Indirubins Inhibit Glycogen Synthase Kinase-3β and CDK5/P25, Two Protein Kinases Involved in Abnormal Tau Phosphorylation in Alzheimer’s Disease

A PROPERTY COMMON TO MOST CYCLIN-DEPENDENT KINASE INHIBITORS?**

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Sophie Leclerc‡, Matthieu Garnier‡, Ralph Hoessel§, Doris Marko§, James A. Bibb¶, Gretchen L. Snyder¶, Paul Greengard¶, Jacek Biernat*, Yong-Zhong Wu*, Eva-Maria Mandelkow*, Gerhard Eisenbrand*, and Laurent Meijer‡ **

From the ‡CNRS, Cell Cycle Group, Station Biologique, BP 74, Roscoff 29682 Cedex, Bretagne, France, the §Department of Chemistry, Division of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Erwin-Schrödinger-Strasse 52, Kaiserslautern 67663, Germany, the ¶Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York 10012, and the ¶¶Max-Planck Unit for Structural Molecular Biology, Notkestrasse 85, Hamburg D-22603, Germany

The bis-indole indirubin is an active ingredient of Danggui Longhui Wan, a traditional Chinese medicine recipe used in the treatment of chronic diseases such as leukemias. The antitumoral properties of indirubine appear to correlate with their antimetotic effects. Indirubins were recently described as potent (IC50 50–100 nM) inhibitors of cyclin-dependent kinases (CDKs). We report here that indirubins are also powerful inhibitors (IC50 5–50 nM) of an evolutionarily related kinase, glycogen synthase kinase-3β (GSK-3β). Testing of a series of indoles and bis-indoles against GSK-3β, CDK1/cyclin B, and CDK5/p25 shows that only indirubins inhibit these kinases. The structure-activity relationship study also suggests that indirubins bind to GSK-3β’s ATP binding pocket in a way similar to their binding to CDKs, the details of which were recently revealed by crystallographic analysis. GSK-3β, along with CDK5, is responsible for most of the abnormal hyperphosphorylation of the microtubule-binding protein tau observed in Alzheimer’s disease. Indirubin-3’-monoxime inhibits tau phosphorylation in vitro and in vivo at Alzheimer’s disease-specific sites. Indirubins may thus have important implications in the study and treatment of neurodegenerative disorders. Indirubin-3’-monoxime also inhibits the in vivo phosphorylation of DARPP-32 by CDK5 on Thr-75, thereby mimicking one of the effects of dopamine in the striatum. Finally, we show that many, but not all, reported CDK inhibitors are powerful inhibitors of GSK-3β. To which extent these GSK-3β effects of CDK inhibitors actually contribute to their antimitic and antitumoral properties remains to be determined. Indirubins constitute the first family of low nanomolar inhibitors of GSK-3β to be described.

Indigoids are bis-indoles derived from various natural sources by fermentation, oxidation, and dimerization in the presence of light. The colorful indirubin (1) and indigo (5) originate from the dimerization of colorless precursors, indoxyl and isatin (4) (see Fig. 1). These indoles are released during the fermentation process from conjugates, the nature of which depends on the plant (indican, isatan B) or mollusc (indoxylsulfate) species from which the dyes are prepared (see Fig. 1). The use of indigoids as textile dyes dates back to the Bronze age (~7000), but indigo (now synthetic) remains the most abundantly produced dye in the world (blue jeans, denims, etc.) (1, 2). Indigo-producing plants have also been used in traditional Chinese medicine (3–5). A well-studied example is Danggui Longhui Wan, a mixture of 11 herbal medicines traditionally utilized against certain types of leukemias. Only one of these ingredients, Qing Dai (Indigo naturalis), a dark blue powder originating from various indigo-producing plants, was found to carry the antileukemic activity (6). Although it is mostly constituted of indigo, a minor constituent, indirubin, was identified as the active component by the Chinese Academy of Medicine (7–9). Preclinical studies performed with indirubin, and more soluble analogues, confirmed that these compounds exhibit good antitumor activity and only minor toxicity (10–14). Several mechanisms of action have been brought forward to explain the antimitotic and antitumoral properties of indirubins (22–24). We recently reported that indirubins are potent inhibitors of cyclin-dependent kinases (CDKs) (25), a family of key cell cycle regulators (26–28). Indirubins act by competing with ATP for binding to the catalytic site of the kinase. The kinase selectivity study showed that indirubins have a strong affinity for CDKs (IC50 values in the range of 50–100 nM) (25). Nevertheless, they are not totally devoid of activity toward a few kinases (IC50 values in the 1–10 μM range) (25). This rather loose selectivity, when compared with the high specificity of purine inhibitors of CDKs, led us to continue to investigate the selectivity of indirubins as kinase inhibitors.

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** To whom correspondence should be addressed: Tel.: 33-29-82-92-339; Fax: 33-29-82-92-342; E-mail: meijer@sb-roscoff.fr.

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Indirubins Inhibit GSK-3β and CDK5/p25

We report here that indirubins are very potent inhibitors (IC_{50} values in the 5–50 nM range) of glycogen synthase kinase-3β (GSK-3β). This kinase is an essential element of the WNT signaling pathway (29). It is involved in multiple physiological processes, including cell cycle regulation by controlling the levels of cyclin D1 (30) and β-catenin (31), dorsal-ventral patterning during development (31–33), insulin action on glycogen synthesis (34, 35), axonal outgrowth (36), HIV-1 Tat-mediated neurotoxicity (37), among others. Furthermore, GSK-3β and CDK5 are responsible for most of the abnormal hyperphosphorylation of the microtubule-binding protein tau observed in the paired helical filaments, which are diagnostic for Alzheimer’s disease (AD) (38, 39). It was recently demonstrated that conversion of p35, the regulatory subunit of CDK5, and CDK5/p25 are very potent inhibitors of CDK5/p25. Furthermore, indirubins are derived from the dimerization of indoxyls and isatins, which are themselves derived from the hydrolysis of either indican and isatans (plants) or indoxyl sulfates (molluscs).

FIG. 1. The bis-indoles indigo (5), indirubin (1), and isoindigo (40) are derived from the dimerization of indoxyls and isatins, which are themselves derived from the hydrolysis of either indican and isatans (plants) or indoxyl sulfates (molluscs).

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Hypotonic Lysis Buffer (HLB)—HLB buffer consisted of 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 10% glycerol, 1% Nonidet-P40, 5 mM DTT, 1 mM EGTA, 20 mM NaF, 1 mM orthovanadate, 5 μM microcystin, and 100 μg/ml each of leupeptin, aprotinin, and pepstatin.

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 mM. Blank values were subtracted, and activities were calculated as picomoles of phosphate incorporated for a 10-min incubation. The activities are usually expressed in percentage of the maximal activity, i.e. in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethyl sulfoxide. In a few cases phosphorylation of the substrate was assessed by autoradiography after SDS-PAGE (see below).

GSK-3β

GSK-3β was expressed in and purified from insect Sf9 cells (42). It was assayed, following a 1/100 dilution in 1 mg/ml BSA, 10 mM DTT, with 5 μl of 40 μM GS-1 peptide as a substrate, in buffer A, in the presence of 15 μM [γ-32P]ATP (3000 Ci/mmol; 1 mCi/ml) in a final volume of 30 μl. After 30-min incubation at 30 °C, 25-μl aliquots of supernatant were spotted onto 2.5- × 3-cm pieces of Whatman P81

**TABLE I**

Structure of the indoles and bis-indoles used in this study

| N° | Indirubin | 3' | 5' | 1 | 5 | 6 |
|----|-----------|----|----|---|---|---|
| 1  | indirubin | -  | -  | - | - | - |
| 4  | 5-iodindirubin | -  | -  | I | - | - |
| 5  | 5-bromindirubin | -  | -  | Br| - | - |
| 6  | 5-chlorindirubin | -  | Cl | - | - | - |
| 7  | 5-fluorindirubin | -  | F  | - | - | - |
| 8  | 5-methylindirubin | -  | -  | CH₃| - | - |
| 9  | 5-nitroindirubin | -  | -  | NO₂| - | - |
| 10 | indirubin-5-sulfonic acid | -  | -  | - | SO₃H | - |
| 11 | 5-bromindirubin | -  | -  | Br| - | - |
| 12 | 5-bromindirubin-5-sulfonic acid | -  | -  | CH₃ | - | - |
| 13 | indirubin-3'-monoxime | =N-OH | -  | - | - | - |
| 14 | 5-iodindirubin-3'-monoxime | =N-OH | -  | - | 1 | - |
| 15 | 6-iodindirubin | -  | -  | - | - | 1 |
| 16 | 1-methylindirubin | -  | -  | - | - | - |
| 17 | 1-phenylindirubin | -  | -  | - | - | - |
| 18 | indirubin-3'-monoxime-5-sulfonic acid | =N-OH | -  | - | SO₃Na | - |
| 19 | indirubin-5-sulfonamide | -  | -  | - | SO₂NH₂ | - |
| 20 | indirubin-5-sulfonic acid dimethylamide | -  | -  | - | SO₂N(CH₃)₂ | - |
| 21 | indirubin-5-sulfonic acid (2-hydroxyethyl)amide | -  | -  | - | SO₂N-(CH₂)OH | - |
| 22 | indirubin-5-sulfonic acid bis-(2-hydroxyethyl)amide | -  | -  | - | SO₂N-(CH₂)OH | - |
| 23 | indirubin-5-sulfonic acid methylimide | -  | -  | - | SO₂NCH₃ | - |
| 24 | indirubin-5-sulfonic acid | -  | -  | - | SO₃Na | - |

| N° | Indigo | 5 | 7 | 5' | 7' |
|----|--------|---|---|----|----|
| 2  | indigo | - | - | - | - |
| 25 | 5,5,7,7'-indigotetrasulfonic acid (potassium salt) | SO₃K | SO₃K | SO₃K | SO₃K |
| 26 | 5,5,7,7'-indigotetrasulfonic acid (potassium salt) | SO₃K | SO₃K | SO₃K | SO₃K |
| 27 | indigo carmine | SO₃Na | SO₃Na | - | - |

| N° | Isatin | 1 | 5 | 6 |
|----|--------|---|---|---|
| 3  | isatin | - | - | - |
| 28 | 5-iodoisatin | - | - | - |
| 29 | 5-fluorosatin | - | F | - |
| 30 | 5-bromosatin | - | Br | - |
| 31 | 5-chlorosatin | - | Cl | - |
| 32 | 5-methylsatin | - | - | CH₃ | - |
| 33 | isatin-5-sulfonic acid, sodium salt | - | SO₃Na | | |
| 34 | 5-nitroisatin | - | NO₂ | - |
| 35 | 1-methylisatin | CH₃ | - | - |
| 36 | 1-phenylisatin | Phenyl | - | - |
| 37 | isatin-5-sulfonic acid dimethylamide | - | SO₃N(CH₃)₂ | - | - |
| 38 | isatin-5-sulfonic acid bis-(2-hydroxyethyl)amide | - | SO₃N-(CH₂)OH | - | - |
| 39 | 6-iodoisatin | - | - | 1 | - |

**3-indoxyl acetate (X=H)** (43)

5-bromo-3-indoxylacetate (X=Br) (44)
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 Pharmacia Biotech) scintillation fluid.

**CDK1/Cyclin B**—CDK1/cyclin B was extracted in homogenization buffer from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on p9CKShs1-Sepharose beads, from which it was eluted by free p9 CKShs1 as described previously (43, 44). The kinase activity was assayed in buffer C, with 1 mg/ml histone H1, in the presence of 15 μM [γ-32P]ATP (3000 Ci/mmol; 1 mCi/ml) in a final volume of 30 μl. After 10-min incubation at 30 °C, 25-μl aliquots of supernatant were spotted onto P81 phosphocellulose papers and treated as described above.

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

### Table II

**Inhibition of GSK-3β, CDK1, and CDK5 by indoles and bis-indoles**

Numbers refer to structures shown in Table I. Enzyme activities were assayed as described under “Experimental Procedures,” in the presence of increasing concentrations of indole derivatives. IC\(_{50}\) values were calculated from the dose-response curves. ≤0.01 μM (solid black), 0.01–0.1 μM (dark gray), 0.1–1 μM (medium gray), 1–10 μM (light gray), >10 μM (white).

| No. | Compounds | GSK3 | CDK1 | CDK5 |
|-----|-----------|------|------|------|
| 15  | 5-iodoirubin-3'-monoxime | 0.009 | 0.025 | 0.020 |
| 14  | indirubin-3'-monoxime | 0.022 | 0.18 | 0.10 |
| 22  | indirubin-5-sulfonic acid (2-hydroxyethyl)-amide | 0.033 | 0.065 | 0.050 |
| 20  | indirubin-5-sulfonamide | 0.040 | 0.11 | 0.075 |
| 9   | 5-nitroindirubin | 0.042 | 0.25 | 0.38 |
| 6   | 5-chloroindirubin | 0.050 | 0.28 | 0.23 |
| 5   | 5-bromoindirubin | 0.055 | 0.23 | 0.25 |
| 8   | 5-methylindirubin | 0.062 | 0.28 | 0.21 |
| 4   | 5-iodoindirubin | 0.068 | 0.22 | 0.20 |
| 7   | 5-fluoroindirubin | 0.078 | 0.35 | 0.25 |
| 19  | indirubin-3'-monoxime-5-sulfonic acid | 0.080 | 0.005 | 0.007 |
| 24  | indirubin-5-sulfonic acid methylamide | 0.11 | 0.080 | 0.020 |
| 16  | 6-iodoindirubin | 0.13 | 0.80 | 1.5 |
| 21  | indirubin-5-sulfonic acid dimethylamide | 0.18 | 0.10 | 0.060 |
| 12  | 5'-dibromodindirubin | 0.25 | 600 | 200 |
| 10  | indirubin-5-sulfonic acid | 0.28 | 0.050 | 0.065 |
| 11  | 5'-bromodindirubin | 0.35 | 0.51 | 4 |
| 23  | indirubin-5-sulfonic acid bis-(2-hydroxyethyl)-amide | 0.40 | 0.15 | 0.15 |
| 1   | indirubin | 0.60 | 10 | 5.5 |
| 13  | 5'-bromo-indirubin-5-sulfonic acid | 4 | 0.080 | 0.075 |
| 38  | isatin-5-sulfonic acid bis-(2-hydroxyethyl)-amide | 29 | 600 | 800 |
| 43  | 3-indoxyl acetate | 70 | >1000 | >1000 |
| 39  | 6-iodoisatin | 75 | 600 | 800 |
| 37  | isatin-5-sulfonic acid-dimethylamide | 85 | >1000 | >1000 |
| 42  | 3,3'-diphenyl-2,2'-biindole | 180 | 500 | 500 |
| 18  | 1-phenylindirubin | 200 | 500 | 800 |
| 26  | 5,5',7'-indigotrisulfonic acid (potassium salt) | 280 | >1000 | >1000 |
| 34  | 5-nitroisatin | 310 | >1000 | >1000 |
| 40  | isoidigo | 320 | 40 | 130 |
| 25  | 5,5',7',7'-indigotetrasulfonic acid (potassium salt) | 350 | >1000 | >1000 |
| 27  | indigo carmine | 360 | 400 | >1000 |
| 41  | 2,2'-biindole | 380 | 700 | 300 |
| 44  | 5-bromoisindoxylacetate | 400 | >1000 | >1000 |
| 2   | indigo | 550 | >1000 | >1000 |
| 28  | 5-isoidigo | >1000 | 300 | 800 |
| 31  | 5-bromo-isatin | >1000 | 600 | >1000 |
| 17  | 1-methyl-indirubin | >1000 | >1000 | >1000 |
| 3   | isatin | >1000 | >1000 | >1000 |
| 29  | 5-fluoroisatin | >1000 | >1000 | >1000 |
| 30  | 5-chloroisatin | >1000 | >1000 | >1000 |
| 32  | 5-methylisatin | >1000 | >1000 | >1000 |
| 35  | 5-sulfonic acid, sodium salt dihydrate | >1000 | >1000 | >1000 |
| 36  | 1-phenylisatin | >1000 | >1000 | >1000 |

**In Vitro and in Vivo Tau Phosphorylation**

**Cells and Viruses—**Sf9 cells (Invitrogen, San Diego, CA) were grown at 27 °C in monolayer culture Grace’s medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum and 2.5 μg/ml amphotericin. BaculoGold was obtained from PharMingen (San Diego, CA), pVL1392 was obtained from Invitrogen.

**Tau Transfection—**The gene for htau23, the shortest human tau isoform, was excised from the bacterial expression vector pNO2 (45) with XhoI and BamHI and inserted into the baculovirus transfer vector.
pVL1392 cut with the same restriction endonucleases. The BaculoGold system was used to construct the tau baculovirus-containing vector. The BaculoGold DNA is a modified type of baculovirus containing a lethal deletion. Cotransfection of the BaculoGold DNA with a complementing baculovirus transfer vector rescued the lethal deletion of this virus DNA and reconstituted viable virus particles carrying the htau23 coding sequence. Plasmid DNA used for transfections was purified using Qiagen cartridges (Hilden, Germany). Sf9 cells grown in monolayers (2 × 10^6 cells in a 60-mm cell culture dish) were cotransfected with baculovirus DNA (0.5 μg of BaculoGold DNA) and with vector derivatives of pVL1392 (2 μg) using a calcium phosphate coprecipitation method. The presence of recombinant protein was examined in the infected cells 5 days post-infection by SDS-PAGE and Western blotting.

**TREATMENT OF SF9 CELLS WITH KINASE INHIBITORS**—To determine the effects of aminopurvalanol and indirubin-3′-monoxime on tau phosphorylation, SF9 cells infected with baculovirus expressing htau23 protein were treated 36 h post-infection (when cells have already expressed levels of tau sufficient for the outgrowth of cell processes (46)) with 20

**FIG. 2. Comparisons of the inhibitory activity of indirubins on GSK-3β, CDK5/p25, and CDK1/cyclin B.** GSK-3β and CDKs were assayed using the GS-1 peptide or histone H1 as substrates, respectively, with 15 μM ATP and in the presence of increasing concentrations of indirubins. IC_{50} values toward each enzyme, determined graphically, were plotted against the IC_{50} values for the other two kinases. The dose-response curves for compounds 12, 14, 15, and 19 are presented in Fig. 3.

**FIG. 3. Inhibition of GSK-3β, CDK5/p25, and CDK1/cyclin B by indirubins.** GSK-3β and CDKs were assayed using the GS-1 peptide or histone H1 as substrates, respectively, with 15 μM ATP and in the presence of increasing concentrations of indirubins. Activity is presented as the percentage of maximal activity (no inhibitors). Dose-response are shown curves for the most active inhibitor toward GSK-3β (5-iodo-indirubin-3′-monoxime) (15) (A), the most GSK-3β-selective compound (5,5′-dibromoindirubin) (12) (B), the most CDK-selective compound (5-sulfonic acid-indirubin-3′-monoxime) (19) (C), and the most frequently used compound in the studies of indirubins’ cellular effects (indirubin-3′-monoxime) (14) (D).
Indirubins inhibit GSK-3β and CDK5/p25

In situ inhibition of CDK5 in the striatum

Adult mouse brain striatal slices were prepared using standard methodology (47). Following equilibration in Krebs' bicarbonate buffer oxygenated with continuous aeration (95% O2/5% CO2), slices were treated with various concentrations of indirubin-3'-monoxime or 10 μM roscovitine for 60 min or were left in Krebs' bicarbonate buffer for the same period of time. Slices were homogenized by sonication in boiling 1% SDS and 50 mM NaF. Protein concentrations were determined by the BCA method using a BSA standard curve. Equal amounts of protein (80 μg) were subjected to SDS-PAGE using a 15% acrylamide gel, electrophoretically transferred to nitrocellulose membrane, and immunoblotted with a phosphorylation state-specific antibody that selectively detects DARPP-32 phosphorylated at Thr-75 (41).

RESULTS

Indirubins inhibit GSK-3β and CDK5/p25—In the course of studying the CDK inhibitory properties of indirubin, we synthesized a series of indole derivatives and dimers (Table I). While further investigating the kinase inhibition selectivity of indirubin-3'-monoxime, the indirubin used in our cellular studies, we noticed that this compound was a powerful inhibitor of GSK-3β (see below). Our collection of indoles/bis-indoles was further evaluated for inhibition against purified GSK-3β, CDK5/p25, and CDK1/cyclin B. Kinase activities were assayed with an appropriate substrate (GSK-3β, GS1 peptide; CDKs, histone H1) in the presence of 15 μM ATP and increasing concentrations of compounds. IC50 values were calculated from the dose-response curves and are presented in Table II. The GSK-3β and CDK inhibition activity was limited to the indirubins family. Neither indigo nor isatin, and their derivatives, displayed a significant effect on any of the three kinases. To compare the effects of active compounds on GSK-3β and CDKs, the IC50 values toward each enzyme were plotted against the IC50 values for the other two kinases (Fig. 2). This analysis shows that the efficacies of indirubins toward CDK1 and CDK5 are closely related, whereas the efficacies toward GSK-3β and CDKs are less so. This probably reflects the closer evolutionary proximity between CDK1 and CDK5 compared with that between GSK-3β and CDKs (48). The dose-response curves for the most active compound on the three kinases (5-iodo-indirubin-3'-monoxime), the most GSK-3β-selective compound (5,5'-dibromoindirubin), the most CDK-selective compound (5-sulfonic acid-indirubin-3'-monoxime) and the most frequently used compound in the studies of indirubins' cellular effects (indirubin-3'-monoxime) are presented in Fig. 3.

Indirubins act by competition with ATP at the catalytic site—To investigate the mechanism of indirubins' action on GSK-3β, kinetic experiments were performed by varying both ATP levels (0.1, 0.15, 0.25, and 0.5 mM) and indirubin-3'-monoxime concentrations (0, 0.5, 1, 1.5, and 2 μM) (Fig. 4). Double-reciprocal plotting of the data suggests that indirubin-3'-monoxime acts as a competitive inhibitor of ATP binding. The apparent Kᵢ was 0.85 μM. The apparent Kᵢ was 110 μM. Because recombinant GSK-3β was used throughout this study, this preparation is likely to contain an unknown proportion of inactive, misfolded enzyme as well as some proteolytic degradation fragments. Therefore, we feel that Kᵢ and Kᵢ values are only estimates.

Indirubins inhibit in vitro and in vivo tau phosphorylation by GSK-3β—To confirm the inhibitory effects of indirubins on GSK-3β activity (assessed with a peptide substrate) we tested indirubin-3'-monoxime on the phosphorylation of a more physiological substrate, the microtubule-binding protein tau. Bacterially expressed recombinant human tau was indeed phosphorylated in vitro by GSK-3β, and this phosphorylation was inhibited in a dose-dependent manner by indirubin-3'-monoxime, with an IC50 value of around 100 nM (Fig. 5, A and B).

We next investigated the effect of indirubin-3'-monoxime on the phosphorylation of human tau23 expressed in S99 cells (Fig. 5C). Cells were left untreated (control) or exposed to 20 μM indirubin-3'-monoxime or aminopurvalanol, a CDK-selective inhibitor. Htau23 was resolved by SDS-PAGE followed by immunoblotting with various antibodies (Fig. 5D).

AT100 recognizes tau phosphorylated at Thr-212 and Ser-214; this reaction is highly specific for Alzheimer tau but occurs in S99 cells as well, provided both sites are phosphorylated (49). The epitope is formed by sequential phosphorylation, first of Thr-212 by GSK-3β, then of Ser-214 by PKA (49). Indirubin-3'-monoxime completely inhibits the phosphorylation of the AT100 epitope. The phosphorylation of these two sites may be indirectly dependent on CDK5, because aminopurvalanol, which is a very poor inhibitor of GSK-3β (Table III) or PKA (50), completely abolishes the AT100 epitope phosphorylation. AT8, AT180, and PHF-1 are specific for different phosphorylated SP or TP motifs, respectively, Ser-202 and Thr-205,
Indirubins Inhibit GSK-3β and CDK5/p25

**In vitro - GSK3**

| tau phosphorylation vs. indirubin-3'-monoxime concentration (nM) |
|---------------------------------------------------------------|
| % Tau phosphorylation                                         |
| 110                                                          |
| 100                                                          |
| 90                                                           |
| 80                                                           |
| 70                                                           |
| 60                                                           |
| 50                                                           |
| 40                                                           |
| 30                                                           |
| 20                                                           |
| 10                                                           |
| 0                                                            |

**Coomassie, K9JA, AT100, PHF-1, AT8, AT180**

**htau23 and htau40**

**D**

- **Tau-1 + P**
- **AT8 + P**
- **AT100 + P**
- **AT270 + P**
- **SP 265 + P**
- **TP 265 + P**
- **TP 252 + P**
- **AT 180 + P**
- **AT 231 + P**
- **SP 396 + P**
- **PHF-1 + P**
- **SMI 34 + P**

**Fig. 5. Indirubin-3'-monoxime inhibits tau phosphorylation by GSK-3β in vitro and in vivo.** A and B, bacterially expressed recombinant human tau was phosphorylated in vitro with GSK-3β in the presence of increasing indirubin-3'-monoxime concentrations and resolved by SDS-PAGE, followed by autoradiography (A) and quantification (B). C, Sf9 cells expressing htau23 were left untreated (control) or exposed to indirubin-3'-monoxime or aminopurvalanol for 3 h. Cell lysates (3 μg of htau23) were resolved by SDS-PAGE, stained with Coomassie Blue, or immunoblotted with various antibodies: K9JA (a pan-tau antibody) recognizes tau independently of phosphorylation; AT100 recognizes tau phosphorylated at Thr-212 and Ser-214, a highly specific reaction for Alzheimer tau; PHF-1 (phosphorylated Ser-396/Ser-404); AT8 (phosphorylated Ser-202/Thr-205); AT180 (phosphorylated Thr-231/Ser-235). D, diagram of tau isoforms, antibody epitopes, and preferred phosphorylation sites (as numbered in htau40, the longest human tau isoform). htau23 lacks the N-terminal inserts and the second repeat. The repeats are shown in light gray, the flanking regions are in the dark shade. Some antibody epitopes are indicated.

Thr-231 and Ser-235, and Ser-396 and Ser-404. The primary target of GSK3β is the PHF-1 site, followed by the AT-8 site (51). Indirubin-3'-monoxime moderately inhibits the phosphorylation of the PHF-1 epitope. Both inhibitors completely inhibit phosphorylation at the AT-8 epitope and partially at the AT180 epitope. These sites are efficiently phosphorylated by CDKs (e.g. cdk5) and to a lesser extent by GSK-3β.

Altogether the results suggest that indirubin-3'-monoxime inhibits both the CDKs and GSK3β.

**Indirubins Inhibit DARPP-32 Phosphorylation by CDK5 in Vivo—**The neuronal protein DARPP-32 has been recently identified as a physiological substrate of CDK5/p25 (41). When phosphorylated by this kinase on Thr-75, DARPP-32 becomes an inhibitor of PKA. Phosphorylation on this site does not occur in p35−/− tissue (41). To assess the ability of indirubin-3'-monoxime to inhibit CDK5 in the brain, slices of striatum (an area of the brain enriched in DARPP-32) were prepared and treated with different concentrations of indirubin-3'-monoxime or roscovitine (Fig. 6). Homogenates from these slices were then probed with a phosphorylation state-specific antibody that only detects DARPP-32 phosphorylated at Thr-75. Indirubin-3'-monoxime was able to inhibit phosphorylation of DARPP-32 in situ and in the same range of concentrations previously found to be effective for roscovitine (41).

**Many CDK Inhibitors Are Potent Inhibitors of GSK-3β—**

Intrigued by the efficiency of indirubins to inhibit both CDKs and GSK-3β, we decided to test the effects of reported CDK inhibitors on purified GSK-3β (Table III). The results confirmed the strong selectivity of the olomoucine/roscovitine/purvalanol series, which was essentially inactive on GSK-3β. The non-selective isopentenyladenine was active on both CDKs and GSK-3β. Butyrolactone-I was inactive on GSK-3β. Both staurosporine and its 7-hydroxy analogue (UCN-01), two rather non-selective kinase inhibitors, were found to inhibit GSK-3β quite efficiently (Table III; Fig. 7). The GSK-3β inhibitory properties of UCN-01 are particularly interesting, in view of the development of this compound as an anticancer agent (phase II trials). UCN-01 has been reported to inhibit various kinases, including protein kinase C (52), CDK2 (53), and the chk1 kinase (54). The well-studied flavopiridol is also in phase II as an antitumor agent (55). It inhibits both CDK1/2 and CDK4 (56). We here report that it also inhibits GSK-3β at similar concentrations (Table III; Fig. 7). The recently described paullones (57, 58) were also found to be excellent GSK-3β inhibitors.

Another recently described CDK inhibitor derived from marine sponges, hymenialdisine, is equally efficient on GSK-3β and CDKs (59). Finally, the fungi-derived CDK inhibitor toyocamycin (60) has also some GSK-3β inhibitory activity. In summary, except for butyrolactone and the purines, all reported CDK inhibitors are potent inhibitors of GSK-3β.

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Indirubins Inhibit GSK-3β

We have previously described the inhibition of CDKs by indirubin and analogues (25). In this article, we further investigated CDK5 inhibition and we also report that GSK-3β is an excellent target for indirubins. Other indigoids are inactive (Table II). CDK5 is very closely related to CDK1 and CDK2 (73% and 75% identity, respectively) (61). GSK-3β is one of the evolutionarily closest enzymes to the CDK family (42). We have confirmed that indirubin-3'-monoxime inhibits GSK-3β by competing with ATP for binding to the catalytic site (Fig. 4). The crystal structure of CDK2/indirubin-3'-monoxime shows that the inhibitor binds, through three hydrogen bonds, to the backbone atoms of Glu-81 and Leu-83, two residues located in the ATP-binding pocket of the enzyme (25). The corresponding amino acids are Glu and Cys in CDK5, and Asp and Val, in GSK-3β. These observations support the idea that indirubins bind to GSK-3β as they do to CDKs. This is further supported by the structure-activity relationship study (Table II, Fig. 2): good CDK inhibitors are good GSK-3β inhibitors. There are two noticeable exceptions, 5,5'-dibromoindirubin (12), which is quite active on GSK-3β but poorly active on CDKs, and 5-SO₃Na-indirubin-3'-monoxime (19), which is over 10-fold less active on GSK-3β than on CDKs (Fig. 2). Although the 5-SO₃Na substitution on indirubin-3'-monoxime stimulates the inhibitory activity toward CDKs, it does not seem to operate the same way with GSK-3β. In contrast, a 5-iodo substitution is very favorable for inhibitory activity toward GSK-3β. These two exceptions suggest that it might be

**Table III**

Many CDK inhibitors are good inhibitors of GSK-3β

Enzymes activities were assayed as described under “Experimental Procedures,” in the presence of increasing concentrations of the reported CDK inhibitors. IC₅₀ values were calculated from the dose-response curves and are expressed in μM. References refer to the first description of the compounds as CDK inhibitors. Compounds that were selective for CDKs are in light gray. Compounds that were equally active on GSK-3β and CDK1 or more active on GSK-3β are in dark gray.

| Compound                  | GSK-3β | CDK5/p25 | CDK1/Cyclin B | Reference on compound |
|---------------------------|--------|----------|---------------|-----------------------|
| olomoucine                | 100    | 3        | 7             | 81                    |
| roscovitine               | 130    | 0.16     | 0.45          | 43                    |
| purvalanol A              | 13     | 0.075    | 0.004         | 62                    |
| aminopurvalanol           | 13     | 0.02     | 0.033         | 50                    |
| butyrostatine-1           | 100    | 0.15     | 1.1           | 83                    |
| isopenetylidenine         | 60     | 90       | 55            | 82                    |
| staurosporine             | 0.015  | 0.004    | 0.005         | 82                    |
| UCN-01 (7-hydroxystaurosporine) | 0.07   | 0.15     | 0.06          | 53                    |
| flavopiridol              | 0.45   | 0.17     | 0.20          | 84                    |
| indirubin                 | 0.60   | 5.5      | 9             | 25                    |
| indirubin-3'-monoxime     | 0.022  | 0.10     | 0.18          | 25                    |
| kenpaullone               | 0.023  | 0.85     | 0.40          | 57                    |
| alsterpaullone            | 0.004  | 0.04     | 0.035         | 58                    |
| hynemialdisine            | 0.015  | 0.028    | 0.022         | 59                    |
| toxyacumin                | 3.5    | 1.2      | 0.50          | 60                    |

**Fig. 6.** DARPP-32 phosphorylation by CDK5 on Thr-75 is inhibited in vivo by indirubin-3'-monoxime. Striatum slices were incubated with 0, 1, 10, and 50 μM indirubin-3'-monoxime or 10 μM roscovitine for 60 min. The level of DARPP-32 phosphorylation on Thr-75 was monitored by Western blotting with a phosho-specific antibody (top panel) and estimated by quantification of the blots (bottom panel).

**Fig. 7.** Inhibition of GSK-3β by flavopiridol, UCN-01, and staurosporine. GSK-3β activity was assayed using the GS-1 peptide as substrate with 15 μM ATP and in the presence of increasing concentrations of inhibitors. Activity is presented as the percentage of maximal activity (no inhibitors).
possible to obtain indirubin derivatives highly selective for either GSK-3β or CDKs. The synthesis of compounds specific for either CDK1 or CDK5 appears to be less likely.

Selectivity of Indirubins—Although indirubins have a strong affinity for CDKs, we had previously noticed that they were slightly less selective than purines (25). The identification of GSK-3β as a very sensitive target of indirubins raises some questions about the range of cellular targets of these compounds. We intend to purify and identify the indirubin-binding proteins, from various tissues, by affinity chromatography on immobilized indirubin-3’-monoxime. The crystal structures of indirubins in complex with CDK2 provides precious information with respect to the orientation of the two indole rings within the ATP-binding pocket. Carbons 6’ and 7’ clearly point toward the outside of the kinase catalytic site and are accessible to solvent. This is where a linker could be attached to tether the inhibitor to a solid matrix while maintaining free access of the inhibitor to its kinase targets. Using this approach with purvalanol, based on the CDK2/purvalanol crystal structure (62), we have recently been able to identify the intracellular targets of purvalanol in a variety of cells and tissues (63).

Selectivity of Other CDK Inhibitors—Most reported CDK inhibitors are powerful GSK-3β inhibitors (Table III). This observation has several important consequences: First, the range and identity of the exact cellular targets of these compounds must be seriously evaluated. We believe that the affinity chromatography approach described above is the most straightforward approach available at present. We are currently developing it with purines, indirubins, paullones, and hymenialdisine. Most of these compounds have been crystallized in the ATP-binding pocket of CDK2 (64), and this will allow proper orientation of the inhibitors on the matrix. Second, previously published papers on the cellular effects of these CDK inhibitors need to be re-evaluated. In the case of flavopiridol, several authors (65, 66) had convincingly suggested that it might act on targets other than CDKs while inducing apoptosis. Third, it remains to be determined whether GSK-3β inhibition is a favorable or a negative property of these compounds in their potential use as antitumor agents, as well as for any other application, including neuroprotection. This is an important question, because it will orient the search for and optimization of these inhibitors either toward highly CDK- or GSK-3β-selective compounds or toward dual-specificity agents. In the case of antitumor properties, GSK-3β inhibition (expected to favor cell division) (30), and CDK inhibition (expected to arrest the cell cycle) (67, 68) may turn out to create an “intracellular conflict of interest,” which might only be solved by the induction of apoptosis. This is clearly a desired effect in cancer therapy. However, GSK-3β inhibition may reduce or even mask therapeutically interesting properties of CDK inhibitors. Alternatively, GSK-3β inhibitors might be more efficient in cells if devoid of CDK inhibitory properties.

Indirubins Inhibit Tau Phosphorylation: Implication in Alzheimer’s Disease—Three groups of proteins are known to play a major role in the development of AD: presenilins, the amyloid β peptides, and the microtubule-binding protein tau. Mutations in the presenilin genes are the most common cause of early onset familial AD (69, 70). Amyloid β peptides, derived from proteolytic cleavage of the amyloid precursor protein, form the extracellular senile neuritic plaques, a diagnostic feature of AD (69).

Numerous reports describe the abnormal hyperphosphorylation of tau in AD (38, 39, 70). When hyperphosphorylated, tau aggregates into paired helical filaments (PHP), which form the typical neurofibrillary tangles, a hallmark feature of AD. Four recent discoveries have recently helped in understanding the link between tau hyperphosphorylation and AD: 1) the existence of tau mutations in AD-related diseases (39, 72) the functions of tau in regulating intracellular traffic along microtubules (71, 3) the accumulation of p25 and increase in CDK5 kinase activity in the brains of AD patients (40), and 4) the positioning of tau hyperphosphorylation downstream of presenilin and amyloid-β (70). Tau hyperphosphorylation occurs on more than 20 sites and is essentially carried out by two proline-directed kinases, GSK-3β and CDK5/p25, and by PKA (38, 39, 70, 72), and indirectly carried out by casein kinase 1, which phosphorylates and activates CDK5 (73). Presenilin associates with GSK-3β, and presenilin 1 mutations that cause AD increase the ability of presenilin 1 to bind and activate GSK-3β (74). Exposure of hippocampal neurons to amyloid β peptides leads to GSK-3β stimulation and enhanced tau phosphorylation (75). CDK5 potentiates GSK-3β-stimulated tau phosphorylation (76). CDK5 is increased in AD brains (40). Lithium, a recently reported GSK-3β inhibitor (IC50 in the millimolar range), reduces AD-like tau phosphorylation in cultured cells (77–80). Altogether, these data suggest that CDK5 and GSK-3β inhibition can be expected to impact severely on the hyperphosphorylation of tau observed in AD and possibly also on the outcome of this disease. Indirubins, and many of the previously reported CDK inhibitors (flavopiridol, paullones, UCN-01, hymenialdisine) constitute lead compounds with great potential for the treatment of AD and other “taupathies.”

In summary, we have identified indirubins as very potent inhibitors of GSK-3β and CDK5, two major kinases involved in tau hyperphosphorylation. Indirubins therefore constitute a promising family of lead compounds, which deserve to be evaluated as therapeutic agents in Alzheimer’s disease and other neurodegenerative disorders.

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