *J* = 5.9 Hz, H-2''), 2.50 (4H, brs, H-3'', H-6''), overlapped with DMSO, 2.31 (4H, brs, H-4'', H-5''), 2.13 (3H, s, -NCH₃). APCI-MS (+) *m/z* 482, 484 (M + H)⁺. Anal. (C₂₃H₂₄N₅O₂Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-[4-(2-hydroxyethyl)piperazin-1-yl]ethyl)oxime] (13). mp 187 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 8.52 (1H, d, *J* = 8.5 Hz, H-4), 8.16 (1H, d, *J* = 7.6 Hz, H-4'), 7.43 (2H, m, H-6', H-7'), 7.13 (1H, dd, *J* = 8.5/1.8 Hz, H-5), 7.05 (1H, m, H-5'), 7.02 (1H, d, *J* = 1.8 Hz, H-7), 4.69 (2H, t, *J* = 5.7 Hz, H-1''), 3.45 (2H, t, *J* = 6.3 Hz, H-8''), 2.85 (2H, t, *J* = 5.7 Hz, H-2''), 2.50 (4H, H-3'', H-6''), overlapped with DMSO, 2.42 (4H, H-4'', H-5''), 2.34 (2H, t, *J* = 6.3 Hz, H-7''). APCI-MS (+) *m/z* 512, 514 (M + H)⁺. Anal. (C₂₄H₂₆N₅O₃Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-[4-(2-methoxyethyl)piperazin-1-yl]ethyl)oxime] (14). mp 184 °C. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.70 (1H, s, H-1'), 10.90 (1H, s, H-1), 8.50 (1H, d, *J* = 8.5 Hz, H-4), 8.16 (1H, d, *J* = 7.6 Hz, H-4'), 7.44 (2H, m, H-6', H-7'), 7.15 (1H, dd, *J* = 8.5, 1.7 Hz, H-5), 7.07 (2H, m, H-5', H-7), 4.70 (2H, t, *J* = 5.6 Hz, H-1''), 3.40 (2H, H-8'', overlapped with water), 3.21 (3H, s, -OCH₃), 2.87 (2H, brt, H-2''), 2.66–2.40 (H-4'', H-5'', H-3'', H-6'', H-7'', overlapped with DMSO). APCI-MS (+) *m/z* 526, 528 (M + H)⁺. Anal. (C₂₅H₂₈N₅O₃Br) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-[4-[2-(2-hydroxyethoxy)-ethyl]piperazin-1-yl]ethyl)oxime] (15). mp 183 °C. ¹H NMR (400 MHz, pyridine-*d*₅, δ ppm, *J* in Hz) 12.31 (1H, s, H-1'), 12.25 (1H, s, H-1), 8.89 (1H, d, *J* = 8.3 Hz, H-4), 8.39 (1H, d, *J* = 7.9 Hz, H-4'), 7.45–7.33 (3H, m, H-5, H-7, H-6'), 7.09 (2H, m, H-5', H-7), 4.78 (2H, t, *J* = 5.8 Hz, H-1''), 3.96 (2H, t, *J* = 5.0 Hz, H-10''), 3.70 (2H, t, *J* = 5.0 Hz, H-9''), 3.66 (2H, t, *J* = 5.8 Hz, H-8''), 2.94 (2H, t, *J* = 5.8 Hz, H-2''), 2.68 (2H, brs, H-3'', H-6''), 2.57 (8H, t, *J* = 5.8 Hz, H-4'', H-5'', H-7''). APCI-MS (+) *m/z* 556, 558 (M + H)⁺. Anal. (C₂₆H₃₀N₅O₄Br) C, H, N.

General Procedure for the Preparation of the Amine Salts 16–26. The appropriate indirubin derivative 5–15 (0.10 mmol) was dissolved in anhydrous THF (50 mL) and 0.2 mL of a saturated solution of hydrochloric acid in ether was added dropwise. The reaction mixture was left to cool in an ice bath and the precipitate formed was collected by filtration.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-dimethylaminoethyl)oxime] Hydrochloride (16). *S_w* (g/L) 0.141. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.70 (1H, s, H-1'), 10.97 (1H, s, H-1), 8.49 (1H, d, *J* = 8.3 Hz, H-4), 8.22 (1H, *J* = 7.4 Hz, H-4'), 7.46 (2H, m, H-7, H-6'), 7.20 (1H, dd, *J* = 8.3/1.7 Hz, H-5), 7.05 (2H, m, H-5', H-7), 4.95 (2H, brs, H-1''), 3.58 (2H, m, H-2''), 2.81 (6H, brs, -N(CH₃)₂). Anal. (C₂₀H₂₀N₄O₂BrCl) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-diethylaminoethyl)oxime] Hydrochloride (17). *S_w* (g/L) 0.192. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.70 (1H, s, H-1'), 10.98 (1H, s, H-1), 8.49 (1H, d, *J* = 8.6 Hz, H-4), 8.21 (1H, d, *J* = 7.4 Hz, H-4'), 7.47 (2H, m, H-7, H-6'), 7.21 (1H, dd, *J* = 8.6/1.9 Hz, H-5), 7.09–7.04 (2H, m, H-5', H-7), 5.00 (2H, brs, H-1''), 3.58 (2H, brs, H-2''), 3.24 (4H, brs, -N(CH₂CH₃)₂), 1.21 (6H, t, *J* = 7.0 Hz, -N(CH₂CH₃)₂). Anal. (C₂₂H₂₄N₄O₂BrCl) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-pyrrolidin-1-ylethyl)oxime] Hydrochloride (18). *S_w* (g/L) 0.195. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.71 (1H, s, H-1'), 10.97 (1H, s, H-1), 8.48 (1H, d, *J* = 8.6 Hz, H-4), 8.22 (1H, d, *J* = 7.4 Hz, H-4'), 7.44–7.52 (2H, m, H-7, H-6'), 7.20 (1H, dd, *J* = 8.6/1.9 Hz, H-5), 7.07 (2H, m, H-5', H-7), 4.94 (2H, brs, H-1''), 3.64 (2H, brs, H-2''), 3.13 (4H, m, H-3'', H-6''), 2.02 (4H, m, H-4'', H-5''). Anal. (C₂₂H₂₂N₄O₂BrCl) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-morpholin-1-ylethyl)oxime] Hydrochloride (19). ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.69 (1H, s, H-1'), 10.98 (1H, s, H-1), 8.47 (1H, d, *J* = 8.5 Hz, H-4), 8.22 (1H, d, *J* = 7.8 Hz, H-4'), 7.45 (2H, m, H-7, H-6'), 7.21 (1H, dd, *J* = 8.3, 1.8 Hz, H-5), 7.06 (2H, m, H-5', H-7), 5.05 (2H, brs, H-1''), 3.95 (2H, m, H-2''), 3.75 (4H, m, H-4'',

H-5''), 3.27 (4H, H-3'', H-6'', overlapped with water). Anal. (C₂₂H₂₂N₄O₃BrCl) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-(N,N-(2-hydroxyethyl)aminoethyl)oxime)] (20). ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.71 (1H, s, H-1'), 10.98 (1H, s, H-1), 8.48 (1H, d, *J* = 8.3 Hz, H-4), 8.22 (1H, d, *J* = 7.9 Hz, H-4'), 7.46 (2H, m, H-7, H-6'), 7.21 (1H, dd, *J* = 8.3/1.8 Hz, H-5), 7.06 (2H, m, H-5', H-7'), 5.35 (2H, brs, OH) = 5.03 (2H, brs, H-1''), 3.84 (2H, brs, H-2''), 3.78 (4H, brs, -N(CH₂CH₂OH)₂), 3.38 (4H, m, -N(CH₂CH₂OH)₂, overlapped with water). Anal. (C₂₂H₂₄N₄O₄BrCl) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-(O-[2-[N-methyl,N-(2,3-dihydroxypropyl)amino]ethyl]oxime) Hydrochloride (21). *S_w* (g/L) 1.45. ¹H NMR (400 MHz, DMSO-*d*₆, δ ppm, *J* in Hz) 11.69 (1H, s, H-1'), 10.96 (1H, s, H-1), 8.48 (1H, d, *J* = 8.5 Hz, H-4), 8.20 (1H, d, *J* = 7.9 Hz, H-4'), 7.4 (2H, m, H-7, H-6'), 7.19 (1H, dd, *J* = 8.5/1.8 Hz, H-5), 7.05 (2H, m, H-5', H-7'), 4.95 (2H, brs, H-1''), 3.89 (1H, brs, H-4''), 3.38 (4H, H-3'', H-5'', overlapped with water), 2.83 (2H, brs, H-3''), 2.50 (3H, -N(CH₃), overlapped with DMSO). Anal. (C₂₂H₂₄N₄O₄BrCl) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-piperazine-1-yl-ethyl)oxime] Dihydrochloride (22). *S_w* (g/L) 1.61. ¹H NMR (400 MHz, D₂O, δ ppm, *J* in Hz) 7.65 (1H, d, *J* = 8.5 Hz, H-4), 7.55 (1H, d, *J* = 7.5 Hz, H-4'), 7.26 (1H, t, *J* = 7.5 Hz, H-6'), 6.85 (1H, t, *J* = 7.2 Hz, H-5'), 6.76 (1H, d, *J* = 8.5 Hz, H-5), 6.72 (1H, d, *J* = 7.5 Hz, H-7'), 6.54 (1H, s, H-7), 4.42 (2H, brt, H-1''), 3.38 (4H, brt, H-4'', H-5''), 3.11 (6H, brs, H-3'', H-6'', H-2''). Anal. (C₂₂H₂₄N₅O₂BrCl₂) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-[2-(4-methylpiperazin-1-yl)ethyl]oxime] Dihydrochloride (23). *S_w* (g/L) 1.50. ¹H NMR (400 MHz, D₂O, δ ppm, *J* in Hz) 7.63 (1H, d, *J* = 8.2 Hz, H-4), 7.53 (1H, d, *J* = 7.5 Hz, H-4'), 7.25 (1H, t, *J* = 7.6 Hz, H-6'), 6.84 (1H, t, *J* = 7.6 Hz, H-5'), 6.75 (1H, d, *J* = 8.2 Hz, H-5), 6.70 (1H, d, *J* = 7.5 Hz, H-7'), 6.53 (1H, s, H-7), 4.39 (2H, brs, H-1''), 3.39 (4H, brs, H-3'', H-6''), 3.11 (6H, brs, H-2'', H-4'', H-5''), 2.90 (3H, s, -NCH₃). Anal. (C₂₃H₂₆N₅O₂BrCl₂) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-(O-[2-[4-(2-hydroxyethyl)piperazin-1-yl]ethyl]oxime) Dihydrochloride (24). *S_w* (g/L) 1.14. ¹H NMR (400 MHz, D₂O, δ ppm, *J* in Hz) 7.68 (1H, d, *J* = 8.5 Hz, H-4), 7.56 (1H, d, *J* = 7.4 Hz, H-4'), 7.27 (1H, brt, *J* = 7.2 Hz, H-6'), 6.85 (1H, brt, *J* = 7.2 Hz, H-5'), 6.78 (1H, d, *J* = 8.5 Hz, H-5), 6.73 (1H, d, *J* = 7.4 Hz, H-7'), 6.57 (1H, s, H-7), 4.42 (2H, brs, H-1''), 3.89 (2H, brs, H-8''), 3.39 (4H, brs, H-3'', H-6''), 3.26 (2H, brs, H-7''), 3.12 (6H, brs, H-2'', H-4'', H-5''). Anal. (C₂₄H₂₈N₅O₃BrCl₂) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-(O-[2-[4-(2-methoxyethyl)piperazin-1-yl]ethyl]oxime) Dihydrochloride (25). *S_w* (g/L) 0.57. ¹H NMR (400 MHz, D₂O, δ ppm, *J* in Hz) 7.70 (1H, brs, H-4), 7.61 (1H, brs, H-4'), 7.28 (1H, brt, *J* = 7.2 Hz, H-6'), 6.88 (1H, brt, *J* = 7.5 Hz, H-5'), 6.77 (2H, brs, H-5, H-7'), 6.58 (1H, s, H-7), 4.52 (2H, brs, H-1''), 3.74 (2H, brs, H-8''), 3.55–3.22 (12H, H-2'', H-3'', H-4'', H-5'', H-6'', H-7''), 3.35 (3H, s, -OCH₃). Anal. (C₂₅H₃₀N₅O₃BrCl₂) C, H, N.

Data for (2'Z-3'E)-6-Bromoindirubin-3'-[O-(2-[4-[2-(2-hydroxyethoxy)-ethyl]piperazin-1-yl]ethyl]oxime) Dihydrochloride (26). *S_w* (g/L) 4.253. ¹H NMR (400 MHz, D₂O, δ ppm, *J* in Hz) 7.54 (1H, d, *J* = 8.1 Hz, H-4), 7.44 (1H, d, *J* = 7.2 Hz, H-4'), 7.21 (1H, brt, *J* = 7.6 Hz, H-6'), 6.78 (1H, brt, *J* = 7.2 Hz, H-5'), 6.69 (1H, d, *J* = 7.2 Hz, H-7'), 6.61 (1H, d, *J* = 8.1 Hz, H-5), 6.34 (1H, s, H-7), 4.28 (2H, brs, H-1''), 3.82, 3.72, 3.63, (6H, H-8'', H-9'', H-10''), 3.46–2.72 (12H, H-2'', H-3'', H-4'', H-5'', H-6'', H-7''). Anal. (C₂₆H₃₂N₅O₄BrCl₂) C, H, N.

Molecular Modeling. All compounds were designed and minimized. Compounds carrying nitrogen in their side chain were designed in their free and protonated states as well. Ligand partial charges were calculated in a semiempirical level with the AM1 Hamiltonian using MOPAC6.⁴² The receptor was prepared from the PDB entry of 6BIO bound to GSK-3β (1UV5). Protons were added, and all crystallographic water molecules were deleted except waters 47 and 79, which mediate the interaction of the oxime with thr138 and water 1, which is buried in the cavity.²⁸ The kinase

was minimized in order to relieve the crystallographic strain in the absence of the ligand and then all molecules were manually docked in the active site by superimposition on crystallographic 6BIO coordinates. Docking was performed with the Monte Carlo Multiple Minimum search algorithm as implemented in MacroModel 9 software.⁴³ The three key hydrogen bonds of each complex were restrained at their crystallographic values by application of harmonic distance and angle constraints. Force constraints were applied on waters 49 and 79. The remaining atoms within 6 Å around the ligand were free to move. All calculations were performed with a truncated Newton conjugate gradient minimizer, a convergence criterion of 0.05 kJ/Å-mol, the AMBER* forcefield, including protein partial charges, and the GB/SA implicit solvent model. Values of LogD and pK_a were calculated by Pallas 3.1 software.

Biology. Kinase Preparation and Assays. Kinase activities were assayed in buffer A (10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 25 mM Tris-HCl pH 7.5, 50 μg heparin/mL) or C (homogenization buffer but 5 mM EGTA, no NaF and no protease inhibitors), at 30 °C, at a final ATP concentration of 15 μM. Blank values were subtracted and activities were expressed in % of the maximal activity, i.e., in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide. Phosphorylation of the substrate was assessed by the P81 phosphocellulose binding assay.

CDK1/cyclin B was extracted in homogenization buffer (60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg leupeptin/mL, 10 μg aprotinin/mL, 10 μg soybean trypsin inhibitor/mL, and 100 μM benzamide) from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on p9^{CKShs1}-sepharose beads, from which it was eluted by free p9^{CKShs1} as previously described.²⁵ The kinase activity was assayed in buffer C, with 1 mg histone H1/mL, in the presence of 15 μM [γ-³³P] ATP (3000 Ci/mmol; 10 mCi/mL) in a final volume of 30 μL. After 30 min of incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto 2.5 cm × 3 cm pieces of Whatman P81 phosphocellulose paper and, 20 s later, the filters were washed five times (for at least 5 min each time) in a solution of 10 mL of phosphoric acid/liter of water. The wet filters were counted in the presence of 1 mL of ACS (Amersham) scintillation fluid.

CDK5/p25 was reconstituted by mixing equal amounts of recombinant human 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. L.H. Tsai) (p25 is a truncated version of p35, the 35 kDa CDK5 activator). Its activity was assayed with histone H1 in buffer C as described for CDK1/cyclin B.

GSK-3α/β was purified from porcine brain by affinity chromatography on immobilized axin.⁴⁷ It was assayed, following a 1/100 dilution in 1 mg BSA/mL 10 mM DTT, with 4 μM GS-1 (YRRAAVPPSPSLSRHSSPHQSpEDEEE), a GSK-3 specific substrate obtained from Millegen (Labege, France), in buffer A, in the presence of 15 μM [γ-³³P] ATP (3000 Ci/mmol; 10 mCi/mL) in a final volume of 30 μL. After 30 min of incubation at 30 °C, 25 μL aliquots of supernatant were processed as described above.

Cellular Assays. Cell Culture Conditions and Cell Survival Assessment. SH-SY5Y human neuroblastoma cell line (kindly provided by Dr. Jacint Boix, Lleida) was grown at 37 °C with 5% CO₂ in DMEM supplemented with 2 mM L-glutamine (Invitrogen, Cergy Pontoise, France), plus antibiotics (penicillin–streptomycin) from Lonza, and a 10% volume of fetal calf serum (FCS) (Invitrogen). Drug treatments were performed on exponentially growing cultures at the indicated time and concentrations. Control experiments were carried also using appropriate dilutions of DMSO. Cell viability was determined by means of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method after 48 h of treatment as previously described.⁴⁸

β -Catenin Phosphorylation in SH-SY5Y Human Neuroblastoma Cells. Nearly confluent SH-SY5Y human neuroblastoma cells were grown in 96 plates in DMEM (supplemented with 10% FCS and antibiotics). Cells were cotreated with tested compounds and 2 μ M MG132 (to allow accumulation of phosphorylated β -catenin) for 6 h. Final DMSO concentration did not exceed 1%. Cells were then subjected to an ELISA assay using antibodies directed against Ser33/Ser37/Thr41-phosphorylated (1:1000) β -catenin obtained from Cell Signaling Technology. Results are expressed in percentage of maximal β -Catenin phosphorylation, i.e., in untreated cells exposed to MG132 only as positive control (100% phosphorylation).

Cell Culture and Luminescence Assay of Circadian Rhythmicity. These experiments used Rat-1 fibroblasts that have been stably transfected with a $P_{Per2}::Fluc$ reporter construct that shows a robust circadian rhythm of luminescence as a gauge of clock-controlled $Per2$ promoter (P_{Per2}) activity.⁴⁶ The cells were cultured in DMEM (11965-092, GIBCO/Invitrogen) supplemented with 5% FBS, 50 units/mL penicillin, and 50 μ g/mL streptomycin in a 5% CO_2 incubator at 37 °C. Approximately 5×10^5 cells were seeded in a 35 mm dish at least 5 days before the experiment. Three days after the cells reached 100% confluence, the cells were treated with 0.1 μ M dexamethasone (Sigma) for 1 h to synchronize the oscillators among the cells in the population. At the end of the treatment, the medium was replaced with assay medium (DMEM without phenol red, supplemented with bicarbonate (350 mg/L), 5% FBS, 10 mM HEPES (pH 7.2), antibiotics (25 units/mL penicillin, 25 μ g/mL streptomycin), and 0.1 mM luciferin (Promega)). Culture dishes were sealed with a 40 mm microscope glass coverslip and high-vacuum grease to prevent the evaporation of culture medium. The luminescence rhythm was monitored in a LumiCycle (Actimetrics Inc., Evanston, IL). Before being sealed, drugs were added to the culture dishes to different final concentrations and left continuously with the cells thereafter while the luminescence patterns were recorded for 5 days or more. DMSO was used as a solvent control. Regression analyses to determine period and phase of the luminescence rhythms were performed with the Chrono II program (courtesy of Dr. Till Roenneberg).

Electrophoresis and Western blotting. Cells were resuspended, lysed for 30 min at 4 °C in homogenization buffer, and sonicated. After centrifugation (14000 rpm for 15 min at 4 °C), the protein concentration was determined in the supernatants by the Bradford protein assay (Bio-Rad). Following heat denaturation for 5 min, proteins were separated by 10% NuPAGE precast Bis-Tris acetate polyacrylamide mini gel (Invitrogen) with MOPS SDS running buffer. Proteins were transferred to 0.45 μ m nitrocellulose filters (Schleicher and Schuell). These were blocked with 5% low fat milk in Tris-buffered saline-Tween-20, incubated overnight at 4 °C with antibodies directed against Ser33/Ser37/Thr41-phosphorylated β -catenin (1:1000) (Cell Signaling Technology), and analyzed by enhanced chemiluminescence (ECL, Amersham).

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Supporting Information Available: Elemental analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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