Neurotoxicology and Teratology 32 (2010) 212–219

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

# Indirubins deplete striatal monoamines in the Intact and MPTP-treated mouse brain and block kainate-induced striatal astrogliosis

Prokopios Magiatis [a], Panagiotis Polychronopoulos [a], Alexios-Leandros Skaltsounis [a,*], Olivier Lozach [b], Laurent Meijer [b], Diane B. Miller [c], James P. O'Callaghan [c,*]

[a] Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Athens, Panepistimiopolis Zografou, GR-15771 Athens, Greece
[b] C.N.R.S., Protein Phosphorylation & Human Disease Group, Station Biologique, 29682 ROSCOFF cedex, Bretagne, France
[c] Molecular Neurotoxicology and Chronic Stress & Neurotoxicology Laboratories, Health Effects Laboratory Division, Centers for Disease Control and Prevention-NIOSH, 1095 Willowdale Road, Morgantown, WV 26505, USA

ARTICLE INFO

*Article history:*
Received 13 August 2009
Received in revised form 3 December 2009
Accepted 16 December 2009
Available online 23 December 2009

*Keywords:*
Indirubin
MPTP
Kainate
Astrogliosis
Dopamine
Serotonin

ABSTRACT

The indirubins long have been used in Chinese medicine for treatment of myelocytic leukemia. Among the many more recently described biological activities of the indirubins, attention has been directed toward the ability of these compounds to inhibit GSK-3 and CDKs, kinases implicated in neurodegenerative conditions. Little information is available on effects of indirubins on chemically-induced neurodegeneration. Here we examined the influence of three indirubins on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and kainic acid (KA)-induced neurotoxicity in the mouse. The three indirubins examined were 6-bromoindirubin-3′-oxime (6BIO), 5-bromoindirubin-3′-oxime (5BIO) and 5-amino-6-bromoindirubin (5A6BI). The first two derivatives were previously described indirubins with low nanomolar inhibitory activity against GSK-3 and CDKs. The third compound was synthesized by the dimerization of 5-amino-6-bromoisatin with 3-acetoxyindol. The synthesis of the key compound 5-amino-6-bromoisatin was based on the bromination of the ketal of 5-amino-isatin. All indirubins examined decreased various measures associated with dopaminergic neurotransmission in striatum. These effects occurred alone or over and above the decrements seen following administration of the dopaminergic neurotoxicant, MPTP. Striatal serotonin and serotonin turnover were decreased by the indirubins in MPTP-treated mice. None of these striatal effects of the indirubins alone were associated with evidence of astrogliosis, an indicator of underlying neuropathology, nor did they potentiate the astrogliosis accompanying administration of MPTP. In general, the indirubins reduced KA-associated mortality and striatal but not hippocampal astrogliosis due to this toxicant. The data suggest that indirubins affect striatal biogenic amine levels and turnover in intact mice. The data do not indicate a neuroprotective action of indirubins in mice treated with MPTP but that they do suggest that they may be neuroprotective against KA-induced injury of the neostriatum.

Published by Elsevier Inc.

The indirubins, have long been mainstays of Traditional Chinese Medicine [18], Interest in indirubin and its analogues (collectively referred to as indirubins) from an experimental perspective strongly increased when they were discovered to inhibit cyclin-dependent kinases (CDKs) [9], glycogen synthase kinase-3 (GSK-3) [15], glycogen phosphorylase b [12], and to bind and activate the Aryl Hydrocarbon Receptor (AhR), known also as the dioxin receptor [1,7,13]. 6-Bromoindirubin-3′-oxime (6BIO) has been identified as a strong and selective inhibitor of GSK-3, [17,27] while 5-bromoindirubin-3′-oxime (5BIO) is a strong inhibitor of both GSK-3 and CDKs. Recently, GSK-3 and CDKs, especially CDK5, have been shown to be involved in cell-signaling events associated with injury-induced neural damage and glial activation [5,19]. These observations raised the possibility that indirubins may afford protective actions against chemically-induced injury of the CNS. To address this issue we evaluated the effects of three indirubins on two mechanistically distinct neurotoxicity models, the dopaminergic terminal damage due to the known dopaminergic neurotoxicant, MPTP, [26], and limbic and striatal neuropathology induced by the rigid analogue of glutamate, KA [3,4]. CDK5 has been implicated in the neurotoxic effects of both of

*Abbreviations:* 6BIO, 6-bromoindirubin-3′-oxime; 5BIO, 5-bromoindirubin-3′-oxime; 5A6BI, 5-amino-6-bromoindirubin; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin/5-hydroxytryptamine; AhR, aryl hydrocarbon receptor; BSA, bovine serum albumin; CDK, cyclin-dependent kinase; CI-MS, chemical ionization mass spectroscopy; DA, dopamine; DMSO, dimethyl sulfoxide; DOPAC, 3,4-dihydroxyphenylacetic acid; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; GSK-3, glycogen synthase kinase-3; GST, glutathione-S-transferase; HPLC, high performance liquid chromatography; HVA, homovanillic acid; KA, kainic acid; NMR, nuclear magnetic resonance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Pd/C, palladium on carbon; TH, tyrosine hydroxylase.

* Corresponding authors. O'Callaghan is to be contacted at Tel.: +1 304 285 6079; fax: +1 304 285 6220. Skaltsounis, Tel.: +30 2107274598; fax: +30 2107274594.
*E-mail addresses:* skaltsounis@pharm.uoa.gr (A.-L. Skaltsounis), jdo5@cdc.gov (J.P. O'Callaghan).

0892-0362/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.ntt.2009.12.005

these compounds [29,31,35], observations further strengthening the potential for CDK inhibitory action to affect neurotoxic outcomes following exposure to MPTP and KA. The data obtained do not support a neuroprotective role of indirubins in MPTP neurotoxicity but do suggest a potential protective role in KA neurotoxicity.

## 1. Methods

### 1.1. Chemistry

#### 1.1.1. General chemistry procedures

All chemicals were purchased from Aldrich Chemical Co. NMR spectra were recorded on Bruker DRX 400; chemical shifts are expressed in ppm downfield from TMS. The $^{1}H$–$^{1}H$ and the $^{1}H$–$^{13}C$ NMR experiments were performed using standard Bruker microprograms. CI-MS spectra were determined on a Finnigan GCQ *Plus* ion-trap mass spectrometer using $CH_4$ as the CI ionization reagent. Column chromatography was conducted using flash silica gel 60 Merck (40–63 μm) with an overpressure of 300 mbars. All the compounds gave satisfactory combustion analyses (C, H, N, within ±0.4% of calculated values). 6-Bromoindirubin-3′-oxime (Polychronopoulos et al., 2004) and 5-bromoindirubin-3′-oxime [30] were synthesized as previously described.

#### 1.1.2. 5-Nitroisatin (2)

A solution of $NaNO_3$ (5.78 g, 0.068 mol) in $H_2SO_4$ (100 ml) at 0 °C was added dropwise over 1 h to a solution of isatin (**1**) (10.0 g, 0.068 mol) in $H_2SO_4$ (120 ml). The reaction mixture then was poured onto ice and the resulting precipitate was filtered and washed with water to yield 5-nitroisatin (**2**) (11.88 g, 91%) with NMR data identical to that previously described [8].

#### 1.1.3. 5,5-Dimethyl-5′-nitrospiro[1,3-dioxan-2,3′-indol]-2′(1′H)-one (3)

2,2′-Dimethylpropan-1,3-diol (2.69 g, 25.9 mmol) and a catalytic amount of p-toluenesulfonic acid (5%) was added to a suspension of 5-nitroisatin (5.0 g, 26.0 mmol) (**2**) in cyclohexane (35 mL). The reaction mixture then was refluxed for 12 h using a Dean-Stark apparatus. The mixture then was cooled, the precipitate was filtered, washed with $NaHCO_3$ (sat.), and dried to give the ketal of 5-nitroisatin (**3**) (6.5 g, 90%).$^{1}H$ NMR data are: (DMSO, 400 MHz, δ ppm, *J* σε Hz) 8.28 (1H, dd, $J=2.3$, 8.6 Hz, H-6′), 8.07 (1H, d, $J=2.3$ Hz, H-4′), 7.03 (1H, d, $J=8.6$ Hz, H-7′), 4.48 (2H, d, $J=10.9$ H-6α, H-4α), 3.54 (2H, d, $J=11.1$ Hz, H-6β, H-4β), 1.33 (3H, s, 5α-$C\underline{H}_3$), 0.83 (3H, s, 5β-$C\underline{H}_3$); CI-MS *m/z* 279 $(M+H)^+$.

#### 1.1.4. 5′-amino-5,5-dimethylspiro[1,3-dioxan-2,3′-indol]-2′(1′H)-one (4)

The ketal of 5-nitroisatin (**3**) (3.0 g, 10.8 mmol) was suspended in methanol (25 ml) and then 450 g of Pd/C (10%) were added. The mixture was stirred at room temperature under $H_2$ for 5 h. The solution then was filtered with celite and activated carbon and the filtrate was evaporated to yield the ketal of 5-amino-isatin (**4**) (2.57 g, 96%). $^{1}H$ NMR data are: (DMSO, 400 MHz, δ ppm, *J* σε Hz) 10.09 (1H, s, N′-H), 6.77 (1H, d, $J=2.1$ Hz, H-4′), 6.59 (1H, dd, $J=2.1$, 8.1 Hz, H-6′), 6.54 (1H, d, $J=8.1$ Hz, H-7′), 4.47 (2H, d, $J=10.8$ H-6α, H-4α), 3.43 (2H, d, $J=11.0$ Hz, H-6β, H-4β), 3.93 (2H, brs, $NH_2$), 1.28 (3H, s, 5α-$C\underline{H}_3$), 0.80 (3H, s, 5β-$C\underline{H}_3$); CI-MS *m/z* 249 $(M+H)^+$.

#### 1.1.5. 5′-amino-6′-bromo-5,5-dimethylspiro[1,3-dioxan-2,3′-indol]-2′(1′H)-one (5)

The ketal of 5-amino-isatin (**4**) (2.0 g, 8.06 mmol) was dissolved in absolute ethanol (200 ml) and the solution was cooled to 0 °C. A solution of $Br_2$ (410 ml, 8.06 mmol) in chloroform (0.5 ml $Br_2$ dissolved in 500 ml $CHCl_3$) was added dropwise and the mixture was stirred for a total of 3 h. The pH then was neutralized with $NaHCO_3$. The mixture was filtered and the filtrate was evaporated to dryness. The solid residue was submitted to flash chromatography using silica gel and 15:75 ratio of EtOH/cyclohexane as the eluent to yield the ketal of 5-amino-6-bromoisatin (**5**) (1.2 g, 45%). $^{1}H$ NMR data are: (acetone-$d_6$, 400 MHz, δ ppm, *J* σε Hz) 9.12 (1H, brs, N′-H), 7.03 (1H, s, H-7′), 6.91 (1H, s, H-4′), 4.76 (2H, brs, $NH_2$), 4.62 (2H, d, $J=10.8$ H-6α, H-4α), 3.45 (2H, d, $J=11.0$ Hz, H-6β, H-4β), 1.33 (3H, s, 5α-$C\underline{H}_3$), 0.83 (3H, s, 5β-$C\underline{H}_3$); CI-MS *m/z* 327, 329 $(M+H)^+$.

#### 1.1.6. 6-Bromo-5-acetamidoisatin (7)

The ketal of 5-amino-6-bromoisatin (**5**) (1.08 g, 3.30 mmol) was dissolved in pyridine (10 ml). Acetic anhydride (311 μl, 3.30 mmol) then was added and the reaction mixture was stirred for 12 h at 0 °C. The solvent was removed under reduced pressure to yield the ketal of 6-bromo-5-acetamidoisatin (**6**). This latter compound was suspended in a saturated solution of oxalic acid (30 ml) and heated to 60 °C for 12 h. The mixture then was filtered and washed with water to yield the 6-bromo-5-acetamidoisatin (**7**) (690 mg, 81%). $^{1}H$ NMR data are: (DMSO, 400 MHz, δ ppm, *J* σε Hz) 11.17 (1H, s, N-H), 9.64 (1H, s, AcN-H),7.61 (1H, s, H-7), 7.20 (1H, s, H-4), 2.14 (3H, s, $C\underline{H}_3CO$); CI-MS *m/z* 283, 285 $(M+H)^+$.

#### 1.1.7. 5-Amino-6-bromoisatin (8)

6-Bromo-5-acetamidoisatin (**7**) (680 mg, 2.40 mmol) was dissolved in $H_2SO_4$ (9.5 ml, 30%) and refluxed for 1.5 h . The solution then was cooled and stirred to yield the 6-bromo-5-amino-isatin sulfate (645 mg) as a precipitate. After filtration and an acetone wash, the sulfate salt was dissolved in hot water (25 mL) and boiling $Na_3BO_3$ (520 mg) was added. The resulting precipitate was recovered by filtration after washing with water (500 mg, 86%). $^{1}H$ NMR data are: (DMSO, 400 MHz, δ ppm, *J* σε Hz) 10.74 (1H, br s, N-H), 6.96 (1H, s, H-7), 6.91 (1H, s, H-4), 5.28 (2H, s, $N\underline{H}_2$); CI-MS *m/z* 241, 243 $(M+H)^+$.

#### 1.1.8. 5-Amino-6-bromoindirubin (9)

Methanol (80 ml) was stirred under $N_2$ for 20 min followed by addition of 5-amino-6-bromoisatin (**8**) (500 mg, 2.07 mmol) and 3-acetoxyindol (350 mg, 2.0 mmol). After 5 min, dry $Na_2CO_3$ (450 mg) was added and the reaction was allowed to continue for 3 h. The resulting precipitate was recovered by and washed with 100 ml of a 1:1 mixture of water and methanol solution. 5-Amino-6-bromoindirubin (9) (650 mg, 90%) was obtained as the end product. $^{1}H$ NMR data are: (DMSO, 400 MHz, δ ppm, *J* σε Hz) 11.01 (1H, s, N′-H), 10.58 (1H, brs, N-H) 8.38 (1H, s, H-4), 7.65 (1H, d, $J=7.5$ Hz, H-4′), 7.58 (1H, t, $J=7.5$ Hz, H-6′), 7.42 (1H, d, $J=7.5$, Hz, H-7′), 7.02 (1H, t, $J=7.5$ Hz, H-5′), 6.86 (1H, s, H-7), 4.98 (2H, s, 5-$NH_2$); CI-MS *m/z* 356, 358 $(M+H)^+$.

### 1.2. Protein kinase assays

#### 1.2.1. Biochemical reagents

Sodium ortho-vanadate, EGTA, EDTA, Mops, ß-glycerophosphate, phenylphosphate, sodium fluoride, dithiothreitol (DTT), glutathione-agarose, glutathione, bovine serum albumin (BSA), nitrophenylphosphate, leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, benzamidine, histone H1 (type III-S) were obtained from Sigma Chemicals. [γ-$^{33}P$]-ATP was obtained from Amersham. The GS-1 peptide (YRRAAVPPSPSLSRHSSPHQSpEDEEE) was synthesized by the Peptide Synthesis Unit, Institute of Biomolecular Sciences, University of Southampton, Southampton SO16 7PX, U.K.

#### 1.2.2. Buffers

The Homogenization Buffer contained 60 mM ß-glycerophosphate, 15 mM p-nitrophenylphosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mMDTT, 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 benzamidine. Buffer A contained 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris–HCl

pH 7.5, 50 µg heparin/ml. Buffer C contained homogenization buffer and 5 mM EGTA but did not contain NaF or protease inhibitors.

*1.2.3. Kinase preparations and assays*

Kinase activities were assayed in Buffer A or C, at 30 °C, at a final ATP concentration of 15 µM. Blank values were subtracted and activities calculated as pmoles of phosphate incorporated for a 10 min incubation. The activities are usually expressed in % of the maximal activity, i.e. in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide.

*CDK5/p25* was reconstituted by mixing equal amounts of recombinant mammalian CDK5 and p25 expressed in *E. coli* as GST (glutathione-S-transferase) fusion proteins and purified by affinity chromatography on glutathione-agarose (vectors kindly provided by Dr. J.H. Wang) (p25 is a truncated version of p35, the 35 kDa CDK5 activator). The kinase activity was assayed in buffer C, with 1 mg histone H1/ml, in the presence of 15 µM [$\gamma$-$^{33}$P] ATP (3000 Ci/mmol; 10 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 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 phosphoric acid/liter of water. The wet filters were counted in the presence of 1 ml ACS (Amersham) scintillation fluid. [15].

*GSK-3α/β* was purified from porcine brain by affinity chromatography on immobilized axin [28]. It was assayed, following a 1/100 dilution in 1 mg BSA/ml 10 mM DTT, with 5 µl 4 µM GS-1 peptide substrate, in buffer A, in the presence of 15 µM [$\gamma$-$^{33}$P] ATP (3000 Ci/mmol; 10 mCi/ml) in a final volume of 30 µl. After 30 min incubation at 30 °C, 25 µl aliquots of supernatant were processed as described above.

*1.3. Neurotoxicity experiments*

*1.3.1. Animals*

Female C57BL/6 J mice (Jackson Laboratories, Bar harbor, ME), 3–4 months of age were used for all experiments. The colony room was controlled for temperature (22 ± 2 °C) and humidity (30–40%) and was maintained on a 12-h light/12-h dark cycle. Access to chow and water was allowed *ad libitum*. The animal protocol governing the experiments was approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention-National Institute for Occupational Safety and Health. The Animal Care and Use Facility is accredited by the American Association for Accreditation of Laboratory Animal Care.

*1.3.2. Toxicant administration and brain tissue preparation*

Female mice were used in all experiments based on our historical data for single dose effects of MPTP & KA in this gender and because larger number of females can be group-housed for future long-term time point evaluations. In our hands, the neurotoxic effects of single doses of MPTP or KA do not differ between male and female C57Bl6J mice [20,21,32,33]. Mice were administered a single dose of MPTP (12.5 mg/kg, s.c.) or KA (20 mg/kg, s.c.) or vehicles (0.9% saline or DMSO for the indirubins) alone or in combination with 6BIO, 5BIO or 5A6BI (50 mg/kg, s.c.). A dosage of 50 mg/kg was used for the three indirubins based on dose-range evaluations (10, 25, and 50 mg/kg) showing this to be an effective dosage level for effects of all indirubins on all endpoints examined (data not shown). DMSO was limited to 50 µl per injection; no effects of DMSO alone were observed for any measures compared to data obtained for saline vehicle or uninjected mice. For the combination dosing experiments, the indirubins or vehicle were given once daily for four days; MPTP or KA were given 1 h after the second dose of indirubin. Mice were sacrificed by focused microwave irradiation [24] 72 h after administration of MPTP, KA or vehicle; this time point yields maximum increases in GFAP after exposure to either toxicant [3,21]. Focused microwave irradiation sacrifice was used because it preserves steady-state protein phosphorylation by heating brain tissue to a level sufficient to abolish enzymatic action of kinases and phosphatases [25,32]. A portion of the SDS tissue homogenates (see below) was retained for future analysis of specific phosphoproteins. After sacrifice, brains were allowed to cool and then were dissected free-hand. The selected regions from one side of the brain were frozen for subsequent analysis of brain amines and the regions from the other side of the brain were homogenized in hot (80–90 °C) 1% (w/v) SDS for subsequent analysis by specific ELISA.

*1.3.3. GFAP and TH ELISAs*

Glial fibrillary acidic protein (GFAP) and tyrosine hydroxylase (TH) were assayed using the detergent-based sandwich enzyme-linked immunosorbent assays (ELISAs) developed in this laboratory. Detailed protocols for the GFAP ELISA procedure have been described [22,24]. The modifications used for assaying TH also have appeared [32]. Briefly, "capture" antibodies were coated on the wells of Immunlon-2 microtiter plates (Thermo Labsystems, Franklin, MA). The SDS homogenates of specific brain regions and standards were diluted in PBS containing 0.5% Triton X-100. Nonspecific binding was blocked with 5% nonfat dry milk in PBS and then aliquots of the homogenates and standards were added to the wells and incubated. Following 4 sequential washes, a different host specifies antibody to GFAP or TH from the ones used as "capture" were added to "sandwich" the antigen between the antibodies. An alkaline phosphatase or peroxidase conjugated secondary with fluorogenic substrate was used as a reporter for quantification of GFAP and TH, respectively, via spectrophotometry and fluorometry.

*1.3.4. HPLC measurement of DA, 5HT and metabolites*

Dopamine (DA), serotonin (5-HT) and their metabolites were quantified by HPLC with electrochemical detection (Waters, Milford, MA). The frozen striata were homogenized in 300 µl of ice-cold 0.2 M perchloric acid, containing 1 µM dihydroxybenzylamine as internal standard, and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.2 µm membrane, and an aliquot (10 µl) of the sample was injected via an automatic sample injector (Waters 717 plus Autosampler) connected to a Waters 515 HPLC pump. Reverse phase C18 chromatography was used for sample separation followed by electrochemical detection as described previously [32].

## 2. Statistics

Analyses were performed using the JMP (version 6.0.3) statistical analysis software package. The test of significance for individual variables was performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. Mortality data were evaluated using the Chi Square statistic. In all cases, the alpha level was set at 0.05.

## 3. Results

In addition to 6BIO and 5BIO, known inhibitors of GSK-3 and CDK5, we synthesized 5-amino-6-bromoindirubin (**9**) (5A6BI) (Fig. 1). In the past we have clearly shown [17,27], that the 6-bromo substitution is responsible for increased selectivity of 6BIO towards GSK-3. Recently, it was found [2] that the introduction of an amino functional group at position 5 of the indirubin skeleton favors inhibitory activity against CDK-5. In this context, we envisaged the combination of the 5-amino substitution with a bromine atom at position 6.

*3.1. Synthesis of 5-amino-6-bromoindirubin*

5-Amino-6-bromoindirubin (9) (5A6BI) was synthesized by the dimerization of 5-amino-6-bromoisatin with 3-acetoxyindol (Fig. 1).

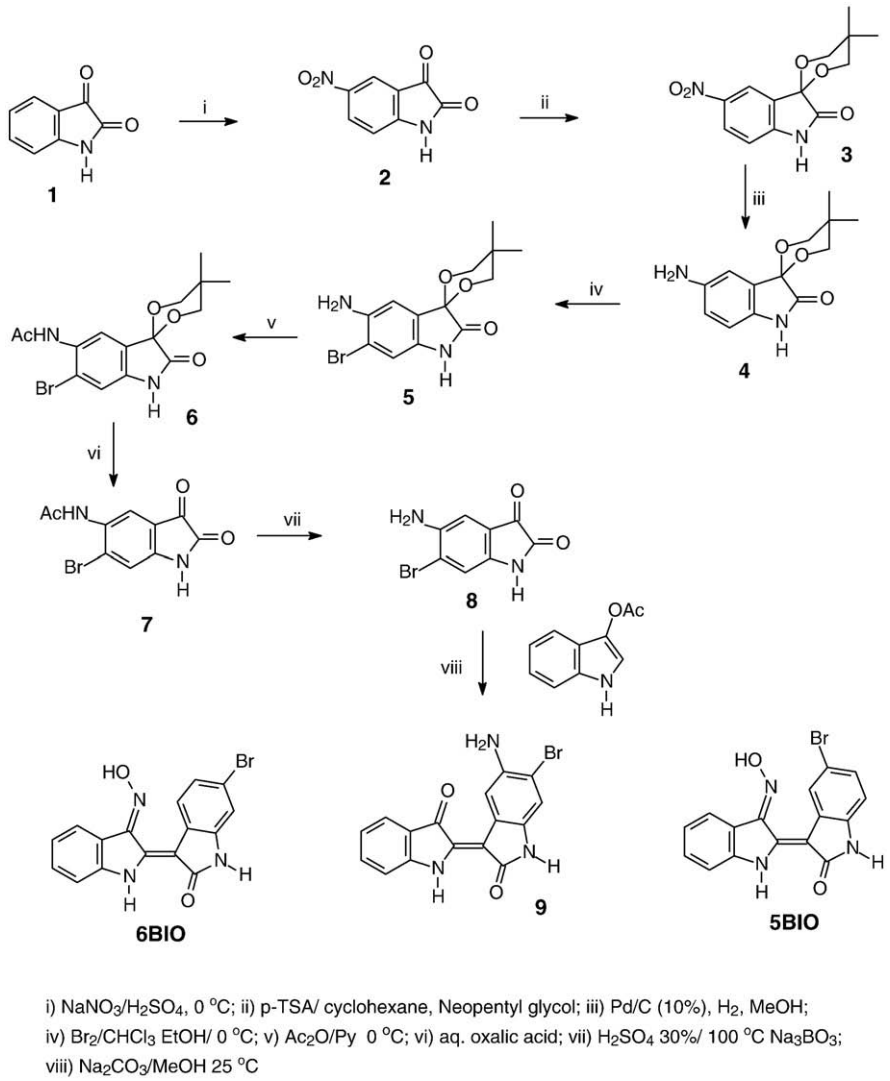


**Fig. 1.** Synthetic scheme for synthesis of 5-amino-6-bromoindirubin (5A6BI). The structures of 6BIO and 5BIO are shown for comparison.

The synthesis of the key compound 5-amino-6-bromoisatin was based on the bromination of the ketal of 5-amino-isatin. Nitration of isatin led selectively and in high yield to 5-nitroisatin which then was protected by the formation of a ketal at position 3. Subsequent catalytic hydrogenation led to the ketal of 5-aminoisatin (**4**). Careful bromination of **4** with $Br_2$ in chloroform resulted in a 45% yield to the ketal of 5-amino-6-bromoiasatin. Direct deprotection of this compound was highly problematic and for this reason the amino group was acetylated and the ketal was hydrolyzed with oxalic acid. Final hydrolysis of the acetamido group with sulfuric acid led to 5-amino-6-bromoisatin (**8**) followed by formation of 5A6BI (**9**) via reaction with 3-acetoxyindol. The structures of 6BIO and 5BIO are shown for comparison (Fig. 1).

### 3.2. In vitro evaluation for GSK-3α/ß and CDKs inhibition

The in *vitro* inhibitory activity of the indirubins against GSK-3α/ß and CDK5 is summarized in Table 1. The presence of an amino group in position 5 enhanced the selectivity of the compounds towards CDK5/p25 versus GSK-3α/ß. 5A6BI showed reduced overall kinase inhibitory activity against GSK-3α/ß in comparison to the actions of 5BIO and 6BIO (Table 1). On the other hand, 5A6BI was found to be very soluble in apolar solvents like dichloromethane (data not shown). This is the first example of an indirubin with this property among the hundreds of derivatives of indirubins previously synthesized. This solubility feature of 5A6BI should make it more practical to administer for *in vivo* experiments, in contrast to 6BIO, 5BIO and their corresponding ketones, which exhibit poor solubility in most solvents. Whether the solubility of 5A6BI affects it bioavailability for CNS targets remains to be evaluated.

### 3.3. Effect of indirubins on MPTP-induced neurotoxicity

The indirubins alone and in combination with MPTP affected a variety of striatal measures (Fig. 2). The three indirubins alone significantly decreased dopamine (DA) while the 5BIO and 5A6BI compounds decreased the dopamine metabolite, DOPAC; 6BIO also slightly decreased HVA. Striatal levels of 5-HT and its metabolite, 5-HIAA, were not affected by the indirubins alone Except for a small

**Table 1**
Inhibitory activity against GSK-3 and CDK5/p25 ($IC_{50}$ values in μM).

| Compound | GSK-3 | CDK5/p25 |
|---|---|---|
| 5BIO | 0.018 | 0.028 |
| 6BIO | 0.005 | 0.083 |
| 5A6BI | 0.600 | 0.054 |

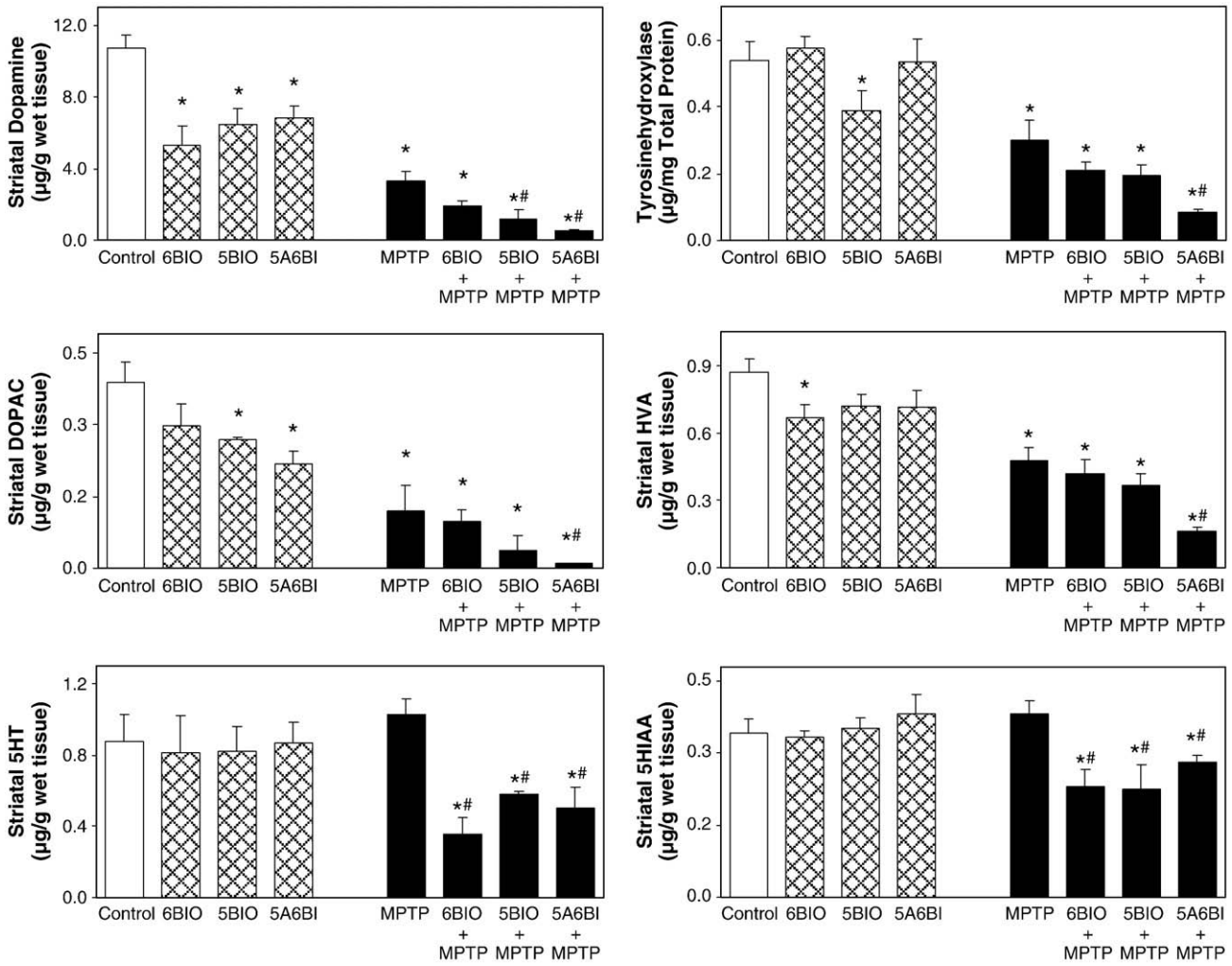


**Fig. 2.** Indirubins affect striatal biogenic amines alone and after administration of the dopaminergic neurotoxicant, MPTP. Mice were administered either saline or DMSO as vehicle controls for MPTP (12.5 mg/kg, s.c.) or indirubin (50 mg/kg, s.c.), respectively. Saline and DMSO controls did not differ; DMSO controls are shown. Indirubins were given once daily for four days; MPTP was given 1 h after the second dose of indirubins. Biogenic amines and metabolites were quantified by HPLC with electrochemical detection; tyrosine hydroxylase was quantified by sandwich ELISA. Values represent the mean ± S.E.M. for five independent observations. *Significantly different from control. #Significantly different from MPTP alone.

decrease observed for 5BIO, the levels of the striatal dopaminergic terminal marker, TH, were not affected by the indirubins alone. These findings are suggestive of a regulatory action of the indirubins because DA levels/metabolism were altered in the absence of corresponding changes in a dopaminergic nerve terminal marker. The known dopaminergic neurotoxicant, MPTP, caused the expected decrease in all dopaminergic indices. Levels of 5-HT and 5-HIAA, as expected, were not affected by MPTP. When given in combination with MPTP, the three indirubins resulted in greater decreases in dopamine, with the effects of 5BIO and 5A6BI being significantly greater than the dopamine decrements seen with MPTP alone. The 5A6BI compound, when given with MPTP, further reduced levels of the DA metabolites, DOPAC and HVA, and caused a further decrease in TH. With respect to the two serotoninergic measures, all three indirubins resulted in decreases in 5-HT and 5-HIAA, when given in combination with MPTP. Thus, in essence, the indirubins rendered MPTP a 5-HT as well as a dopaminergic acting agent. Despite the effects of indirubins on striatal DA and TH, when given alone or in combination with MPTP, they did not appear to initiate or exacerbate damage, respectively, because damage-related astrogliosis (induction of GFAP) observed with MPTP was not seen with the indirubins alone and the increases in GFAP seen with MPTP were not further enhanced by the indirubins (Fig. 3). Moreover, consistent with the known selective effects of MPTP on nigro-striatal dopaminergic neurons, induction of GFAP by MPTP in the presence and absence of indirubins was confined to striatum and did not extend to hippocampus (Fig. 3).

### 3.4. Effect of indirubins on KA-induced neurotoxicity

Consistent with our previous findings [3,4], systemic administration of KA was associated with significant mortality (Table 2). All indirubins examined reduced KA-associated mortality (Table 2). We also observed a significant increase in striatal and hippocampal GFAP after a single dose of the excitotoxic compound, KA (Fig. 4). These findings are consistent with our prior data and the known neuropathology and astrogliosis associated with exposure to this agent [3,4]. The KA-induced astrogliosis in striatum was prevented by all three indirubins (Fig. 4). Together, these findings were suggestive of a protective action of the indirubins against KA toxicity, in general, and a region-selective neuroprotective effect against neurotoxicity. These latter findings underscore the possibility that region-specific substrates of the indirubins may play a role in their neuroprotective actions.

## 4. Discussion

A variety of biological and medicinal activities have been attributed to the indirubins [18]. Among these actions, the recently

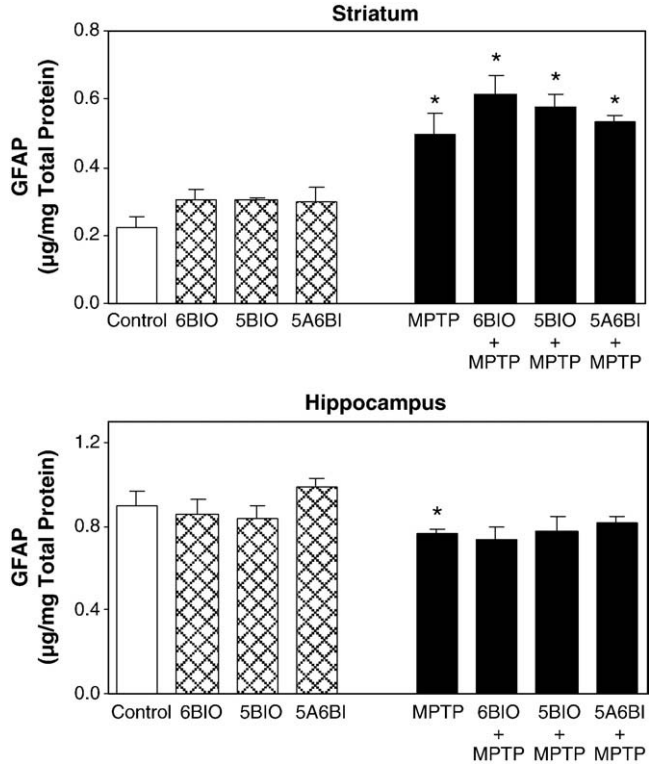


**Fig. 3.** The astroglial reaction to MPTP-induced neurotoxicity is not affected by the indirubins. Mice were administered either saline or DMSO as vehicle controls for MPTP (12.5 mg/kg, s.c.) or indirubin (50 mg/kg, s.c.), respectively. Saline and DMSO controls did not differ; DMSO controls are shown. Indirubins were given once daily for four days; MPTP was given 1 h after the second dose of indirubins. GFAP was quantified by sandwich ELISA. Values represent the mean ± S.E.M. for five independent observations. *Significantly different from control.

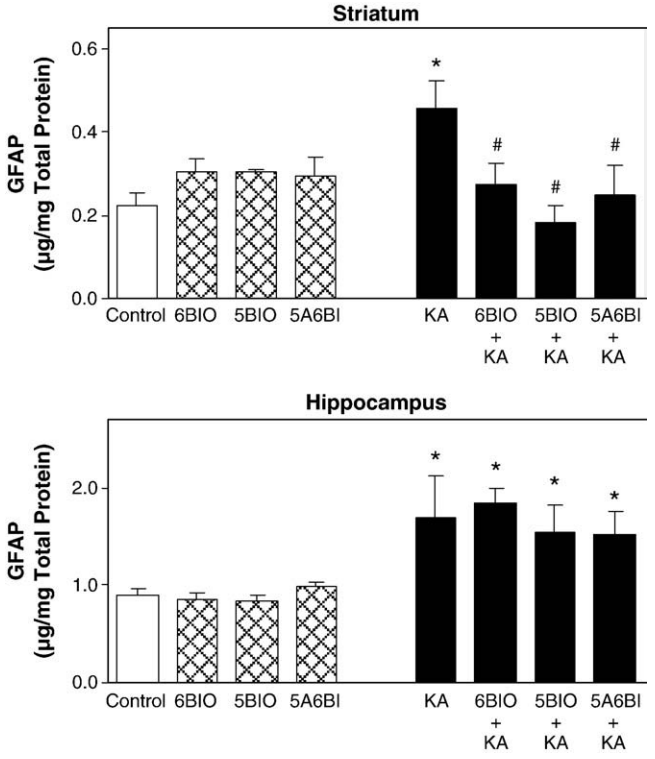


**Fig. 4.** The astroglial reaction to KA-induced neurotoxicity is attenuated by indirubins in the striatum but not the hippocampus. Mice were administered either saline or DMSO as vehicle controls for KA (20 mg/kg, s.c.) or indirubin (50 mg/kg, s.c.), respectively. Saline and DMSO controls did not differ; DMSO controls are shown. Indirubins were given once daily for four days; KA was given 1 h after the second dose of indirubins. GFAP was quantified by sandwich ELISA. Values represent the mean ± S.E.M. for five independent observations. *Significantly different from corresponding control. # Significantly different from KA alone.

identified inhibitory properties of indirubins against GSK-3 and CDK5, kinases implicated in neurodegenerative diseases, such as AD and PD [6,29], as well as in neurotoxic responses [29,31], suggest that indirubins may have therapeutic actions on the CNS. The two existing indirubins, and the novel indirubin for which we now have provided the synthetic scheme, were shown to have marked inhibitory activity *in vitro* against GSK3 and CDK5. Therefore, we chose to evaluate the effects of these compounds in two models of neurotoxicity that we have extensively characterized in our laboratories and for which CDK5 had been implicated in their effects. These models, MPTP- and KA-induced neurotoxicity, affect different brain regions and primarily affect dopaminergic nerve terminals in striatum and glutamatergic pyramidal neurons in hippocampus, respectively. Thus, these agents would seem to serve as useful regional and subcellular denervation tools associated with CDK5 activity with which to examine potential neuroprotective actions of the indirubins. The data obtained are among the first to show neurobiological actions, *in vivo*, for the indirubins.

**Table 2**
Mortality of animals for the tested compounds.

| Treatment group | Number of animals | Survival | Mortality | % Mortality |
|---|---|---|---|---|
| Sal | 5 | 5 | 0 | 0% |
| DMSO | 5 | 5 | 0 | 0% |
| 6BIO | 5 | 5 | 0 | 0% |
| 5BIO | 5 | 5 | 0 | 0% |
| 5A6BI | 5 | 5 | 0 | 0% |
| MPTP | 5 | 5 | 0 | 0% |
| 6BIO/MPTP | 5 | 5 | 0 | 0% |
| 5BIO/MPTP | 5 | 5 | 0 | 0% |
| 5A6BI/MPTP | 5 | 5 | 0 | 0% |
| KA* | 14 | 5 | 9 | 64% |
| 6BIO/KA | 5 | 5 | 0 | 0% |
| 5BIO/KA | 8 | 5 | 3 | 38% |
| 5A6BI/KA | 8 | 6 | 2 | 25% |

*When compared to Saline controls, KA caused a significant increase in mortality ($\chi^2 = 10.3$; $0.01 < P < 0.001$). 6BIO completely protected against KA-induced mortality ($\chi^2 = 10.3$; $0.01 < P < 0.001$); 5A6BI provided some protection ($\chi^2 = 3.14$; $0.01 < P < 0.05$) and 5BIO was not protective ($\chi^2 = 1.5$; $0.5 < P < 0.10$).

In the striatum, our results demonstrated significant effects of the indirubins alone and in combination with MPTP that were not indicative of neuroprotection. We found a novel depleting action of all three indirubins on striatal DA and metabolites. When administered in combination with MPTP, decreases in 5-HT and its metabolite also were observed. Decrements in dopaminergic measures were seen with the compounds alone and these effects were generally additive to the loss of the same dopaminergic markers after co-administration with the dopaminergic neurotoxicant, MPTP. This additivity over and above the known toxic action of MPTP on dopaminergic nerve terminals was suggestive of a regulatory action of indirubins on dopaminergic metabolism. This possibility is consistent with the observation that inhibition of GSK-3 activation can prevent loss of nigral dopamine neurons while only minimally affecting striatal dopamine levels due to dopamine synthesis impairment [34]. Although speculative, the possibility exists for serine-directed phosphorylation and activation of tyrosine hydroxylase by GSK-3, which would be inhibited by the indirubins, leading to declines in dopamine levels. On the other hand, another conclusion that could tentatively be drawn from the data is that inhibition of GSK-3 might not be critical for MPTP toxicity, given the potentiated rather than protective effects of the indirubins (especially 5A6BI) against dopaminergic measures. Surprisingly, and in contrast to effects on dopaminergic markers, decreases in 5-HT and metabolites only occurred when indirubins were combined with MPTP. While

analogues of MPTP are known to deplete and perhaps damage 5-HT-containing neurons (e.g. see [16]), broadening the selectivity of MPTP to include 5-HT as well as DA systems by co-administration of another drug has not been documented. While speculative again, a possible mechanism through with acute depletion of 5-HT and 5-HIAA may have occurred could be related to the structural similarities between the indirubins and 5-HT, which have two indole-like moieties and a single indoleamine, respectively. This possibility seems unlikely, however, because effects on 5-HT measures should have been seen with the indirubins alone and not just in combination with MPTP. In aggregate, our data suggest that biogenic amine depletion is a novel biologic effect of the indirubins examined. One also could posit that the data reflect damaging aspects of indirubins on both the dopaminergic and serotonergic pathways. This possibility seems unlikely for two reasons. First, the aforementioned additive depleting effects of indirubins when combined with MPTP suggest different mechanisms are involved to achieve the decreases observed. Second, because the dopaminergic nerve terminal damage-related increase in striatal astrogliosis (increase in GFAP) by MPTP was not exacerbated by the indirubins, nor did the indirubins alone result in an astroglial activation, strongly argues for a depleting rather than a neurodegenerative action of the indirubins. Also consistent with these views is the fact that depletion of striatal biogenic amines with reserpine does not result in evidence of astrogliosis [23].

Systemic injection of KA is associated with significant neuronal loss and attendant astrogliosis. Dose-related increases in GFAP, a biomarker of astrogliosis, have been shown to reflect dose-related damage to known neurotoxicants, including those that selectively damage nerve terminals or neuronal perikarya (reviewed in [26]). We assessed striatal and hippocampal GFAP levels as an index of KA-induced astrogliosis and underlying neuronal damage [3,4]. The KA-induced astrogliosis in striatum, but not hippocampus, was prevented by all three indirubins. Presumably, similar excitotoxic mechanisms result in neural damage and astrogliosis in striatum and hippocampus, therefore, the apparent neuroprotective effects of the indirubins for striatum alone may reflect actions of these compounds on substrates distinct from shared actions of KA across striatum and hippocampus. KA-induced excitotoxicity and neurodegeneration are engendered by binding of KA to KA and AMPA subtypes of the glutamate receptor. Glutamate receptor-evoked neural damage can involve GSK-3-mediated intracellular signaling potentially affected by indirubin pretreatments [10]. Finally, the distribution of KA and AMPA receptor subtypes differs between the striatum and hippocampus [14]. Accordingly, it is possible that these different distribution profiles may contribute to the divergent neuroprotective action of the indirubins in striatum and hippocampus. While the 50 mg/kg dosage we used to achieve effects on multiple CNS endpoints may seem high, the bioavailability of the indirubins for CNS targets has not been established. Reduced entrance of these compounds into the brain may have dictated the need for high pretreatment dosages. We also note that, with respect to the relevance of the present findings to potential therapeutic actions of indirubins, our short-term pretreatment may not be of sufficient duration to afford neuroprotection, given that a clinical course of indirubin therapy would presumably be given on a months-long basis [18].

In summary, our data are indicative of complex neurobiological actions of indirubins on CNS substrates in striatum. While our data do not point to a unifying mechanism of action, the known inhibitory properties of indirubins on GSK-3 and CDK5, kinases implicated in neurodegenerative responses in various brain areas, including striatum [11,19], suggest that these kinases and their downstream effectors [11], should be the subject of future investigation of indirubin actions. The apparently unique propensity of the indirubins to broaden the action of MPTP to serotonergic systems also deserves further scrutiny.

## Conflict of interest statement

No conflicts of interest are reported.

## Acknowledgments

This research was supported by intramural funds from CDC-NIOSH (J.O. and D.M.) and grants from the EEC (FP6-2002-Life Sciences & Health, PRO-KINASE Research Project) (L.M.) and the Canceropole Grand-Ouest (L.M.). The authors thank Fang Ma, Christopher Felton and Brenda Billig for excellent technical assistance. The critical review of the manuscript by Dr. Lawrence P. Wennogle is gratefully acknowledged.

## References

[1] J. Adachi, Y. Mori, S. Matsui, H. Takigami, J. Fujino, H. Kitagawa, C.A. Miller III, T. Kato, K. Saeki, T. Matsuda, Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine, J. Biol. Chem (2001) 27631475–27631478.
[2] A. Beauchard, Y. Ferandin, S. Frere, O. Lozach, M. Blairvacq, L. Meijer, V. Thierya, T. Besson, Synthesis of novel 5-substituted indirubins as protein kinases inhibitors, Bioorg. Med. Chem. 14 (2006) 6434–6443.
[3] S.A. Benkovic, J.P. O'Callaghan, D.B. Miller, Sensitive indicators of injury reveal hippocampal damage in C57BL/6 J mice treated with kainic acid in the absence of tonic–clonic seizures, Brain Res. 1024 (2004) 59–76.
[4] S.A. Benkovic, J.P. O'Callaghan, D.B. Miller, Regional neuropathology following kainic acid intoxication in adult and aged C57Bl/6 J mice, Brain Res. 1070 (2006) 215–231.
[5] J.C. Cruz, H.-C. Tseng, J.A. Goldman, H. Shiih, L.-H. Tsai, Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles, Neuron 40 (2003) 471–483.
[6] O. Engmann, K.P Giese, Crosstalk between Cdk5 and GSK3β: implications for Alzheimer's Disease. Front Mol Neurosci 2009;2:2 (in press), doi:10.3389/neuro.02.002.2009.
[7] P.F. Guengerich, M.V. Martin, W.A. McCormick, L.P. Nguyen, E. Glover, C.A. Bradfield, Aryl hydrocarbon receptor response to indigoids in vitro and in vivo, Arch. Biochem. Biophys. 423 (2004) 309–316.
[8] G.K. Jnaneshwar, V.H. Deshpande, Synthesis of convolutamydine A from isatin, J. Chem.Res. (1999) 632–633 S.
[9] R. Hoessel, S. Leclerc, J. Endicott, M. Noble, A. Lawrie, P. Tunnah, M. Leost, E. Damiens, D. Marie, D. Marko, E. Niederberger, W. Tang, G. Eisenbrand, L. Meijer, Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases, Nat. Cell Biol. 1 (1999) 60–67.
[10] M. Kajta, H. Domin, G. Grynkiewicz, W. Lason, Genistein inhibits glutamate-induced apoptoci process in primary neuronal cell cultures: an involvement of aryl hydrocarbon receptor and estrogen receptor/glycogen synthase kinase-3beta intracellular signaling pathway, Neuroscience 145 (2007) 592–604.
[11] D. Kim, C.L. Frank, M.M. Dobbin, R.K. Tsunemoto, W. Tu, P.L. Peng, J.-S. Guan, B.-H. Lee, L.Y. Moy, P. Giusti, N. Broodie, R. Mazitschek, I. Delalle, S.J. Haggarty, R.L. Neve, L.-H. Tsai, Deregulation of HDAC1 by p25/Cdk5 in neurotoxicit, Neuron 60 (2008) 803–817.
[12] M.N. Kosmopoulou, D.D. Leonidas, E.D. Chysina, N. Bischler, G. Eisenbrand, C.E. Sakarellos, R. Pauptit, N.G. Oikonomakos, Binding of the potential antitumour agent indirubin-5-sulphonate at the inhibitor site of rabbit muscle glycogen phosphorylase b. Comparison with ligand binding to pCDK2–cyclin A complex, Eur. J. Biochem. 271 (2004) 2280–2290.
[13] M. Knockaert, M. Blondel, S. Bach, M. Leost, C. Elbi, G. Hager, S.R. Naggy, D. Ham, M. Denison, M. Ffrench, X.P. Ryan, P. Magiatis, P. Polychronopoulos, P. Greengard, L. Skaltsounis, L. Meijer, Independent actions on cyclin-dependent kinases and aryl hydrocarbon receptor mediate the anti-proliferative effects of indirubins, Oncogene 23 (2004) 4400–4412.
[14] V.C. Kurschner, R.L. Petruzzi, G.T. Golden, W.H. Berrettini, T.N. Ferraro, Kainate and AMPA receptor binding in seizure-prone and seizure-resistant inbred mouse strains, Brain Res. 780 (1998) 1–8.
[15] S. Leclerc, M. Garnier, R. Hoessel, D. Marko, J.A. Bibb, G.L. Snyder, P. Greengard, J. Biernat, E.-M. Mandelkow, G. Eisenbrand, L. Meijer, Indirubins inhibit glycogen synthase kinase-3β and CDK5/p25, two kinases involved in abnormal tau phosphorylation in Alzheimer's disease — a property common to most CDK inhibitors ? J. Biol. Chem. 276 (2001) 251–260.
[16] B.A. Luellen, D.B. Miller, A.C. Chisnell, D.L. Murphy, J.P. O'Callaghan, Anne M. Andrews, Neuronal and astroglial responses to the serotonin and norepinephrine neurotoxin: 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydro-pyridine, J. Pharmacol. Exp. Ther. 307 (2003) 923931.
[17] L. Meijer, A.L. Skaltsounis, P. Magiatis, P. Polychonopoulos, M. Knockaert, M. Leost, X.P. Ryan, C.D. Vonica, A. Brivanlou, R. Dajani, A. Tarricone, A. Musacchio, S.M. Roe, L. Pearl, P. Greengard, GSK-3 selective inhibitors derived from Tyrian purple indirubins, Chem. & Biol. 10 (2003) 1255–1266.
[18] L. Meijer, N. Guyard, L.A. Skaltsounis, G. Eisenbrand (Eds.), Indirubin, the red shade of indigo, Editions «Life in Progress», Station Biologique, Roscoff, ISBN: 9-782951-802902, 2006, 27 chapters, 297. pp.
[19] D.A. Meyer, E. Richer, S.A. Benkovic, K. Hayashi, J.W. Tansy, C.F. Hale, L.Y. Moy, Y. Kim, J.P. O'Callaghan, L.H. Tsai, P. Greengard, A.C. Nairn, C.W. Cowan, D.B. Miller, P. Antich, J.A. Bibb, Striatal dysregulation of Cdk5 alters locomotor responses to cocaine, motor learning, and dendritic morphology, PNAS 105 (2008) 18561–18566.
[20] D.B. Miller, J.P. O'Callaghan, Environment-, drug- and stress-induced alterations in body temperature affect the neurotoxicity of substituted amphetamines in the C57Bl/6 J mouse, J. Pharmacol. Exp. Ther. 270 (1994) 752–760.

[21] J.P. O'Callaghan, D.B. Miller, J.F. Reinhard Jr, Characterization of the origins of astrocyte response to injury using the dopaminergic neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Brain Res (1990) 73–80.

[22] J.P. O'Callaghan, Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA, Neurotoxicol. Teratol. 13 (1991) 275–281.

[23] J.P. O'Callaghan, Quantitative features of reactive gliosis following toxicant-induced damage of the CNS, Ann. NY Acad. Sci. 679 (1993) 195–210.

[24] J.P. O'Callaghan, Measurement of glial fibrillary acidic protein, in: L.G. Costa (Ed.), Current Protocols in Toxicology, John Wiley & Sons, New York, 2002, pp. 12.81.1–12.8.12.

[25] J.P. O'Callaghan, K. Sriram, Focused microwave irradiation of the brain preserves *in vivo* protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins, J. Neurosci. Methods 135 (2004) 159–168.

[26] J.P. O'Callaghan, K. Sriram, GFAP and other glial proteins as biomarkers of neurotoxicity, Expert Opinion in Drug Safety 4 (2005) 433–442.

[27] P. Polychronopoulos, P. Magiatis, L. Skaltsounis, V. Myrianthopoulos, E. Mikros, A. Tarricone, A. Musacchio, S.M. Roe, L. Pearl, M. Leost, P. Greengard, L. Meijer, Structural basis for the synthesis of indirubins as potent and selective inhibitors of glycogen synthase kinase-3 and cyclin-dependent kinases, J. Med. Chem. 47 (2004) 935–946.

[28] A. Primot, B. Baratte, M. Gompel, A. Borgne, S. Liabeuf, J.L. Romette, F. Costantini, L. Meijer, Purification of GSK-3 by affinity chromatography on immobilised axin, Protein Expr. & Purif. 3 (2000) 394–404.

[29] D. Qu, J. Rashidian, M.P. Mount, H. Aleyasin, M. Parsanejad, A. Lira, E. Haque, Y. Zhang, S. Callaghan, M. Daigle, M.W.C. Rousseaux, R.S. Slack, P.R. Albert, I. Voincent, J.M. Woulfe, D.S. Park, Role of Cdk5-mediated phosphorylation of Prx2 in MPTP toxicity and Parkinson's disease, Neuron 55 (2007) 37–52.

[30] J. Ribas, K. Bettayeb, Y. Ferandin, M. Knockaert, F. Totzke, C. Schächtele, J. Mester, P. Polychronopoulos, P. Magiatis, A.L. Skaltsounis, J. Boix, L. Meijer, 7-bromo-indirubins induce caspase-independent cell death, Oncogene 25 (2006) 6304–6318.

[31] P.D. Smith, S.J. Crocker, V. Jackson-Lewis, K.L. Jordan-Sciutto, S. Hayley, M.P. Mount, M.J. O'Hare, S. Callaghan, R.S. Slack, S. Przedborski, H. Anisman, D.S. Park, Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease, Proc. Natl. Acad. Sci. USA 100 (2003) 13650–13655.

[32] K. Sriram, S.A. Benkovic, M.A. Hebert, D.B. Miller, J.P. O'Callaghan, Activation of the gp130-JAK/STAT3 pathway in astrocytes precedes the induction of GFAP in the MPTP model of dopaminergic neurodegeneration: key signaling pathway for astrogliosis? J. Biol. Chem. 279 (2004) 19936–19947.

[33] K. Sriram, D.B. Miller, J.P. O'Callaghan, Minocycline attenuates microglial activation but fails to mitigate striatal dopaminergic neurotoxicity : role of tumor necrosis factor-α, J. Neurochem. 96 (2006) 706–718.

[34] W. Wang, Y. Yang, C. Ying, W. Li, H. Ruan, X. Zhu, Y. You, Y. Han, R. Chen, Y. Wang, M. Li, Inhibition of glycogen synthase kinase-3beta protect dopaminergic neurons from MPTP toxicity, Neuropharmacology 52 (2007) 1678–1684.

[35] F.-Y. Wei, K. Tomizawa, T. Ohshima, Akiko Asada, T. Saito, C. Nguyen, J.A. Bibb, K. Ishiguro, A.B. Kulkarni, H.C. Pant, K. Mikoshiba, H. Matsui, S.J. Hisanaga, Control of cyclin-dependent kinase 5 (Cdk5) activity by glutamatergtic regulation of p35 stability, Neurochem. 93 (2005) 502–512.