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Synthesis of novel 5-substituted indirubins as protein kinases inhibitors

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Abstract—In an effort to identify new pharmacological inhibitors of disease-relevant protein kinases with increased potency and selectivity, we synthesized and evaluated new 5-substituted indirubins. The effects of 34 indirubin derivatives on CDK1/cyclin B, CDK5/p25, and GSK-3, as well as on SH-SY5Y human neuroblastoma cell survival, were investigated.

1. Introduction

Because of their involvement in all major human diseases protein kinases have become a major screening target in the pharmaceutical industry[1]. Cyclin-dependent kinases (CDKs) constitute a family of highly conserved protein kinases targeted for the therapy of cancer, viral infections and neurodegenerative diseases. Glycogen synthase kinases (GSK-3) play a key role in neurodegenerative disorders (Alzheimer's disease), diabetes, and cardiovascular diseases. Both families of kinases have thus been extensively used as targets to identify small molecular weight pharmacological inhibitors of potential therapeutic interest.[2,3] Over 100 CDK inhibitors and 40 GSK-3 inhibitors have been identified, most of which act by competition with ATP for binding at the kinase catalytic site.[4,5] Among these inhibitors, the bisindole indirubin (Fig. 1, **I**) and its analogs has raised considerable interest.[5] Indirubin is a red-colored isomer of the famous blue dye indigo (Fig. 1, **II**). Interestingly indirubin is the active constituent of a traditional Chinese medicine, Danggui Longhui Wan, reported as a treatment for chronic myelocytic leukemia.[5,6] Indirubin-3′-oxime (Fig. 1, **III**) and indirubin 5-sulfonate show a high selectivity for CDK1 and CDK2.[4] Among indirubin isomers isolated from marine organisms, the natural product 6-bromoindirubin and its synthetic, cell-permeable derivative, 6-bromoindirubin-3′-oxime (Fig. 1, **IV**), display increased selectivity for inhibition of GSK-3 versus CDKs.[7,8]

In the course of our studies of small molecular weight kinase inhibitors of potential therapeutic interest, we synthesized new series of 5-substituted indirubins, with the aim of improving the pharmacological properties of this promising scaffold. The effects of these new indirubins on CDKs (CDK1, CDK5) and GSK-3 were investigated. As this work was in progress, similar oxindole-based CDK inhibitors were described.[9,10]

2. Results and discussion

2.1. Chemistry

In an effort to optimize new kinase inhibitors with increased potency and selectivity, we synthesized various new 5-nitro-indirubins, 5-amino-indirubins, and 5-halogeno-indirubins.

The preparation of substituted indirubins **12**–**27** was accomplished by a route analogous to that described in the literature, that is, by condensation of isatins **1**–**9** with indoxyl acetates **10** or **11** in the presence of Na_2CO_3

Keywords: Indirubin; Cyclin-dependent kinase; Glycogen synthase kinase-3.
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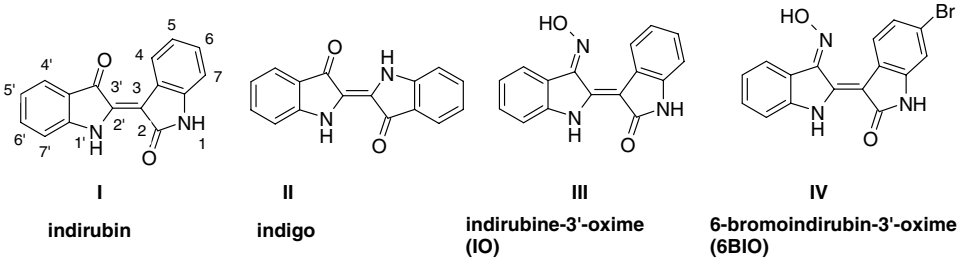
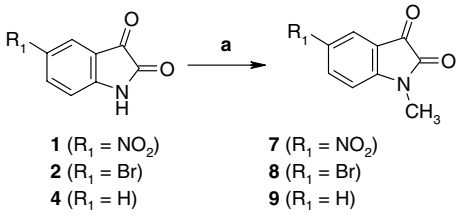


Figure 1. Structure of indirubin, its isomer indigo, and frequently indirubin derivatives.

in methanol (Scheme 1).[4,8,10] The bis-indoles were obtained in a *Z* form. The structure of the *Z* isomers was unambiguously established by an X-ray diffraction study of compound **17**. Because of poor water solubility and low bioavailability of indirubins **12**–**27**, their oxime analogs **28**–**43** were also synthesized with hydroxylamine in pyridine under microwave irradiation[11] (Scheme 1). According to literature data, the oximes were selectively prepared in a (2′*Z*, 3′*E*) form. In order to establish the structure of the different indirubin regioisomers unambiguously, we prepared some previously described compounds **12** and **20** which were used as biological references.

5-Aminoisatin **5** was obtained from commercially available 5-nitroisatin **1** by reduction in the presence of iron in acidic media in 79% yield. Treatment of aminoisatin **5** with acetic anhydride yielded 91% of the corresponding amide **6** (Scheme 2).

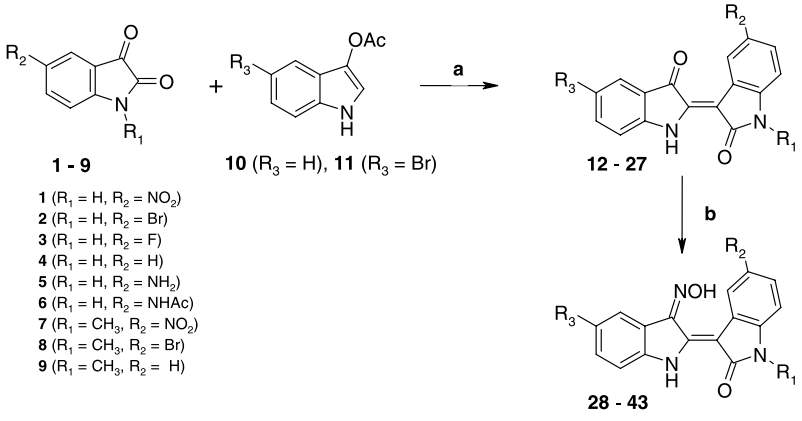
Alkaline treatment of indole-2,3-diones **1**, **2**, **4** with iodomethane afforded the *N*-methylisatin derivatives **7**–**9** in quantitative yield (Scheme 3).



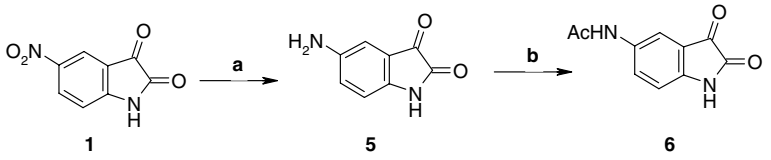
Scheme 3. Reagents and condition: (a) NaH, CH_3I, THF, rt.

2.2. Biology

Indirubin analogs were next tested for their potential inhibitory action on three protein kinases, CDK1/cyclin B, CDK5/p25, and GSK-3α/β. Kinases were purified and assayed in the presence of 15 μM ATP and appropriate protein substrates (histone H1 for CDKs, GS-1 peptide for GSK-3) as previously described.[12–14] For those compounds exhibiting some inhibitory activity at a 10 μM concentration, IC_{50} values were determined from dose–response curves and are provided in Tables 1–3.

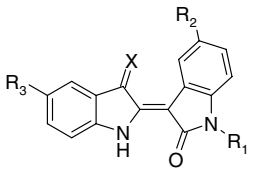


Scheme 1. Reagents and conditions: (a) Na_2CO_3, MeOH, rt, 30–95%; (b) $NH_2OH{\cdot}HCl$, pyridine, μW, 25–90%.



Scheme 2. Reagents and conditions: (a) Fe, HCl 36%, 70 °C; (b) Ac_2O, pyridine, 0 °C.

Table 1. Kinase inhibition by 5-nitroindirubin analogs



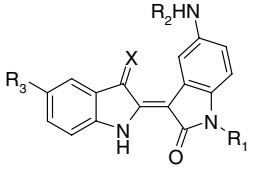
Compound	R_1	R_2	R_3	X	CDK1	CDK5	GSK-3	SH-SY5Y survival	
								24 h	48 h
12[a]	H	NO_2	H	O	40	25	19	>10	>10
13	CH_3	NO_2	H	O	>10	>10	>10	>10	>10
14[b]	H	NO_2	Br	O	>10	>10	>10	>10	>10
15	CH_3	NO_2	Br	O	>10	>10	>10	>10	>10
28[a,b]	H	NO_2	H	NOH	0.019	0.006	0.0021	1.8	1.2
29	CH_3	NO_2	H	NOH	2.2	1.4	0.53	>10	>10
30[b]	H	NO_2	Br	NOH	0.16	0.05	0.055	1.8	1.2
31	CH_3	NO_2	Br	NOH	8	3.3	9.3	>10	1.2

Effects of 5-nitroindirubins on the activity of three protein kinases and on SH-SY5Y cell survival. Kinase assays: IC_{50} values were determined for compounds displaying some activity at an initial 10 μM concentration. SH-SY5Y cells were exposed for 24 h to increasing concentrations of 5-nitroindirubins. Cell survival was estimated by the MTS reduction assay and is expressed in % of survival in untreated cells. Average ± SE of at least four independent experiments with three independent measurements per experiment.

[a] CDK/GSK-3 inhibition also reported in Ref. 7.

[b] Anti-proliferative activity and CDK2 inhibition also reported in Ref. 10.

Table 2. Kinase inhibition by 5-aminoindirubin analogs



Compound	R_1	R_2	R_3	X	CDK1	CDK5	GSK-3	SH-SY5Y survival	
								24 h	48 h
16	H	H	H	O	1.5	0.59	0.08	>10	>10
17	H	Ac	H	O	0.05	0.018	0.0075	>10	>10
18	H	H	Br	O	0.6	0.55	0.44	>10	>10
19	H	Ac	Br	O	0.055	0.11	0.073	>10	>10
32	H	H	H	NOH	0.1	0.15	0.36	6	5
33[a]	H	Ac	H	NOH	0.088	0.16	0.35	10	>10
34	H	H	Br	NOH	0.41	1	6.6	4.8	2
35	H	Ac	Br	NOH	1.4	3.3	40	5.3	4

Effects of 5-aminoindirubins on the activity of three protein kinases and on SH-SY5Y cell survival. Kinase and cellular assays as described in Table 1.

[a] Anti-proliferative activity and CDK2 inhibition also reported in Ref. 10.

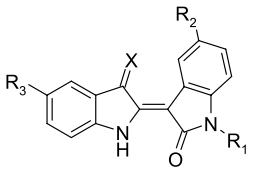
In previously published results, substitution at position 6 contributed to an increased selectivity.[7] Among the known 6-substituted indirubins, the bromo analog (Fig. 1, **III**) exhibited the highest activity against GSK-3. Although it had also been demonstrated that some 5-nitroindirubin derivatives showed an enhanced inhibitory activity on CDK1 or CDK2, no indications on GSK-3 had been reported.[9,10]

First, we considered the nitro-indirubin derivatives (Table 1). 5-Nitroindirubins **12**–**15** displayed little, if any, kinase inhibitory activity. Substitution by an oxime on position 3′ greatly enhanced the kinase inhibitory activity (compounds **28**–**31**). For example, compared to 5-nitro-indirubin **12**, 5-nitro-indirubin-3′-oxime **28** presented a much higher inhibitory activity on CDK1/cyclin B, CDK5/p25, and GSK-3 (0.019, 0.006, and 0.0021 μM vs 40, 25, and 19 μM, respectively). Addition of a bromine in position 5′ led to a reduction in kinase inhibition (compounds **30** and **31**).

Unexpectedly, the 1-*N*-methyl-5-nitro-indirubin-3′-oxime **29** and the 1-*N*-methyl-5-nitro-3′-monoxime-5′-bromo-indirubin **31** displayed modest but significant inhibitory activity on the three kinases (Table 1). 1-Methyl derivatives have so far provided kinase inactive control compounds[7]. The reason for the residual activity of compounds **29** and **31** remains to be identified.

We next considered the amino-indirubin derivatives (Table 2). Replacement of the nitro group on position 5 by the electron-donating amino group leads to kinase active

Table 3. Kinase inhibition by 5-halogenoindirubin analogs



Compound	R_1	R_2	R_3	X	CDK1	CDK5	GSK-3	SH-SY5Y survival	
								24 h	48 h
20[a]	H	Br	H	O	0.4	0.25	0.21	>10	>10
21	CH_3	Br	H	O	>10	>10	>10	>10	>10
22	H	Br	Br	O	>10	>10	>10	>10	>10
23	CH_3	Br	Br	O	>10	>10	>10	>10	>10
24[b]	H	F	H	O	>10	>10	>10	>10	>10
25	H	F	Br	O	>10	>10	>10	>10	>10
26	CH_3	H	Br	O	>10	>10	>10	>10	>10
27	CH_3	H	H	O	>10	>10	>10	>10	>10
36[b]	H	F	H	NOH	0.53	0.46	1.3	>10	5
37	H	F	Br	NOH	7.1	23	15	>10	2
38[b]	H	Br	H	NOH	0.033	0.016	0.053	>10	7
39	CH_3	Br	H	NOH	>10	>10	>10	>10	>10
40	H	Br	Br	NOH	0.23	0.1	24[c]	>10	>10
41	CH_3	Br	Br	NOH	>10	>10	>10	>10	>10
42	CH_3	H	H	NOH	>10	>10	>10	>10	>10
43	CH_3	H	Br	NOH	>10	>10	>10	>10	>10

Effects of 5-halogenoindirubins on the activity of three protein kinases and on SH-SY5Y cell survival. Kinase and cellular assays as described in Table 1.

[a] CDK/GSK-3 inhibition also reported in Ref. 7.

[b] Anti-proliferative activity and CDK2 inhibition also reported in Ref. 10.

[c] Precipitates.

indirubins (Table 2). In addition a somewhat enhanced selectivity for GSK-3 over CDKs was generally observed. Among all 5-NH-substituted indirubins reported in Table 2, the acetamidoindirubin 17 appeared to be the most potent GSK-3 inhibitor (IC_{50}: 7.5 nM). In this amino series, addition of a bromo substitution on position 5′ led to a major reduction in GSK-3 inhibition. This contrasted with the enhancement of GSK-3 inhibitory potency observed with 5′-bromo-5-nitro derivatives (Table 1). As expected, the selectivity for CDK versus GSK-3 inhibition was slightly enhanced for 5-amino indirubin substituted with an oxime on 3′ (compounds **32**–**35**). In all cases, addition of an amino or acetamido group on position 5 led to an overall increase in kinase inhibitory effects (Table 2).

We next tested the consequence of substitution at position 5 by a weaker electron-withdrawing group such as a bromine or a fluorine atom. This mainly resulted in a loss of kinase inhibitory activity, except for 5-fluoro-indirubin-3′-oxime (**36**) which exhibited modest kinase inhibitory activity, and for the known, potent 5-bromo-indirubin-3′-oxime **38** (Table 3). Addition of the bromine atom on position 5′ or alkylation on *N*1 did not increase activity. All *N*1-methylated compounds were inactive.

2.3. Cellular viability assays

The synthesized indirubins were next evaluated for their effects on the survival of human neuroblastoma SH-SY5Y cells estimated with an MTS assay as previously described.[15] For those compounds exhibiting some inhibitory activity at a 10 μM concentration, IC_{50} values were determined from dose–response curves and are provided in Tables 1–3. Results show that: (1) all indirubins that are not substituted on 3′ were inactive; (2) all *N*1-methylated indirubins were inactive with the exception of compound **31** which showed some activity after 48 h of treatment; (3) 5-nitroindirubin-3′-oxime (**28**) and 5-nitro-5′-bromo-indirubin-3′-oxime (**30**), 5-amino-indirubin-3′-oxime (**32**), 5-amino-5′-bromoindirubin-3′-oxime (**34**), and 5-acetamino-5′-bromoindirubin-3′-oxime (**35**) comprised cell survival, even after 24 h of treatment; (4) 5-halogeno-indirubin-3′oximes (**36**–**38**) had modest effects; (5) no correlation could be established between the effects of the presented indirubins on kinases and their effects on cell survival.

3. Conclusion

During the past decade, several groups have been interested in indirubin bis-indoles as a protein kinase inhibitory scaffold. We here reported the synthesis of 5-substituted indirubin analogs. These derivatives were evaluated against the catalytic activity of CDK1, CDK5, and GSK-3. Compounds **17**, **19**, **28**, **30**, and **38** are potent, low nanomolar inhibitors of GSK-3. Low but maybe significant kinase inhibitory activity was observed with 1-*N*-methyl indirubin compounds

29 and **31**, in contrast with previously described indirubins substituted in *N*1.

4. Experimental

4.1. Chemistry

Commercial reagents were used as received without additional purification. Melting points were determined using a Köfler melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Paragon 1000PC instrument. ^{1}H and ^{13}C NMR were recorded on a JEOL NMR LA400 (400 MHz) spectrometer in the 'Centre Commun d'Analyses, Université de la Rochelle'. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) which was used as internal standard. Coupling constants J are given in Hz. The mass spectra (HRMS) were recorded on a Varian MAT311 spectrometer in the 'Centre Régional de Mesures Physiques de l'Ouest' (CRMPO), Université de Rennes. Column chromatography was performed by using Merck silica gel (70–230 mesh) at medium pressure. Light petroleum refers to the fraction boiling point 40–60 °C. Other solvents were used without purification. Analytical thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F254 aluminium-backed plates. Focused microwave irradiations were carried out with a CEM Discover™ focused microwave reactor (300 W, 2450 MHz, monomode system), having in situ magnetic variable speed rotation, irradiation monitored by PC computer, infrared measurement and continuous feedback temperature control. Experiments may be performed at atmospheric pressure or in a sealed tube in pressure-rated reaction tubes with continuous pressure measurement.

Starting materials **1**, **2**, **3**, **4**, and **9** are commercially available. Spectral data for compounds **12**, **20**, **24**, and **38** are consistent with assigned structures as previously described.[7] Spectral data for compounds **14**, **28**, **33**, **36** are consistent with assigned structures as previously described.[10]

4.1.1. Synthesis of 5-*N*-substituted-isatins

4.1.1.1. 5-Aminoisatin (5). Under an inert atmosphere of argon, 5-nitroisatin **1** (0.50 g, 2.6 mmol) was dissolved in warm ethanol (10 mL), and reduced iron powder (0.43 g, 7.8 mmol) was added. Concentrated hydrochloric acid (2 mL) was added gradually and the reaction mixture refluxed for 30 min. When the reaction mixture turned colorless, the residue was treated with a saturated solution of sodium carbonate $NaHCO_3$ to pH 8 and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and the filtrate was concentrated under reduced pressure. The crude residue was purified by chromatography on silica gel, with dichloromethane/MeOH (95:5) as eluent to give the amino derivative **5**. Yield: 79%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.60 (s, NH), 6.82 (dd, 1H, J = 2.4, 8.4 Hz, H-6), 6.71 (d, 1H, J = 2.4 Hz, H-4), 6.63 (d, 1H, J = 8.4 Hz, H-7), 5.12 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 185.31, 159.28, 44.76, 140.73, 123.46, 117.99, 112.60, 108.98. IR = 3196, 1730, 1616, 1499, 1335, 1200, 834 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_8H_6N_2O_2$): calcd 162.0429, found 162.0421.

4.1.1.2. 5-Acetamidoisatin (6). Under an inert atmosphere of argon, to a solution of amine **5** (0.17 g, 1.05 mmol) in pyridine (3 mL) was added dropwise acetic anhydride (0.10 mL, 1.05 mmol). After 3 h under stirring at 0 °C, the residue was treated with a saturated solution of sodium carbonate $NaHCO_3$ and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and the filtrate was concentrated under reduced pressure. The crude residue was purified by chromatography on silica gel, with dichloromethane/ethyl acetate (5:5) as eluent to give the acetamide **3**. Yield: 91%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.88 (s, NH), 9.940 (s, NH), 7.78 (d, 1H, J = 2.4 Hz, H-4), 7.64 (dd, 1H, J = 2.4, 8.4 Hz, H-6), 6.86 (d, 1H, J = 8.4 Hz, H-7), 2.02 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 184.99, 169.32, 159.99, 146.44, 134.87, 129.46, 117.99, 115.86, 112.90, 24.07. IR = 3095, 1736, 1498, 1289, 1197 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{10}H_8N_2O_3$): calcd 204.05349, found 204.0530.

4.1.2. General procedure for the synthesis of 1-*N*-methyl isatin derivatives. Under an inert atmosphere of argon, to a suspension of isatin (5 mmol) and sodium hydride at 60% dispersion in oil (0.17 g, 5 mmol) in DMF (10 mL) was added dropwise methyl iodide (0.7 mL, 5 mmol). After 12 h under stirring at room temperature, the residue was treated with water and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and the filtrate was concentrated under reduced pressure. The crude residue was recrystallized from ethanol.

4.1.2.1. 1-*N*-Methyl-5-nitroisatin (7). Yield: 93%. Mp 202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.53 (dd, 1H, J = 2.4, 8.8 Hz, H-6), 8.22 (d, 1H, J = 2.4 Hz, H-4), 7.35 (d, 1H, J = 8.8 Hz, H-7), 3.31 (s, 3H, CH_3). IR = 3110, 1717, 1656, 1507, 1335, 1276, 1132, 754 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_9H_6N_2O_4$): calcd 206.03276, found 206.0322.

4.1.2.2. 1-*N*-Methyl-5-bromoisatin (8). Yield: 99%. Mp 170 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.83 (dd, 1H, J = 2.0, 8.8 Hz, H-6), 7.68 (d, 1H, J = 8.8 Hz, H-6), 7.11 (d, 1H, J = 8.8 Hz, H-7), 3.12 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 184.99, 169.32, 159.99, 146.44, 134.87, 129.46, 117.99, 115.86, 112.90, 24.07. IR = 3095, 1736, 1498, 1289, 1197 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_9H_6NO_2{}^{79}Br$): calcd 238.95819, found 238.9565.

4.1.3. General procedure for the synthesis of indirubin derivatives. Under an inert atmosphere of argon, a solution of isatin derivatives **1**–**9** (3 mmol), indoxyl acetate **10** or **11** (5 mmol) in methanol (15 mL) was vigorously stirred with Na_2CO_3. After 3 h under stirring at room temperature, the dark violet residue was filtered and successively and intensively washed with methanol and cold

water. The solid was dried over P_2O_5 under reduced pressure (30–95%).

4.1.3.1. (2′Z)-5-Nitroindirubin (12). Yield: 89%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.48 (s, 1H, N′H), 11.18 (s, 1H, NH), 9.68 (s, 1H, H-4), 8.18 (d, 1H, J = 8.4 Hz, H-6), 7.70 (d, 1H, J = 8.0 Hz, H-4′), 7.61 (t, 1H, J = 8.0 Hz, H-6′), 7.44 (d, 1H, J = 8.4 Hz, H-7), 7.05–7.10 (m, 2H, H-7′, H-5′). IR = 3314, 3094, 2874, 1687, 1593, 1522 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_3O_4$): calcd 307.05931, found 307.0565.

4.1.3.2. (2′Z)-1-Methyl-5-nitroindirubin (13). Yield: 90%. Mp > 260 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 10.54 (s, 1H, N′H), 9.82 (d, 1H, J = 2.0 Hz, H-4), 8.29 (dd, 1H, J = 7.6 Hz, H-6), 7.80 (d, 1H, J = 7.6 Hz, H-4′), 7.57 (t, 1H, J = 7.6 Hz, H-6′), 7.10 (t, 1H, J = 7.6 Hz, H-6′), 7.04 (d, 1H, J = 7.5 Hz, H-5′), 6.97 (d, 1H, J = 8.8 Hz, H-7), 3.43 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 181.98, 157.87, 152.75, 151.68, 124.80, 121.82, 118.18, 105.07, 26.30. IR = 3294, 3098, 1675, 1612, 1522, 1473, 1089, 749 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{12}N_3O_4$): calcd 321.07496, found 321.0772.

4.1.3.3. (2′Z)-5′-Bromo-5-nitroindirubin (14). Yield: 93%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.32 (s, 1H, N′H), 9.65 (d, 1H, J = 2.4 Hz, H-4), 8.19 (dd, 1H, J = 2.4, 8.4 Hz, H-6), 7.84 (d, 1H, J = 2.4 Hz, H-4′), 7.76 (dd, 1H. J = 2.4, 8.4 Hz, H-6′), 7.42 (d, 1H, J = 8.4 Hz, H-7), 7.07 (d, 1H, J = 8.4 Hz, H-7′). IR = 3312, 1687,1605, 1464, 1287, 1193, 818 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_3O_4{}^{79}Br$): calcd 384.96982, found 384.9725.

4.1.3.4. (2′Z)-1-Methyl-5′-bromo-5-nitroindirubin (15). Yield: 70%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.33 (s, 1H, N′H), 9.68 (d, 1H, J = 2.0 Hz, H-4), 8.28 (dd, 1H, J = 2.0, 8.8 Hz, H-6), 7.85 (d, 1H, J = 2.0 Hz, H-4′), 7.77 (dd, 1H, J = 2.0, 8.8 Hz, H-6′), 7.43 (d, 1H, J = 8.8 Hz, H-7′), 7.30 (d, 1H,..J = 8.8 Hz, H-7). IR = 3300, 1680, 1610, 1466, 1117, 824 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{11}N_2O_2{}^{79}Br$): calcd 398.98547, found 398.9886.

4.1.3.5. (2′Z)-5-Aminoindirubin (16). Yield: 90%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.96 (s, 1H, N′H), 10.48 (s, 1H, NH), 8.17 (d, 1H, J = 2.0 Hz, H-4), 7.68 (d, 1H, J = 7.2 Hz, H-4′), 7.60 (t, 1H, 7.2 Hz, H-6′), 7.34 (d, 1H, J = 8.4 Hz, H-7′), 7.06 (t, 1H, J = 7.2 Hz, H-5′), 6.70 (d, 1H, J = 8.0 Hz, H-7), 6.61 (dd, 1H, J = 2.0, 8.0 Hz, H-6), 4.74 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 189.15, 171.48, 152.71, 143.46, 138.47, 138.00, 132.76, 125.06, 122.48, 122.02, 119.58, 116.70, 113.69, 112.26, 110.68,1 08.59. IR = 3226, 1767, 1730, 1713, 1612, 1480, 1295, 1195, 1002 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{11}N_3O_2$): calcd 277.08513, found 277.0838.

4.1.3.6. (2′Z)-5-Acetamidoindirubin (17). Yield: 90%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.91 (s, N′H), 9.85 (s, NH), 8.88 (s, 1H, H-4), 7.68 (d, 1H, J = 7.2 Hz, H-4′), 7.60 (t, 2H, J = 8.0 Hz, H-5′, H-7′), 7.50 (d, NH, J = 8.0 Hz, NH-acetamide), 7.41 (d, 1H, J = 8.0 Hz, H-6), 7.07 (t, 1H, J = 7.2 Hz, H-5′), 6.88 (d, 1H, J = 8.0 Hz, H-7), 2.06 (s, 3H, CH_3). IR = 3277, 1732, 1665, 1475, 1313, 1196, 1004 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{18}H_{13}N_3O_3$): calcd 319.09569, found 319.0961.

4.1.3.7. (2′Z)-5-Amino-5′-bromoindirubin (18). Yield: 97%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.01 (s, 1H, N′H), 10.47 (s, 1H, NH), 8.13 (d, 1H, J = 2.0 Hz, H-4), 7.75 (d, 1H, J = 2.0 Hz, H-4′), 7.71 (dd, 1H, J = 2.0, 8.4 Hz, H-6′), 7.40 (d, 1H. J = 8.4 Hz, H-7′), 6.59 (d, 1H, J = 8.4 Hz, H-7), 6.55 (dd, 1H, J = 2.0, 8.4 Hz, H-6). IR = 3278, 1664, 1464, 1278, 1195, 1120, 1015, 819 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{10}N_3O_2{}^{79}Br$): calcd 354.99564, found 354.9973.

4.1.3.8. (2′Z)-5-Acetamido-5′-bromoindirubin (19). Yield: 85%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 11.12 (s, 1H, N′H), 10.83 (s, 1H, NH), 9.91 (s, 1H, NH), 8.81 (s, 1H, H-4′), 7.76 (s, 1H, H-4), 7.71 (dd, 1H. J = 2.0, 8.8 Hz, H-6′), 7.56 (dd, 1H, J = 2.0, 8.8 Hz, H-6), 7.37 (d, 1H, J = 8.8 Hz, H-7′), 6.82 (d, 1H, J = 8.8 Hz, H-7), 2.03 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 187.26, 170.87, 167.89, 151.42, 139.09, 137.94, 137.10, 133.31, 126.41, 121.77, 121.29, 120.81, 117.23, 115.72, 112.82, 109.27, 107.73, 23.78. IR = 3267, 1733, 1667, 1463, 1190, 1121, 1016, 820 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{18}H_{12}N_3O_3{}^{79}Br$): calcd 397.00620, found 397.051.

4.1.3.9. (2′Z)-5-Bromoindirubin (20). Yield: 95%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.02 (s, 1H, N′H), 8.93 (d, 1H, J = 1.9 Hz, H-4), 7.66 (d, 1H, J = 7.3 Hz, H-4′), 7.56 (t, 1H. J = 8.3 Hz, H-6), 7.38–7.44 (m, 2H, H-5′, H-7′), 7.04 (t, 1H, J = 7.3 Hz, H-6′), 6.86 (d, 1H, J = 8.3 Hz, H-7). IR = 3152, 1673, 1590, 1466, 1214, 1004, 801, 580 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_2O_2Br$): calcd 321.07496, found 321.0772.

4.1.3.10. (2′Z)-1-Methyl-5-bromoindirubin (21). Yield: 30%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.14 (s, 1H, N′H), 8.96 (s, 1H, H-4), 7.68 (d, 1H, J = 8.0 Hz, H-4′), 7.60 (t, 1H, J = 8.0 Hz, H-6′), 7.50 (d, 1H, J = 8.4 Hz, H-6), 7.43 (d, 1H, J = 8.4 Hz, H-7), 7.04–7.06 (m, 2H, H-5′, H-7′), 3.28 (d, 3H, CH_3). IR = 3305, 1653, 1598, 1462, 1320, 1103, 1081, 753 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{11}N_2O_2{}^{79}Br$): calcd 354.00039, found 354.0011.

4.1.3.11. (2′Z)-5,5′-Dibromoindirubin (22). Yield: 79%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.11 (s, 1H, N′H), 8.90 (s, 1H, H-4), 7.77 (s, 1H, H-4′), 7.73 (d, 1H, J = 8.4 Hz, H-6), 7.41 (s, 2H, H-6′, H-7′), 6.85 (d, 1H, J = 8.4 Hz, H-7). IR = 3488, 1669, 1611, 1462, 1270, 1206, 1123, 1017, 822 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_8N_2O_2{}^{79}Br$): calcd 417.89525, found 417.89892.

4.1.3.12. (2′Z)-5-Fluoroindirubin (24). Yield: 95%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.09 (s, 1H, N′H), 10.89 (s, 1H, NH), 8.57 (dd, 1H, J = 2.8, 7.2 Hz, H-6′), 7.67 (d, 1H, J = 7.2 Hz, H-4′), 7.59 (t, 1H. J = 7.2 Hz, H-5′), 7.44 (d, 1H, J = 7.6 Hz, H-4), 7.09 (td, 1H, J = 2.8, 7.6 Hz, H-6), 7.04 (d, 1H, J = 7.2 Hz, H-7′), 6.88 (m, 1H, H-7). IR = 3331, 3196, 1667, 1600, 1468, 1309, 1187, 1148, 1003, 818, 753 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_2O_2F$): calcd 280.06481, found 280.0644.

4.1.3.13. (2′Z)-5′-Bromo-5-fluoroindirubin (25). Yield: 80%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.01 (s, 1H, N′H), 8.53 (dd, 1H, J = 2.4, 8.4 Hz, H-6′), 7.77 (s, 1H, H-4′), 7.73 (d, 1H, J = 8.4 Hz, H-7′), 7.39 (d, 1H, J = 8.4 Hz, H-4), 7.08 (td, 1H, J = 2.4, 4.4 Hz, H-6), 6.86 (m, 1H, H-7), 4.09 (s, 1H, NH). IR = 3159, 1737, 1667, 1595, 1466, 1283, 1015, 819 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_8N_2O_2{}^{79}BrF$): calcd 357.97532, found 357.9727.

4.1.3.14. (2′Z)-5′-Bromo-1-methylindirubin (26). Yield: 63%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.11 (s, 1H, N′H), 8.77 (d, 1H, J = 7.8 Hz, H-4), 7.77 (d, 1H, J = 1.9 Hz, H-4′), 7.73 (dd, 1H, J = 1.9, 8.4 Hz, H-6′), 7.40 (d, 1H, J = 8.4 Hz, H-7′), 7.35 (td, 1H, J = 1.9, 7.8 Hz, H-6), 7.07–7.11 (m, 2H, H-5, H-7). IR = 3307, 1672, 1607, 1469, 1127, 752 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{11}N_2O_2{}^{79}Br$): calcd 354.00039, found 354.0022.

4.1.3.15. (2′Z)-1-Methylindirubin (27). Yield: 58%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_{63}) δ ppm 11.04 (s, 1H, N′H), 8.81 (d, 1H, J = 8.3 Hz, H-4), 7.66 (d, 1H, J = 7.3 Hz, H-4′), 7.59 (t, 1H, J = 7.3 Hz, H-6′), 7.43 (d, 1H, J = 7.3 Hz, H-7′), 7.35 (t, 1H, J = 8.3 Hz, H-6), 7.07–7.12 (m, 2H, H-5, H-7), 7.03 (t, 1H, J = 7.3 Hz, H-5′), 3.24 (s, 3H, CH_3). IR = 3305, 1656, 1608, 1482, 1102, 748 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{12}N_2O_2$): calcd 276.08988, found 276.0928.

4.1.4. General procedure for the synthesis of 3′-oxime indirubin derivatives. Under an inert atmosphere of argon, to a stirred solution of indirubin derivatives **16**–**35** (3 mmol) in pyridine (5 mL) was added 2.1 g (30 mmol) of hydroxylamine hydrochloride. The mixture was irradiated during 10 min. The irradiation in CEM oven was programmed to maintain a constant temperature (110 °C) with a maximal power output of 150 W. After cooling, the pyridine was removed under reduced pressure. The crude material was successively and intensively washed with cold water and acetone. The solid was dried over P_2O_5 under reduced pressure (25–90%).

4.1.4.1. (2′Z,3′E)-5-Nitroindirubin-3′-oxime (28). Yield: 92%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.88 (s, 1H, NOH), 11.89 (s, 1H, N′H), 11.39 (s, 1H, NH), 9.47 (d, 1H, J = 2.4 Hz, H-4), 8.27 (d, 1H, J = 8.4 Hz, H-7), 8.09 (dd, 1H, J = 2.4, 8.4 Hz, H-6), 7.49 (d, 1H, J = 7.2 Hz, H-4′), 7.44 (td, 1H, J = 1.6, 7.2 Hz, H-5′), 7.10 (td, 1H, J = 1.6, 7.2 Hz, H-6′), 7.06 (d, 1H, J = 8.4 Hz, H-7′). IR = 3281, 1732, 1671, 1615, 1459, 1328, 1220, 1084, 1019, 747 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{10}N_4O_4$): calcd 322.07020, found 322.0707.

4.1.4.2. (2′Z,3′E)-1-Methyl-5-nitroindirubin-3′-oxime (29). Yield: 44%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.95 (s, 1H, NOH), 11.90 (s, 1H, N′H), 9.50 (d, 1H, J = 2.4 Hz, H-4), 8.26 (d, 1H, J = 8.0 Hz, H-4′), 8.15 (dd, 1H, J = 2.4, 8.8 Hz, H-6), 7.50 (d, 1H, J = 8.0 Hz, H-7′), 7.44 (t, 1H, J = 7.6 Hz, H-5′), 7.26 (d, 1H, J = 8.8 Hz, H-7), 7.10 (t, 1H, J = 7.6 Hz, H-6′), 3.39 (s, 3H, CH_3). IR = 3242, 1602, 1557, 1510, 1465, 1338, 1125, 1078, 1021, 748 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{12}N_4O_4$): calcd 336.08586, found 336.0861.

4.1.4.3. (2′Z,3′E)-5′-Bromo-5-nitroindirubin-3′-oxime (30). Yield: 78%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 14.14 (s, 1H, NOH), 11.90 (s, 1H, N′H), 11.39 (s, 1H, NH), 9.43 (d, 1H, J = 2.4 Hz, H-4), 8.36 (d, 1H, J = 2.0 Hz, H-4′), 8.09 (dd, 1H, J = 2.4, 8.8 Hz, H-6), 7.61 (dd, 1H, J = 2.0, 8.8 Hz, H-6′), 7.46 (d, 1H, J = 8.8 Hz, H-7′), 7.05 (d, 1H, J = 8.8 Hz, H-7). IR = 3279, 1752, 1715, 1452, 1335, 1285, 1024 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_4O_4{}^{79}Br$): calcd 399.98072, found 399.9789.

4.1.4.4. (2′Z,3′E)-1-Methyl-5′-bromo-5-nitroindirubin-3′-oxime (31). Yield: 49%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 14.21 (s, 1H, NOH), 11.93 (s, 1H, N′H), 9.46 (s, 1H, H-4), 8.35 (s, 1H, H-4′), 8.16 (d, 1H, J = 8.4 Hz, H-6′), 7.62 (d, 1H, J = 8.4 Hz, H-7′), 7.47 (d, 1H, J = 8.4 Hz, H-6), 7.25 (d, 1H, J = 8.4 Hz, H-7), 3.38 (s, 3H, CH_3). IR = 3254, 1877, 1554, 1453, 1284, 1079, 1024, 817 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{11}N_4O_4{}^{79}Br$): calcd 413.99637, found 413.9989.

4.1.4.5. (2′Z,3′E)-5-Aminoindirubin-3′-oxime (32). Yield: 84%. Mp > 260 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 13.18 (s, 1H, NOH), 11.67 (s, 1H, N′H), 10.21 (s, 1H, NH), 8.22 (d, 1H, J = 8.4 Hz, H-4′), 8.03 (d, 1H, J = 2.0 Hz, H-4), 7.37 (t, 1H, J = 7.2 Hz, H-5′), 7.33 (d, 1H, J = 7.2 Hz, H-7′), 6.99 (t, 1H, J = 7.2 Hz, H-6′), 6.60 (d, 1H, J = 8.4 Hz, H-7), 6.55 (dd, 1H, J = 2.0, 8.4 Hz, H-6), 4.21 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 170.85, 151.06, 144.82, 144.51, 141.87, 131.79, 130.04, 127.79, 123.17, 120.97, 116.37, 112.54, 111.08, 110.67, 108.68, 30.67. IR = 3296, 1737, 1714, 1573, 1473, 1325, 1238, 999, 747 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{13}N_4O_2$): calcd 293.10385, found 293.10342.

4.1.4.6. (2′Z,3′E)-5-Acetamidoindirubin-3′-oxime (33). Yield: 25%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.26 (s, 1H, NOH), 11.69 (s, 1H, N′H), 10.35 (s, 1H, NH), 8.19 (d, 1H, J = 8.0 Hz, H-4′), 8.07 (s, 1H, H-4), 7.38 (t, 1H, J = 8.0 Hz, H-5′), 7.22 (dd, 1H, J = 4.0 Hz, H-6), 7.03 (t, 1H, J = 7.6 Hz, H-6′), 6.72 (d, 1H, J = 8.0 Hz, H-7), 6.57 (d, 1H, J = 7.6 Hz, H-7′), 2. 05 (s, 3H, CH_3). IR = 3420, 1740, 1660, 1466, 1319, 1200, 1003 cm^{-1}.

4.1.4.7. (2′Z,3′E)-5′-Bromo-5-aminoindirubin-3′-oxime (34). Yield: 78%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.71 (s, 1H, NOH), 10.41 (s, 1H, N′H), 8.32 (d, 1H, J = 2.0 Hz, H-4′), 8.24 (s, 1H, H-4), 7.56 (dd, 1H, J = 2.0, 8.4 Hz, H-6′), 7.36 (d, 1H, J = 8.4 Hz, H-7′), 6.72 (d, 1H, J = 8.4 Hz, H-7), 6.63 (dd, 1H, J = 2.0, 8.4 Hz, H-6), 3.25 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 196.31, 170.64, 167.60, 166.82, 163.55, 149.99, 144.46, 144.00, 134.21, 129.64, 123.10, 117.04, 113.49, 112.38, 108.90, 99.92. IR = 3218, 1746, 1665, 1457, 1303, 1007, 805 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{18}H_{15}N_4O_2{}^{79}BrNa$): calcd 421.02761, found 421.0283.

4.1.4.8. (2′Z,3′E)-5-Acetamido-5′-bromoindirubin-3′-oxime (35). Yield: 41%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.68 (s, 1H, NOH), 10.25 (s, 1H, N′H), 8.33 (s, 1H, H arom, H-4), 8.01 (s, 1H, H arom, H-4′), 7.53 (dd, 1H, H arom, J = 2.0, 8.4 Hz, H-6′), 7.25 (d, 1H, H arom, J = 8.4 Hz, H-7′), 6.63 (d, 1H, H arom, J = 8.0 Hz, H-7), 6.47 (dd, 1H, H arom, J = 2.0, 8.0 Hz, H-6), 2.05 (s, 3H, CH_3). IR = 3243, 2939. 2314. 1735, 1663, 1604, 1456, 1295, 1005, 805 cm^{-1}.

4.1.4.9. (2′Z,3′E)-5-Fluoroindirubin-3′-oxime (36). Yield: 90%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.56 (s, 1H, NOH), 11.78 (s, 1H, N′H), 10.65 (s, 1H, NH), 8.46 (dd, 1H, H arom, J = 2.4, 10.8 Hz), 8.23 (d, 1H, H arom, J = 8.0 Hz), 7.40 (t, 1H, H arom, J = 7.2 Hz), 7.37 (t, 1H, H arom, J = 7.2 Hz), 7.06 (t, 1H, H arom, J = 7.2 Hz), 6.93 (td, 1H, H arom, J = 2.4, 8.0 Hz), 6.85 (dd, 1H, H arom, J = 5.2, 8.0 Hz). IR = 3179, 2360, 1741, 1666, 1573, 1465, 1321, 1234, 1174, 968,753 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{10}N_3O_2F$): calcd 295.07570, found 295.0767.

4.1.4.10. (2′Z,3′E)-5′-Bromo-5-fluoroindirubin-3′-oxime (37). Yield: 68%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.89 (s, 1H, NOH), 11.81 (s, 1H, N′H), 10.74 (s, 1H, NH), 8.44 (dd, 1H, H arom, J = 1.6, 10.8 Hz), 8.32 (s, 1H, H arom), 7.59 (dd, 1H, H arom, J = 1.6, 8.0 Hz), 7.42 (d, 1H, H arom, J = 8.0 Hz), 6.95 (td, 1H, H arom, J = 2.4, 8.8 Hz), 6.84 (dd, 1H, H arom, J = 5.2, 8.8 Hz). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 171.31, 159.09, 156.78, 150.84, 150.02, 145.80, 144.40, 135.29, 134.85, 130.25, 117.60, 114.24, 113.34, 112.66, 112.42, 110.36, 110.09, 109.57, 109.49. IR = 3165, 2792, 1732, 1466, 1290, 1016, 796 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_3O_2$): calcd372.98622, found 372.9867.

4.1.4.11. (2′Z,3′E)-5-Bromoindirubin-3′-oxime (38). Yield: 72%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.82 (s, 1H, NOH), 10.76 (s, 1H, N′H), 8.71 (s, 1H, H-4), 8.19 (d, 1H, J = 8.0 Hz, H-6), 7.39 (t, 1H, J = 7.2 Hz, H-6′), 7.25 (m, 2H, H-4′, H-7′), 7.05 (t, 1H, J = 7.2 Hz, H-5′), 6.88 (d, 1H, J = 8.0 Hz, H-7). IR = 3118, 1726, 1666, 1570, 1467, 1321, 1227, 1152, 748 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{10}N_3O_2{}^{79}Br$): calcd 354.99564, found 354.9940.

4.1.4.12. (2′Z,3′E)-1-Methyl-5-bromoindirubin-3′-oxime (39). Yield: 84%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.73 (s, 1H, NOH), 11.83 (s, 1H, N′H), 8.79 (d, 1H, J = 2.0 Hz, H-4), 8.23 (d, 1H, J = 7.6 Hz, H-4′), 7.39–7.44 (m, 2H, H-6′, H-7′), 7.36 (dd, 1H, J = 2.0, 8.0 Hz, H-6), 7.05 (t, 1H, J = 7.6 Hz, H-5′), 7.01 (d, 1H, J = 8.0 Hz, H-7), 3.31 (s, 3H, CH_3). IR = 3473, 3240, 1769, 1649, 1465, 1333, 1117, 1015, 973, 799 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{12}N_3O_2{}^{79}Br$): calcd 369.0113, found 369.0091.

4.1.4.13. (2′Z,3′E)-5,5′-Bromoindirubin-3′-oxime (40). Yield: 58%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.91 (s, 1H, NOH), 11.83 (s, 1H, N′H), 10.84 (s, 1H, NH), 8.71 (s, 1H, H-4′), 8.34 (s, 1H, H-4), 7.59 (dd, 1H, J = 2.0, 8.4 Hz, H-6′), 7.42 (d, 1H, J = 8.4 Hz, H-7′), 7.28 (dd, 1H, J = 2.0, 8.4 Hz, H-6), 6.83 (d, 1H, J = 8.4 Hz, H-7). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 170.88, 150.88, 145.99, 144.33, 137.89, 134.87, 130.32, 128.75, 125.36, 125.01, 118.64, 114.27, 113.43, 113.12, 110.96, 98.91. IR = 3209, 1726, 1661, 1609, 1568, 1304, 1011, 799 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_3O_2{}^{79}Br_2$): calcd 432.90615, found 432.9035.

4.1.4.14. (2′Z,3′E)-1-Methyl-5,5′-bromoindirubin-3′-oxime (41). Yield: 67%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 14.00 (s, 1H, NOH), 11.86 (s, 1H, N′H), 8.77 (s, 1H, H-4), 8.34 (s, 1H, H-4′), 7.60 (dd, 1H, J = 2.0, 8.0 Hz, H-6′), 7.43 (d, 1H, J = 8.0 Hz, H-7′), 7.38 (dd, 1H, J = 2.0, 8.4 Hz, H-6), 7.02 (d, 1H, J = 8.4 Hz, H-7), 3.27 (s, 3H, CH_3). IR = 3141, 1653, 1568, 1458, 1162, 1023, 797 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{11}N_3O_2{}^{79}Br_2$): calcd 446.92180, found 446.9193.

4.1.4.15. (2′Z,3′E)-1-Methyl-indirubin-3′-oxime (42). Yield: 85%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.52 (s, 1H, NOH), 11.735 (s, 1H, N′H), 8.68 (d, 1H, J = 7.8 Hz, H-4), 8.23 (d, 1H, J = 7.2 Hz, H-4′), 7.40 (m, 2H, H-6′, H-7′), 7.21 (t, 1H, J = 7.8 Hz, H-6), 7.025 (m, 3H, H-5′, H-5, H-7), 3.32 (s, 3H, CH_3). IR = 2979, 1646, 1596, 1454, 1340, 1136, 1011, 748 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{13}N_3O_2$): calcd 291.10078, found 291.1019.

4.1.4.16. (2′Z,3′E)-1-Methyl-5′-bromoindirubin-3′-oxime (43). Yield: 86%. Mp > 260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.70 (s, NOH), 11.73 (s, NH), 8.65 (d, 1H, H arom, J = 8.4 Hz), 8.33 (s, 1H, H arom, H4′), 7.67 (d, 1H, H arom, J = 8.4 Hz), 7.38 (d, 1H, H arom, J = 8.4 Hz), 7.23 (t, 1H, H arom, J = 7.2 Hz, H4 or H5), 7.04 (d, 1H, H arom, J = 7.2 Hz, H2 or H5), 7.00 (d, 1H, H arom, J = 7.2 Hz, H2 or H5), 3.30 (s, 3H, CH_3). IR = 2990, 2846. 1741, 1652, 1600, 1560, 1452, 1303, 1159, 1016 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{17}H_{12}N_3O_2{}^{79}Br$): calcd 369.01129, found 369.0091.

4.2. Preparation and assay of protein kinases

CDKs and GSK-3 were first assayed in the presence of 10 μM of each indirubin. For molecules showing inhib-

itory activity at 10 μM, dose–response curves were performed to calculate the IC_{50} value.

Kinase activities were assayed in buffer A or C (unless otherwise stated), at 30 °C, at a final ATP concentration of 15 μM. Blank values were subtracted and activities calculated as picomoles of phosphate incorporated for a 10 min. incubation. The activities are usually expressed in % of the maximal activity, that is, in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethylsulfoxide.

GSK-3α/β was purified from porcine brain by affinity chromatography on immobilized axin.[12] It was assayed, following a 1/100 dilution in 1 mg BSA/mL of 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-^{33}P]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 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.

CDK1/cyclin B was extracted in homogenization buffer 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.[13] The kinase activity was assayed in buffer C, with 1 mg histone H1/mL, in the presence of 15 μM [γ-^{33}P]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.

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

4.3. Cell biology

Cell Titer 96® kit containing the MTS reagent was purchased from Promega (Madison, WI, USA). The protease inhibitor cocktail was from Roche.

SH-SY5Y human neuroblastoma cell line was grown in DMEM (Biowhittaker) supplemented with 2 mM L-glutamine from Eurobio (Courtaboeuf, France), antibiotics, and 10% volume of FCS from Invitrogen (Cergy Pontoise, France). General culture conditions were an atmosphere of 5% CO_2 and a temperature of 37 °C. Culture dishes and other plastic disposable tools were supplied by Corning (Corning, NY, USA). Indirubin treatments were performed on exponentially growing cultures at the indicated time and concentrations. Control experiments were also carried out using appropriate dilutions of DMSO.

Cell viability was determined by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) as previously described in detail.[15]

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