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Synthesis and kinase inhibitory activity of novel substituted indigoids

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ABSTRACT

The bis-indole indigoids are a promising protein kinase inhibitor scaffold to be further evaluated against the numerous human diseases that imply abnormal regulation of kinases including neurodegenerative disorders. In an effort to identify new pharmacological inhibitors of disease-relevant protein kinases with increased potency and selectivity, we designed, synthesized new 5,7-disubstituted or 6-substituted bis-indole derivatives. On the basis of our previous synthetic work, 22 selected compounds were tested on CDK1/cyclin B, CDK5/p25, DYRK1A, CK1, and GSK-3α/β kinases, five kinases involved in Alzheimer's disease. Some of them were also evaluated for their cytotoxic and antiproliferative activities. 6-Nitro-3′-*N*-oxime-indirubin and 5-amino-3′-*N*-oxime-indirubin derivatives exhibited inhibitory activity in a submicromolar range against CDK1/cyclin B (0.18 and 0.1 μM, respectively), CK1 (0.6 μM and 0.13 μM) and GSK3 (0.04 μM and 0.36 μM).

1. Introduction

Phosphorylation of serine, threonine and tyrosine residues by cellular protein kinases plays an important role in the regulation of various cellular processes.[1] Protein kinases constitute the largest family of human enzymes and are considered to be the largest class amenable to therapeutic intervention by small molecule drugs.[2]

Cyclin-dependent kinases (CDKs) are involved in numerous diseases, including cancers, Alzheimer's disease, Parkinson's disease, stroke, diabetes, polycystic kidney disease, glomerulonephritis, inflammation, and AIDS.[3] Glycogen synthase kinase-3 (GSK-3) plays a key role in a large number of cellular processes, apoptosis control, neurodegenerative disorders (Alzheimer's disease) and cardiovascular diseases. Both families of kinases have been extensively used as targets to identify small molecular weight pharmacological inhibitors of potential therapeutic interest.[3b,4] More than 100 CDK inhibitors and 40 GSK-3 inhibitors have been identified, most of them act by competition with ATP for binding at the catalytic site of the kinase.[5,6] Among these inhibitors, the bis-indole indirubin (Fig. 1, **I**) and its analogs have raised considerable interest[6] since they were discovered to inhibit cyclin-dependent kinases (CDKs), glycogen synthase kinase-3 (GSK-3), and glycogen phosphorylase and to interfere with aryl hydrocarbon receptor (AhR).[7] Casein kinase 1 (CK1) constitutes a conserved family of kinases that plays an important role in the control of cell differentiation, proliferation, apoptosis, circadian rhythms and have been implicated in neurodegenerative diseases.[8,9] Dual specificity, tyrosine phosphorylation regulated kinase 1A (DYRK1A) plays a key role in Alzheimer's disease and Down syndrome. CDKs, GSK-3, CK1s, and DYRK1A are involved in the two key molecular features of Alzheimer's disease, production of amyloid-β peptides (extracellular plaques) and hyperphosphorylation of the microtubule-binding protein Tau (intracellular neurofibrillary tangles).[10]

In previous articles, it was demonstrated that 5-substituted-indirubins displayed high inhibitory activities against various CDKs and GSK-3β.[11,12] Among indirubin isomers isolated from marine organisms, the natural product 6-bromoindirubin and its synthetic, more cell permeable derivative, 6-bromoindirubin-3′-oxime (Fig. 1, **II**), also display enhanced selective inhibition of GSK-3 versus CDKs.[7a,h,13]

The high inhibitory potency for GSK-3 and CDKs of 5-nitroindirubin-3′-oxime (Fig. 1, **III**) led our interest to a series of bi-substituted indirubins, namely on position 5 and 7, thereby possible combining selectivity and high activity.[14] On the other hand, the high selectivity for GSK-3 of 6-bromoindirubin and 6-bromoindirubin-3′-oxime prompted us to investigate more extensively the role of the N-substituent at position 6. With the aim of improving the pharmacological properties of this promising bis-indigoid scaffold, we prepared new series of 5-substituted-7-bromoindirubins, 6-substituted indirubins, and isoindigos (isomer of indirubin). Despite considerable work on the bis-indole indirubins,[11,12,15]

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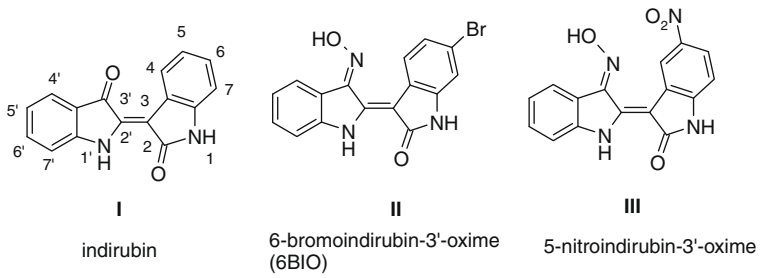


Figure 1. Structure of indirubin, and most studied indirubin derivatives.

CK1 and DYRK1A had never been investigated as potential targets. These compounds were thus screened for potential kinase inhibitory activities on CDK1, CDK5, GSK-3, CK1 and DYRK1A and for antiproliferative effects.

2. Chemistry

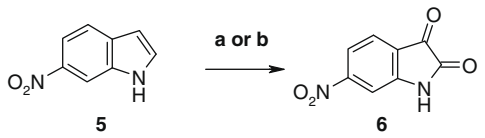
The target 7-bromo-5-nitroisatin **4** was obtained by Sandmeyer reaction from 2-bromoaniline **1** in two steps via the corresponding isonitrosoacetanilide **2** which was treated in concentrated sulfuric acid.[7j,15,16a] The ring closure was followed by a selective nitration of the 7-bromoisatin **3** with $NaNO_3$ in H_2SO_4 (Scheme 1).[16b]

Oxidation of commercially available 6-nitroindole **5** with a catalytic amount of ruthenium trichloride in aqueous acetonitrile with sodium periodate led to a low yield of the expected isatin **6**. Treatment of indole with 2-iodoxybenzoic acid in the presence of indium chloride under microwave irradiation yielded 54% of 6-nitroisatin **6** (Scheme 2).[17]

The preparation of substituted indirubin **12-22** was accomplished as previously described[12] by condensation of isatins **4**–**9** with indoxyl acetates **10** or **11** in the presence of Na_2CO_3 in methanol (Scheme 1, Table 1). Because of the poor water solubility and low bioavailability of indirubins **12–22**, oxime analogs **23–33** were also selectively prepared in a (2′*Z*, 3′*E*) form with hydroxylamine in pyridine. In order to improve the yields of condensation and to reduce the reaction times, reactions were performed under microwave irradiation. The bis-indolyl oximes were obtained in a *E* form under microwave irradiation (Scheme 3, Table 1).

Compared to 5-N-substituted indirubins, 6-N-substituted indirubins were obtained in lower yields, beside ring-opened by-products. Reduction of 6-nitroindirubin **18** in the presence of ammonium formate with Pd on charcoal under microwave irradiation afforded compound **22** in 57% yield. Whatever the experimental conditions, we never succeeded to prepare 6-acetamidoindirubin in pure form.

As part of our ongoing studies focused on the preparation of novel inhibitors of protein kinase, we also decided to synthesise the isomers **35**, **36** of 5-N-substituted isoindigo analogues.[18] The required 5′-nitro or 5′-aminoisoindigos were obtained as single isomers in quantitative yields by heating the 5-substituted isatins with commercially available indolin-2-one in acetic acid in the presence of *para*-toluenesulfonic acid (PTSA) (Scheme 4).



Scheme 2. Reagents and conditions: (a) $RuCl_3{\cdot}2H_2O$, $NaIO_4$, CH_3CN, rt; (b) IBX, $InCl_3$, MeCN–H_2O.

Table 1
Synthesis of substituted indirubins

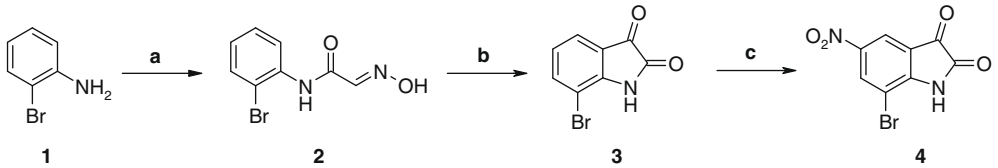
Step a		Step b	
Product	Yield (%)	Product	Yield (%)
12	45	**23**	52
13	35	**24**	14
14	89	**25**	14
15	90	**26**	84
16	90	**27**	25
17	56	**28**	46
18	55	**29**	14
19	93	**30**	78
20	97	**31**	90
21	85	**32**	41
22	57	**33**	84

3. Result and discussion

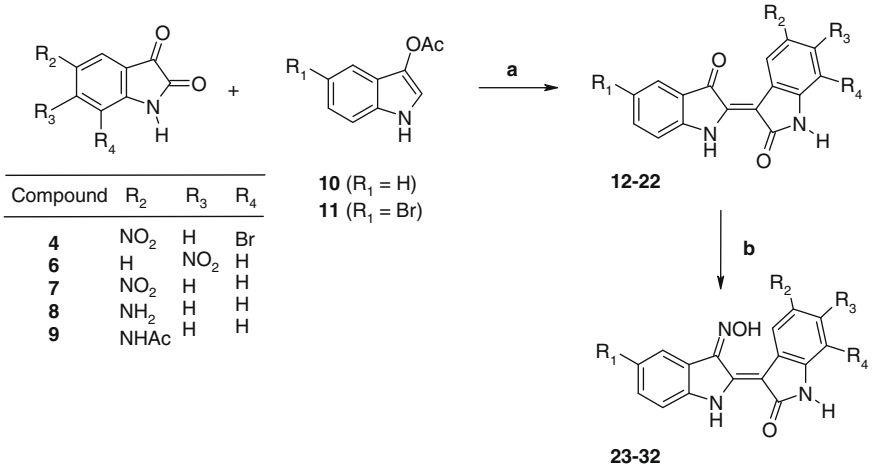
Indirubin analogues **12–33**, **34**, **35** were evaluated for their inhibitory activities on CDK1/cyclin B, CDK5/p25, DYRK1A, CK1 and GSK-3α/β as described (Table 2).[19]

All these kinases are involved at various levels in the development of Alzheimer's disease. 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] For those compounds exhibiting some inhibitory activity at a 10 μM concentration, IC_{50} values were determined from dose-response curves on target enzymes and cell growth activities and are provided in Tables 1 and 2, respectively.

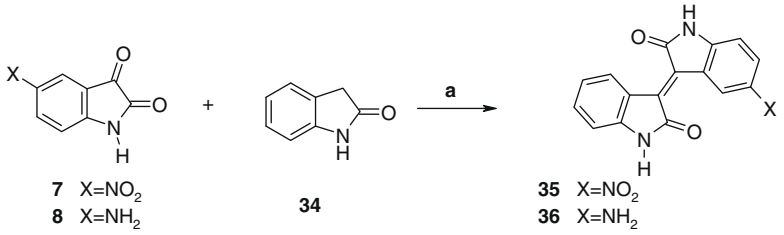
In our previous published work,[12] we demonstrated that 5-nitroindirubin-3′-oxime **25** and 5′-bromo-5-nitroindirubin-3′-oxime **30** were potent, low nanomolar inhibitors of GSK-3 (IC_{50} values: 0.0021 μM and 0.055 μM, respectively).[12] Neither one



Scheme 1. Reagents and conditions: (a) chloral hydrate, Na_2SO_4, $H_2NOH{\cdot}HCl$, H_2O, H^+, 73%; (b) H_2SO_4, 65%; (c) $NaNO_3$, H_2SO_4, 0 °C, 85%.

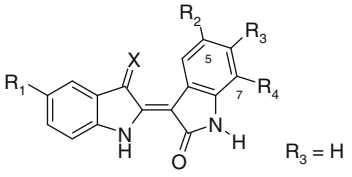


Scheme 3. Reagents and conditions: (a) Na_2CO_3, MeOH, rt, 35–97%; (b) $H_2NOH{\cdot}HCl$, pyridine, μW, 14–92%.



Scheme 4. Reagents and conditions: (a) AcOH, PTSA, μW, 35 (85%), 36 (61%).

Table 2
Kinase inhibition[a] by 5 or/and 7 -substituted indirubin analogues (IC_{50} values in μM)



Compound	R_1	R_2	R_4	X	CDK-1	CDK-5	GSK-3	CK1	DYRK1A
12	H	NO_2	Br	O	>10	>10	>10	>10	>10
14	H	NO_2	H	O	40.0	25	19.0	>10	>10
15	H	NH_2	H	O	1.5	0.59	0.08	0.65	2.5
16	H	NHAc	H	O	0.05	0.018	0.0075	8.0	2.5
17	Br	NO_2	Br	O	>10	>10	>10	>10	>10
19	Br	NO_2	H	O	>10	>10	>10	>10	>10
20	Br	NH_2	H	O	0.6	0.55	0.44	2.7	>10
21	Br	NHAc	H	O	0.055	0.11	0.073	>10	7.3
23	H	NO_2	Br	NOH	>10	>10	>10	>10	>10
25	H	NO_2	H	NOH	0.019	0.006	0.0021	>10	1.3
30	Br	NO_2	H	NOH	0.16	0.05	0.055	>10	>10
28	Br	NO_2	Br	NOH	>10	>10	>10	>10	>10
26	H	NH_2	H	NOH	0.1	0.15	0.36	0.13	4.2
27	H	NHAc	H	NOH	0.088	0.16	0.35	0.4	15.0
31	Br	NH_2	H	NOH	0.41	1.0	6.6	>10	>10
32	Br	NHAc	H	NOH	1.4	3.3	40.0	>10	>10

[a] Kinase assays were carried out at a final ATP concentration of 15 M as described in Section 5.

exhibits activity towards CK1. 5-Nitroindirubin-3′-oxime **25** presents modest inhibitory activity (IC_{50}: 1.3 μM) against DYRK1A. All the other 5-nitro derivatives **14, 19**, **25, 30** are inactive on DYRK1A and CK1. In all cases, addition of a bromine in position 7 (compounds **12**, **17**, **23**, **28**) results in a complete lost of kinase inhibitory activity on CDK1 and GSK3 as previously reported.[7j]

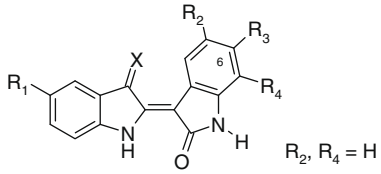
Replacement of the nitro group by the electro-donating amino group in C-5 position for compounds **15**, **20**, **26** leads to enhancement of the inhibitory kinase activity for CK1 and DYRK1A, with a higher affinity for CK1(with respective IC_{50} values of 0.65 μM (**15**); 2.7 μM (**20**); 0.13 μM (**26**)). CDK5 is also sensitive to the three compounds. Their N-acylated counterparts **16**, **21**, and **27** present a much lower affinity for CK1. Almost all amino compounds show submicromolar activities on CDK1 and CDK5. Compared to compounds **26** and **27**, the 3′-*N*-oxime analogues **31**, **32,** bearing a bromo substituent in the C5′ position, are inactive on CK1.

It had been shown that substitution at position 6 turned out to be important for selectivity.[5] Among the known 6-substituted indirubins, the bromo analog (Fig. 1, **II**) exhibited the highest activity against GSK-3. We next considered the 6-substituted derivatives (Table 3). As expected, the selectivity for GSK-3 versus CDK1 was greatly enhanced for the oximes **14** and **29** compared to their keto analogues **13**, **18**. The 6-amino derivative **22** presents selective GSK-3 kinase inhibition (0.36 μM). 6-Nitro-3′-*N*-oxime indirubin **24** shows submicromolar activities on CDK1 (0.18 μM), CK1 (0.6 μM) and GSK3 (0.04 μM) (see Table 3).

The isoindigos **35** and **36** were inactive against the five kinases.

Selected indigoids were also tested for their in vitro inhibition of cell proliferation on a panel of 5 human tumor cell lines, namely, Huh7 (differentiated hepatoma cells), Caco2 (differentiating colon carcinoma) and HCT116 (actively proliferating colon carcinoma), PC3 (prostate cancer), NCI (lung carcinoma) in addition to normal diploid skin fibroblasts as control. Table 4 summarizes the in vitro antiproliferative data activity. It clearly appeared that none molecules were inducing effects onto resting control fibroblastic cells. Isoindigo derivatives, which were inactive on the five kinases, did not induce any growth inhibition on any cell lines. 5-Amino-

Table 3
Kinase inhibition 6-indirubin analogues (IC_{50} values in μM)



Compound	R_1	R_3	X	CDK1	CDK5	GSK3	CK1
13	H	NO_2	O	>10	>10	60.0	>10
18	Br	NO_2	O	>10	>10	>10	>10
22	H	NH_2	O	30.0	4	0.36	16.0
24	H	NO_2	NOH	0.18	0.12	0.04	0.6
29	Br	NO_2	NOH	7.0	0.3	0.7	18.0
33	H	NH_2	NOH	1.0	0.2	0.18	0.5

Table 4
Antiproliferative activity in various cell types (IC_{50} values in μM)

Compound	Huh7	Caco2	HCT116	PC3	NCI	Fibroblasts
15 (5-NH_2)	6 (50%)	2	15	5	20	>25
13 (6-NO_2)	>25	3	5 (40%)	15	2 (40%)	>25
16 (5-NHAc)	2 (50%)	5	2	2	10	>25
22 (6-NH_2)	>25	5	>25	25	25	>25
35 (NO_2iso)	>25	>25	>25	>25	25	>25
36 (NH_2iso)	>25	>25	>25	25	25	>25
Taxol	0.008	0.02	< 0.0008	<0.0008	0.004	0.030 (50%)
Roscovitine	5	6	5	8	6	>25

indirubin **15** and 5-acetamido indirubin **16,** which were very active in the 5 kinase assays, displayed antiproliferative effects on all cell types with major efficiency on Huh7, Caco2 and PC3 cell lines, IC_{50} values ranging between 2 and 5 μM. 6-Nitro-indirubin **13,** poorly active on kinases, displayed weaker cell growth inhibition activity, mainly observed on Caco2, HCT116 and NCI cell lines. 6-Amino-indirubin **22** with low inhibition activity except on GSK3 to some extent was active on one cell line only.

4. Conclusion

During the past decade, several groups have been interested in indirubin bis-indoles as a protein kinase inhibitory scaffold. We here report the synthesis of new series of 7-bromo-5-nitroindirubin, 6-substituted indirubin and isoindigo analogs. 22 Indigoids were synthesized and evaluated against CDK1, CDK5, GSK-3, DYRK1A and CK1. As expected, the selectivity for GSK-3 versus CDKs was slightly enhanced for 6-substituted indirubin. Some dual potent CDKs/GSK-3 inhibitors, the 5-substituted indirubins **15**, **27**, **31,** displayed also potent inhibition of CK1 with IC_{50} values ranging from 0.13 to 0.65 μM. The 6-nitro-3′-*N*-oxime indirubin **24** appears to be a potent CDK1, CK1 and GSK3 inhibitor with respective IC_{50} values of 0.18 μM, 0.6 μM and 0.04 μM.

In order to correlate these enzyme inhibition properties with target biological effects in living cell survival/proliferation, assays were performed by monitoring cell viability and growth activity of 5 different tumor cell lines and normal diploid cells, exposed to increasing concentrations of the molecules. In agreement with the expected antiproliferative biological effects targeted by the molecules, none of the tested molecules were able to induce a specific toxicity or direct apoptotic effect since the diploid control fibroblastic cells, which divided weakly, did not show evidence for any viability changes. In addition, the molecules which displayed high inhibition levels on the 5 kinases in enzymatic assays, were found to be the most effective on the 5 cell lines. Their average bioactivity efficiency was similar or slightly higher to that of a reference CDK inhibitory drug, roscovitine. However, no obvious benefits from the double strategy aiming at targeting simultaneously both the cell cycle kinases and the GSK3 kinase pathway could be detected. Because colon cell lines such as Caco2 have APC complex dysfunction making GSK3 pathway deregulated in these cells, it may be postulated that the antiproliferative inhibition activity observed can be mainly accounted for by inhibition of CDK kinases at least within the set of cell lines studied.

5. Experimental

5.1. Chemical synthesis

5.1.1. Materials and instrumentation

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. ^1H and ^{13}C NMR were recorded on a JEOL NMR LA400 (400 MHz) spectrometer 'Centre Commun d'Analyses, Université de la Rochelle' chemical shifts (δ) are reported in part per million (ppm) downfield from tetramethylsilane (TMS) which was used as internal standard. Coupling constants J are given in hertz. 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 aluminum backed plates. Focused microwave irradiations were carried out with a CEM Discover™ focused microwave reactor (300 W, 2450 MHz, monomode system), has 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**, **5**, **10**, **11**, **34** are commercially available. Spectral data for compounds **7, 8, 9**, **14–16**, **19–21**, **25–27**, **30–32** are consistent with assigned structures as previously described.[12]

5.1.2. Synthesis of 5-N-substituted-isatins

5.1.2.1. *N*-(2-Bromo-phenyl)-2-[(*E*)-hydroxyimino]acetamine (2). Chloral hydrate (1.5 g) and Na_2SO_4 (10.5 g) were dissolved in water (21 mL) in a 500 mL beaker and warmed to 35 °C. A warm solution of commercial 2-bromoaniline **1** (1.42 g, 8.28 mmol) in water (6 mL), and an aqueous solution of concentrated HCl (0.9 mL) was added (a white precipitate of the amine sulfate was formed), followed by a warm solution of hydroxylamine hydrochloride (1.83 g) in water (8.25 mL). The mixture was stirred and heated at 90 °C for 2 h 30 and then allowed to cool, by which time the temperature had fallen to 50 °C and filtered. The pale cream product was washed by stirring with water (100 mL) and filtered. Drying overnight at 40 °C gave the corresponding isonitrosoacetanilide **2**. Yield: 73%. mp = 170 °C. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 12.38 (s, 1H, NOH), 9.38 (s, 1H, NH), 7.93 (d, 1H, J = 7.6 Hz, H-6), 7.68 (d, 1H, J = 7.6 Hz, H-3), 7.64 (s, 1H, CH), 7.40 (t, 1H, J = 7.6 Hz, H arom), 7.13 (t, 1H, J = 7.6 Hz, H arom). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 160.19, 143.23, 135.21, 132.50, 128.19, 126.53, 124.47, 116.05. IR = 3335, 3175, 2884, 1662, 1537, 1023, 755. HRMS (EI) $[M]^{+\cdot}$ ($C_8H_7N_2O_2{}^{79}Br$): calcd 241.96909, found 241.9700.

5.1.2.2. 7-Bromoisatin (3). Sulfuric acid (100 mL) was heated in a 250 mL beaker to 60 °C and then removed. The dry isonitrosoacetanilide **2** (1.76 g, 7.26 mmol) was added in portion with stirring over 30 min so that the temperature did not exceed 65 °C. The mix-

ture was then heated to 80 °C for 15 min, allowed to cool to 70 °C, and cooled on ice. The solution was then poured into ice, and was extracted with ethyl acetate and washed with water. The organic layer was dried ($MgSO_4$) and the filtrate was concentrated under reduced pressure. The crude residue were recrystallized from water. Yield : 65%. mp = 195–200 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.19 (d, 1H, J = 8.0 Hz, H-4), 7.92 (d, 1H, J = 7.2 Hz, H-6), 7.44 (t, 1H, J = 7.2 and 8.0 Hz, H-5). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 183.57, 159.45, 149.30, 140.27, 124.03, 123.43, 119.98, 104.53. IR = 3179, 1737, 1613, 1322, 1130, 757. HRMS (EI) $[M]^{+\cdot}$ ($C_8H_4NO_2{}^{79}Br$) calcd 224.94254, found 224.9425.

5.1.2.3. 7-Bromo-5-nitroisatin (4). To a solution of $NaNO_3$ (0.337 g, 3.97 mmol) in concentrated H_2SO_4 (6.8 mL) was added dropwise a solution of 7-bromoisatin **3** (0.898 g, 3.97 mmol) in concentrated H_2SO_4 (5.7 mL) for a period of 1 h at 0 °C. The reaction mixture was then poured into ice water (30 mL), and the precipitate was collected by filtration and washed with water to give **4**. Yield : 85%. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.92 (s, 1H, NH), 8.61 (s, 1H, H-6), 8.18 (s, 1H, H-4). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 181.60, 160.16, 154.25, 143.00, 134.80, 119.57, 118.20, 140.38. IR = 3185, 1750, 1612, 1528, 1340. HRMS (EI) $[M]^{+\cdot}$ ($C_8H_3NO_4{}^{79}Br$) calcd 269.92762, found 269.9279.

5.1.3. Synthesis of 6-N-substituted-isatins

5.1.3.1. 6-Nitroisatin (6). Under an inert atmosphere of argon, to a solution of 6-nitro-3-indolaldehyde (1.71 g, 9.0 mmol) in acetic acid was added dropwise over a period of 15 min a solution of chromium trioxide (3.6 g, 36.0 mmol) in acetic acid (5 mL) and water (1.6 mL). The dark brown solution was stirred at room temperature for 30 h. The bright orange solid was filtered, washed with water, dried, and recrystallized from ethanol. Yield : 58% . mp >260 °C . ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.31 (s, NH), 7.85 (dd, 1H, J = 2.0 and 8.0 Hz, H-5), 7.76 (d, 1H, J = 8.0 Hz, H-4), 7.54 (d, 1H, J = 2.0 Hz, H-7). IR = 3191, 1750, 1549, 1328, 1213, 736 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_8H_4N_2O_4$): calcd 192.01711, found 192.0180.

5.1.4. General procedure for the synthesis of indirubin derivatives

Under an inert atmosphere of argon, a solution of isatin derivatives **4, 6** (3 mmol), indoxyl acetate **10** or **11** (5 mmol) in methanol (15 mL) was vigorously stirred with Na_2CO_3. After 1 h under stirring at 45 °C, 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 (45–95%).

5.1.4.1. (2′*Z*)-7-Bromo-5-nitroindirubin (12). Yield: 45%. Mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 11.76 (s, 1H, N′H), 11.34 (s, 1H, NH), 9.67 (s, 1H, H-4), 8.30 (s, 1H, H-6), 7.59 (d, 1H, J = 8.0 Hz, H arom), 7.50 (t, 1H, J = 7.2 Hz, H arom), 7.33 (d, 1H, J = 8.0 Hz, H arom), 6.94 (t, 1H, J = 7.2 Hz, H arom). IR = 3103, 1677, 1613, 1465, 1334, 763. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_8N_3O_4{}^{79}Br$): calcd 384.96982, found 384.9687.

5.1.4.2. (2′*Z*)-6-Nitroindirubin (13). Yield: 35%. mp >260 °C . ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.43 (s, NH), 11.27 (s, N′H), 8.88 (d, 1H, J = 8.8 Hz, H-4), 7.91 (dd, 1H, J = 2.0 and 8.8 Hz, H-5), 7.68 (d, 1H, J = 7.6 Hz, H-4′), 7.66 (d, 1H, J = 2.0 Hz, H-7), 7.60 (t, 1H, J = 7.6 Hz, H-5′), 7.40 (t . 1H, J = 7.6 Hz, H-6′), 7.09 (t, 1H, J = 7.6 Hz, H-7′). IR = 3411, 1738, 1673, 1589, 1463, 1331, 1183, 87 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_3O_4$): calcd 307.05931, found 307.0592.

5.1.4.3. (2′*Z*)-5′-7-Dibromo-5-nitroindirubin (17). Yield: 56%. Mp >260 °C. IR = 3282, 3080, 1706, 1679, 1609, 1462, 1285, 823. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_7N_3O_4{}^{79}Br_2$): calcd 462.88033, found 462.8803.

5.1.4.4. (2′*Z*)-5′-Bromo-6-nitroindirubin (18). Yield: 55% . mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.48 (s, NH), 11.29 (s, N′H), 8.85 (d, 1H, J = 8.8 Hz, H-4), 7.94 (d, 1H, J = 10.4 Hz, H-6′), 7.80 (s, 1H, H-7), 7.76 (d, 1H, J = 10.4 Hz, H-7′), 7.60 (s, 1H, H-4′), 7.42 (d, 1H, J = 8.8 Hz, H-5). IR 3296, 1683, 1607, 1460, 1119, 81 cm^{-1}. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_8N_3O_4{}^{79}Br$): calcd 384.96982, found 384.9687.

5.1.4.5. (2′*Z*)-6-Aminoindirubin (22). Yield: 57%. Mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.54 (s, 1H, N′H), 10.43 (s, 1H, NH), 8.57 (d, 1H, J = 8.4 Hz, 4-H), 7.58 (d, 1H, J = 7.6 Hz, H arom), 7,47 (t, 1H, J = 7.6 Hz, H arom), 7,29 (d, 1H, J = 8.4 Hz, 5-H), 6,92 (t, 1H, J = 7.6 Hz, H arom), 6,19 (m, 2H, 7-H et H arom), 5,89 (s, 2H, NH_2). IR = 3176, 1714, 1482, 1274, 1004. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_{11}N_3O_2$): calcd 277.08513, found 277.0838.

5.1.5. General procedure for the synthesis of 3′-monoxime indirubin derivatives

Under an inert atmosphere of argon, to a stirred solution of indirubin derivatives **12, 13, 17, 18, 22** (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 (14–84%).

5.1.5.1. (2′*Z*,3′*E*)-7-Bromo-5-nitroindirubin-3′-oxime (23). Yield: 52%. Mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.99 (s, 1H, N′OH), 12.02 (s, 1H, N′H), 11.59 (s, 1H, NH), 9.47 (s, 1H, H-4), 8.26 (d, 1H, J = 7.6 Hz, H arom), 8.19 (s, 1H, H-6), 7.51 (d, 1H, J = 7.6 Hz, H arom), 7.45 (t, 1H, J = 7.6 Hz, H arom), 7.12 (t, 1H, J = 7.6 Hz, H arom). IR = 3096, 2904, 1718, 1677, 13337, 1226. HRMS (EI) $[M]^{+\cdot}$ ($C_{16}H_9N_4O_4{}^{79}Br$) : calcd 399.98072, found 399.9803.

5.1.5.2. (2′*Z*, 3′*E*)-6-Nitroindirubin-3′-oxime (24). Yield: 14%. Mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.17 (s, 1H, N′H), 11.15 (s, 1H, NH), 8.75 (dd, 1H, J = 4.0 and 8.8 Hz, H arom), 8.24 (d, 1H, J = 8.0 Hz, H arom), 7.83 (d, 1H, J = 3.2 and 8.8 Hz, H arom), 7.64 (s, 1H, H-7), 7.52 (dd, 1H, J = 3.2 and 8.0 Hz, H arom), 7.44 (t, 1H, J = 7.2 Hz, H arom), 7.39 (t, 1H, J = 4.0 Hz), 7.12 (td, 1H, J = 4.0 and 7.2 Hz). IR = 3225, 2917, 1721, 1668, 1566, 1330, 608.

5.1.5.3. (2′*Z*, 3′*E*)-5′-Bromo-6-nitroindirubin-3′-oxime (29). Yield: 14%. Mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.16 (s, 1H, N′H), 11.08 (s, 1H, NH), 8.72 (d, 1H, J = 8.8 Hz, H-4), 8.34 (d, 1H, J = 1.2 Hz, H-7), 7.82 (dd, 1H, J = 1.2 and 8.8 Hz, H-5), 7.65 (d, 1H, J = 1.6 Hz, H-4′), 7.62 (dd, 1H, J = 1.6 and 8.8 Hz, H-6′), 7.48 (d, 1H, J = 8.8 Hz, H-7′). IR = 3186, 2929, 1714, 1569, 1333.

5.1.5.4. (2′*Z*, 3′*E*)-6-Aminoindirubin-3′-oxime (33). Yield: 84%. Mp >260 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.81 (s, 1H, N′H), 8.82 (s, 1H, NH), 8.15 (d, 1H, J = 8.0 Hz, H arom), 7.59 (d, 1H, J = 8.0 Hz, H arom), 7.41 (d, 1H, J = 7.6 Hz, H arom), 7.37 (d, 1H, J = 8.0 Hz, H arom), 7.35 (d, 1H, J = 7.6 Hz, H arom), 7.25 (d, 1H, J = 8.0 Hz, H arom), 6.96 (t, 1H, J = 7.2 Hz, H arom), 6.88 (t, 1H, J = 7.2 Hz, H arom), 3.26 (s large, 2H, NH_2). IR = 3108, 2960, 1713, 1322, 1200. HRMS (EI) [M}]$^{+\cdot}$ ($C_{16}H_{11}N_3O_2$) : calcd 277.08513 found 277.0860.

5.1.5.5. 5-Nitro-1*H*,1′*H*-[3,3′]biindolylidene-2,2′-dione or (3′*Z*)-5-nitroisoindigo (35). Yield: 85%. Mp >260 °C. IR: ν max (cm^{-1}): 3145 (NH), 1705 (C═O), 1682 (C═O) 1525 (NO_2) 1339, 1077. ^{1}H NMR δ (400 MHz, DMSO-d_6): 11.43 (s, 1H, NH), 10.87 (s, 1H, NH), 9.06 (d, 1H, J = 7.6 Hz, 4-H), 8.25 (d, 1H, J = 8.8 Hz, 7-H), 7.38 (t,

1H, J = 7.6 Hz, 6-H), 6.92–7.08 (m, 3H), 6.85 (d, 1H, J = 8.0 Hz). ^{13}C NMR δ (100 MHz, DMSO-d_6): 168.73, 168.57, 148.85, 144.61, 141.61, 135.63, 133.29, 130.25, 129.49, 127.60, 124.50, 121.18, 121.06, 121.01, 109.50, 109.20. HRMS (EI) $[M]^+$: ($C_{16}H_9N_3O_4$), calcd: 307.05931, found: 307.0602.

5.1.5.6. 5-Amino-1*H*,1′*H*-[3,3′]biindolylidene-2,2′-dione or (3′*Z*)-5-aminoisoindigo (36). Yield: 61%; mp >260 °C. IR: ν max (cm^{-1}): 3025, 2876, 2831, 1689, 1677, 1459, 1324. ^{1}H NMR δ (400 MHz, DMSO-d_6): 11.49 (s, 1H, NH), 11.44 (s, 1H, NH), 10.13 (s, 2H, NH_2), 8.96–9.08 (m, 2H, H-4, H-6), 7.25–7.40 (m, 2H), 6.97 (t, 1H, J = 8.0 Hz), 6.89 (d, 1H, J = 8.0 Hz), 6.85 (d, 1H, J = 8.0 Hz). ^{13}C NMR δ (100 MHz, DMSO-d_6): 177.16, 173.47, 149.12, 143.13, 141.35, 128.83, 128.60, 126.63, 126.33, 124.80, 121.18, 118.99, 109.56, 109.05, 74.86, 51.31. HRMS (EI) $[M]^+$: ($C_{16}H_{11}N_3O_2$): calcd: 277.08513, found: 277.0866.

5.2. Biological methods

5.2.1. Preparation and assay of protein kinases

Kinases 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 pmoles 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.

CDK1/cyclin B was extracted in homogenisation 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 (Leclerc et al., 2001; Bach et al., 2005). The kinase activity was assayed in buffer C, with 1 mg histone H1 /mL, in the presence of 15 μM [γ-33P] ATP (3,000 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 *E. coli* as GST (Glutathione-S-transferase) fusion proteins and purified by affinity chromatography on glutathione-agarose (vectors kindly provided by Dr. J.H. Wang) (p25 is a truncated version of p35, the 35 kDa CDK5 activator). Its activity was assayed in buffer C as described for CDK1/cyclin B.

GSK-3α/β was purified from porcine brain by affinity chromatography on immobilised axin (Primot et al., 2000). It was assayed, following a 1/100 dilution in 1 mg BSA/mL 10 mM DTT, with 5 μl 40 μM GS-1 peptide as a substrate, in buffer A, in the presence of 15 μM [γ-33P] ATP (3,000 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/litter of water. The wet filters were counted in the presence of 1 mL ACS (Amersham) scintillation fluid.

CK1 (porcine brain, native) was assayed as described for CDK1 but using the CK1-specific peptide substrate RRKHAAIGpSAYSITA (Reinhardt et al., 2007),[20] obtained from Millegen (Labege, France).

DYRK1A (rat, recombinant, expressed in *E. coli* as a GST fusion protein) was purified by affinity chromatography on glutathione-agarose and assayed as described for CDK1/cyclin B using myelin basic protein (1 mg/ml) as a substrate.

5.2.2. Cell lines and cell cultures

The cytotoxicity and antiproliferative activity of indigoids were studied on 5 distinct cell lines representative of the most frequent solid tumors developing in human. All of them were obtained from the european ECAC collection and skin diploid fibroblasts that were provided by BIOPREDIC International Company. They included two human colon carcinoma cells Caco2 and HCT 116 representative of two distinct differentiated and highly colon tumorigenic tumors, respectively, a differentiated highly growing HUH7 hepatocarcinoma cells, the NCI lung and PC3 prostate tumor cells. They all have adherent cell properties. They were grown according to the providers recommendations.

5.2.2.1. Cytotoxicity and antiproliferative assays. The method is based on an automated imaging analysis. The toxicity test of the compounds on cells was as followed: 4×10^3 cells are seeded in 96 multiwell plates and led for 24 h for attachment, spreading and growth. Then, they were exposed for 24 and 48 h to increasing concentrations of the compounds, ranging from 0.1 to 25 μM in a final volume of 80 μl of culture medium. They were fixed with 4% paraformaldehyde solution and nuclei were stained with Hoechst 3342 and counted according to automated imaging quantification. 4 pictures per well were obtained with a high speed camera and statistical analyses were established using the Simple PCI software. In addition, imaging analysis allowed detection of possible cell morphology changes.

5.2.2.2. Antiproliferative activity. Cells were seeded in 96-wells culture plates (5000 cells/well) in culture medium containing indigoids 10^{-10} to 10^{-6} M. After 72 h growth and exposure to indirubins, viable cells were quantified using the CellTiter 96® Non-radioactive cell proliferation assay (Promega). Antiproliferative activity was expressed as a percentage of growth inhibition in 24 assays from three independent experiments. In parallel, nuclei counting the assay included the comparative tumor cell responses to the molecules according to distinct tissular origins and also with the poorly growing normal diploid cells from human skin origin.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.07.051.

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