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Synthesis and structure–activity relationships of novel indirubin derivatives as potent anti-proliferative agents with CDK2 inhibitory activities

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Abstract—Indirubin, an active ingredient of a traditional Chinese recipe Danggui Longhui Wan, has been known as a CDK inhibitor competing with ATP for binding to the catalytic site of cyclin-dependent kinases (CDKs). Since CDKs, a group of serine/threonine kinases forming active heterodimeric complexes with cyclins, are key regulators of the cell cycle regulation, therapeutic interventions targeting CDKs have been stimulated for the treatment of proliferative diseases, such as cancer, psoriasis, and for the prevention of chemotherapy-associated side effects, such as alopecia. A series of novel indirubin analogs was synthesized and evaluated for anti-proliferative and CDK2 inhibitory activities. Among the indirubin derivatives tested in the growth inhibitions against several human cancer cell lines, 5-nitro, halide, and bulky group containing acylamino substituted analogs showed high anti-proliferative effects. Selected analogs showing potent anti-proliferative activities were evaluated further in the CDK2 enzyme assay, which resulted in the discovery of potent CDK2 inhibitors.

1. Introduction

Abnormal control of cell cycle has been recognized as one of the mechanisms of cancerization. Indeed, oncogenic alterations of cyclins, cyclin-dependent kinases (CDKs), and other upstream regulators of retinoblastoma (Rb) protein, which function as important components of cell cycle take place in a variety of human tumors.[1] CDKs are key regulators among which many of them are related to the cell cycle, the complex process by which cells divide.[2–5] Cell cycle progression is stimulated and controlled by the sequential activation of a series of CDK complexes[6,7] which are binding with specific cyclin through the four phases of the cell cycle.[2,8] A recent report has suggested that CDK2 or cyclin E1/E2-deficient cells could proliferate normally without causing any centrosome duplication defect.[9] These results gave a strong impetus to the research on the development of CDK inhibitors as potential pharmacological agents to treat proliferative diseases, such as cancer, psoriasis, and restenosis,[10–12] and for the prevention of chemotherapy-associated side effects, such as alopecia.[13] A number of selective low-molecular weight inhibitors of CDK1, CDK2, and CDK4, such as Flavopiridol, Roscovitine, UCN-01, and Indirubin[14,15] (Fig. 1), have been developed toward the clinical trials against various cancer diseases.

Among these inhibitors, indirubin, the active ingredient of Danggui Longhui Wan, a traditional Chinese medicinal recipe, has been used to treat chronic myelocytic leukemia.[16] Recently, indirubin and a few indirubin derivatives have been reported to inhibit CDKs by competing in the ATP-binding sites with high selectivity among several kinase families.[17] Although the detailed mechanism of indirubin in the cells is not known completely, the anti-proliferative effect on the human cancer cells was reported to have resulted from inhibiting the genes or proteins of regulating cell cycle progression, arresting G2/M phase of the cell cycle.[18] Additional series of indirubin derivatives were developed aiming at the great potential for the treatment of Alzheimer's disease

Keywords: Indirubin; Anti-proliferative activity; Cyclin-dependent kinases.
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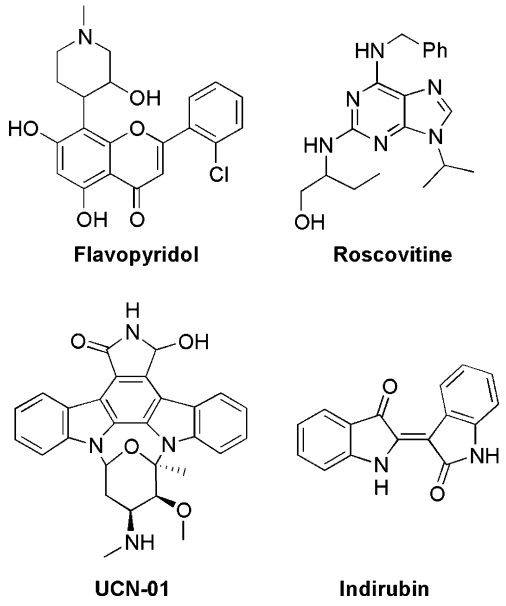


Figure 1.

(AD) by inhibiting GSK-3β and CDK5, which are related to the control of abnormal hyperphosphorylation of the microtubule-binding protein tau, one of the diagnostic features of AD.[19,20] In addition, indirubin-3′-monoxime was reported to up-regulate $p27^{Kip1}$ transcription by acting as an agonist of the aryl hydrocarbon receptor[21–23] and inhibiting c-Jun NH_2-terminal kinase, which is an important regulator of neuronal apoptosis.[24] Moreover, recent reports provide an evidence that indirubin-3′-monoxime can selectively abrogate induced aberrant centriole and centrosome duplication in tumor cells without affecting normal centrosome duplication.[25]

In the present report, we describe the synthesis of novel indirubin derivatives and biological evaluations including anti-proliferative activity against several cancer cell lines (A549, SNU-638, Col2, HT1080, HL-60, and MCF-7) and CDK2 inhibitory activity for selected indirubin derivatives.

2. Results and discussion

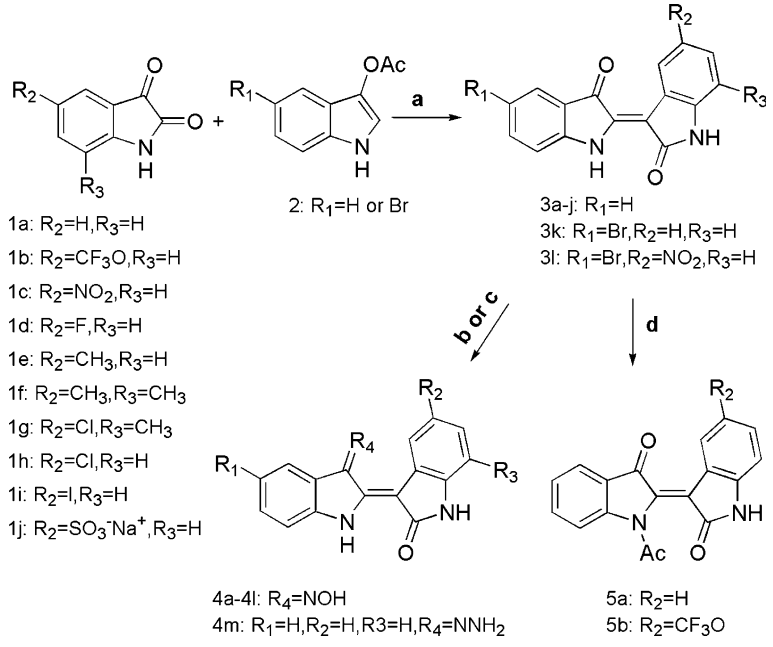
2.1. Chemistry

The general synthetic approach for the substituted indirubin derivatives is shown in Scheme 1. The substituted indirubins (**3a**–**j**) were prepared by condensation of appropriately substituted (mainly 5- and 7-substituted) isatins (**1a**–**j**) with indoxyl acetates (**2**) in the presence of Na_2CO_3 in methanol. Since indirubin-3′-monoxime was reported to exhibit an enhanced inhibitory activity on CDKs compared to indirubin,[17] indirubin-3′-monoxime analogs (**4a**–**l**) were prepared from reactions of the substituted indirubins (**3a**–**l**) with hydroxylamine in pyridine.

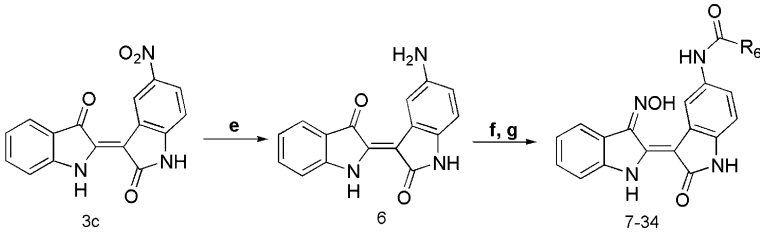
Indirubin-3′-hydrazone derivative, **4m**, was also synthesized from the reaction with hydrazine monohydrate for a comparison with the corresponding monoxime, **4a**. Acetylated compounds **5a**,**b** were prepared to confirm the importance of the proton of secondary amine. To synthesize more diverse analogs with 5-substitutions, for example, 5-acylamino groups, the 5-nitroindirubin, **3c** was first reduced to 5-aminoindirubin, **6** by tin(II) chloride, followed by acylation with acid chlorides in pyridine and subsequent reaction with hydroxylamine to afford various 5-acylamino-indirubin-3′-oxime derivatives (Scheme 2).

2.2. Anti-proliferative activity

The synthesized indirubin derivatives were tested for anti-proliferative activities against several cancer cell



Scheme 1. Reagents and conditions: (a) Na_2CO_3, MeOH, rt; (b) $H_2NOH \cdot HCl$, pyridine, reflux; (c) $H_2NNH_2 \cdot H_2O$, 70–80 °C; (d) acetic anhydride, reflux.

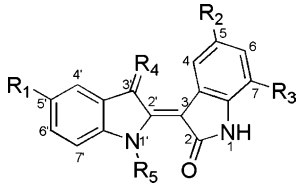


Scheme 2. Reagents and conditions: (e) $SnCl_2 \cdot 2H_2O$, DMF, 70 °C; (f) $ClCOR_6$, pyridine, 0 °C; (g) $H_2NOH \cdot HCl$, pyridine, reflux.

lines including A594, SNU-638, Col2, HT 1080, HL-60, and MCF-7 cells. Tables 1 and 2 show the IC_{50} values of cancer cell growth inhibition by the new indirubin analogs derivatized at various positions of the bis-indole skeleton. Ellipticine, a well-known anticancer agent, was tested together as a positive control in the assays.

Although literatures[17,19] have reported that indirubin-3′-monoxime, **4a**, inhibited CDKs much more efficiently than indirubin, **3a**, with IC_{50} values of 100–200 nM, the anti-proliferative activities of **3a**, **4a**, and the hydrazone analog, **4m**, were similar, except for HT1080 cell lines. Among the series of 5-substituted indirubin-3′-monoxime derivatives given in Table 1, 5-nitro-indiruin-3′-monoxime, **4c**, showed potent inhibitory activity against most of the cancer cells, with IC_{50} values in the range of 1–10 μM. Particularly, it represented an IC_{50} value of 1.2 μM against human stomach cancer cell line (SNU-638) and 1.4 μM against human breast cancer cell line (MCF-7), which are comparable with those of the positive control Ellipticine. However, compound **3j**, the potent CDK inhibitor reported in the literature[17] and its monoxime derivative, **4j**, showed no inhibitory activities, probably because of the low membrane permeability due to the ionic character of 5-sulfonate. Thus, it can be assumed that the partial negative charge by the electron-rich nitro group, which can compete with the phosphate of ATP in the active sites of CDKs, and the membrane permeable character of **4c** enable the potent inhibitory activity. Indeed, further study has demonstrated that **4c** arrested

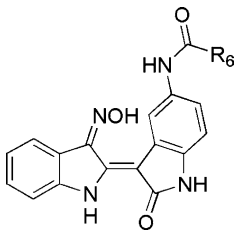
Table 1. Anti-proliferative effects of indirubin derivatives



Compound	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)					
						A549[a]	SNU-638[b]	Col2[c]	HT1080[d]	HL60[e]	MCF-7[f]
3a	H	H	H	O	H	31	>100	>100	42	>100	>100
3b	H	CF_3O	H	O	H	68	>100	>100	>100	>100	>100
3c	H	NO_2	H	O	H	>100	>100	>100	>100	>100	40
3d	H	F	H	O	H	97	40	>100	>100	>100	>100
3j	H	$Na^+SO_3^-$	H	O	H	>100	>100	>100	>100	>100	>100
4a	H	H	H	NOH	H	62	>100	>100	4.8	>100	10
4b	H	CF_3O	H	NOH	H	9.6	23	>100	32	>100	>100
4c	H	NO_2	H	NOH	H	5.4	1.2	25.5	5.9	9.2	1.4
4d	H	F	H	NOH	H	13	2.1	>100	3.4	89	9.0
4e	H	CH_3	H	NOH	H	20	18	>100	37	65	33
4f	H	CH_3	CH_3	NOH	H	>100	91	>100	>100	>100	3.5
4g	H	Cl	CH_3	NOH	H	>100	>100	>100	>100	>100	34
4h	H	Cl	H	NOH	H	12	6.2	17	11	4.8	2.5
4i	H	I	H	NOH	H	>100	>100	>100	>100	>100	>100
4j	H	$Na^+SO_3^-$	H	NOH	H	>100	>100	>100	>100	47	>100
4k	Br	H	H	NOH	H	39	33	11	74	25	12
4l	Br	NO_2	H	NOH	H	2.9	0.79	1.8	1.6	2.6	1.3
4m	H	H	H	$NHNH_2$	H	98	>100	>100	41	>100	>100
5a	H	H	H	O	$COCH_3$	>100	>100	>100	>100	>100	>100
5b	H	CF_3O	H	O	$COCH_3$	>100	>100	>100	>100	>100	>100
Ellipticine (positive control)						0.28	1.6	1.5	3.6	3.1	0.99

[a] Human lung cancer cell line.
[b] Human stomach cell line.
[c] Human colon cancer cell line.
[d] Human fibro sarcoma cell line.
[e] Human leukemia cell line.
[f] Human breast cancer cell line.

Table 2. Anti-proliferative effects of 5-*N*-acyl indirubin derivatives



Compound	R_6	IC_{50} (μM)					
		A549	SNU-638	Col2	HT1080	HL60	MCF-7
7	$-CH_3$	10	38	19	8.4	57	5.4
8	$-CH_2CH_3$	13	61	48	20	>100	14
9	$-(CH_2)_2CH_3$	>100	>100	>100	52	>100	8.2
10	$-(CH_2)_3CH_3$	>100	68	>100	>100	>100	14
11	$-C(CH_3)_3$	6.4	6.5	14	4.2	21	6.7
12	$-CH_2C(CH_3)_3$	11	25	14	11	>100	6.3
13	$-CH{=}C(CH_3)_2$	>100	>100	>100	70	>100	>100
14	–Cyclopropyl	>100	>100	>100	71	>100	38
15	–Cyclohexyl	>100	>100	>100	>100	>100	>100
16	–2-Furane	>100	71	>100	65	>100	4.5
17	–2-Thiophene	90	54	73	73	32	12
18	–Phenyl	>100	>100	>100	>100	>100	19
19	–2-Chlorophenyl	>100	>100	>100	>100	>100	58
20	–4-Chlorophenyl	>100	>100	>100	>100	>100	20
21	2-Fluorophenyl	97	73	>100	55	>100	>100
22	–4-Fluorophenyl	>100	>100	>100	>100	>100	13
23	–2-Trifluoromethylphenyl	17	28	63	17	94	50
24	–3-Nitrophenyl	58	15	>100	99	>100	13
25	–4-Nitrophenyl	58	>100	>100	>100	>100	11
26	–3,5-Dinitrophenyl	98	12	>100	16	>100	2.6
27	–4-Methoxyphenyl	94	>100	>100	>100	>100	21
28	–4-Methylphenyl	>100	79	>100	>100	>100	13
29	–3-Chlorophenoxymethyl	12	52	77	45	97	5.1
30	–4-Chlorophenoxymethyl	18	82	76	35	96	6.8
31	–CH=CH-Ph (trans)	21	53	>100	72	>100	15
32	$-CH_2CH_2Ph$	37	44	>100	29	>100	4.5
33	–Diphenylmethyl	2.7	5.6	6.3	6.6	11	3.5
34	–1-Naphthyl	16	>100	11	44	>100	8.1
Ellipticine (positive control)		1.3	2.9	2.0	1.4	15	0.99

the cell cycle progression at the G_2/M phase and induced apoptosis via p53- and mitochondria-dependent pathway.[26] Other lone pair electron-rich group substituted analogs, such as **4b** (5-OCF_3), **4d** (5-F), and **4h** (5-Cl), also displayed relatively higher inhibitory activities in general. However, 5-iodo analog, **4i**, did not inhibit the cell growth. Bromo substitution (**4k**) at 5′ position (R_1) of indirubin-3′-monoxime, **4a**, resulted in an appreciable increase in activity. Further substitution with nitro group at 5 position (R_2) of **4k** afforded synergistic effect of the anti-proliferative activity, showing 1–2 μM IC_{50} values at all of the tested cancer cell lines (compound 41). On the other hand, acetyl group substituents at R_5 position resulted in a significant decrease in inhibitory activities against A549 and HT1080 cell lines (**3a** versus **5a**, **3b** versus **5b**). These results suggested that the hydrogen bond between secondary amine proton of indirubin and the backbone oxygen of Leu83 of CDK2 as described in the literature[17] was also important for the anti-proliferative activity.

Diversity at 5 position was introduced by acylation of 5-amino-indirubin, **6**, which was obtained by the reduction of **3c** with tin(II) chloride. Among the aliphatic acylamino derivatives of indirubin-monoximes, bulky tertiary butyl group containing compounds, **11** and **12** were particularly active than other alkyanoyl and cycloalkanoyl analogs. Substituted benzoyl analogs were generally inactive, except for trifluoromethyl and dinitro substituted compounds, **23** and **26**. Especially, **26** showed potent anti-proliferative activity against human breast cancer cell line in this series of compounds. Among the series of compounds listed in Table 2, diphenylacetyl analog, **33**, was the most potent compound showing 2–10 μM IC_{50} values at all the tested cancer cell lines. Considering the different electronic environment around the 5 position of the representative potent anti-proliferative indirubin derivatives in Tables 1 and 2, such as **4c** and **33**, we could suppose that similar activity could have resulted from different factors, such as different solubility and cellular uptake, as well as possibly inhibiting different target protein molecules.

In general, the various acylamino derivatives showed better solubility than the compounds listed in Table 1. To investigate the relationship between the anti-proliferative potency and CDK2 inhibitory activity, we further studied

the inhibitory activity of the representative derivatives at CDK2 catalyzed Rb protein phosphorylation.

2.3. CDK2 inhibitory activity

To determine the CDK inhibitory activity in a set of breast cancer cell lines (MCF-7), cells were treated with representative indirubin derivatives (**3a**, **4a**, **4b**, **4c**, **11**, and **33**). CDK2, 4, and 6 from whole cell lysates were immunoprecipitated with a monoclonal antibody and the kinase activity was measured using a C-terminal fragment of pRb as a substrate. Indirubin (**3a**) showed no CDK2, 4, and 6 inhibitory activity, even at 500 μM concentration, whereas indirubin-3′-monoxime (**4a**) inhibited CDK2 more efficiently with 50% inhibition at 30–40 μM concentration (Fig. 2A). In the case of **4b** and **4c**, the kinase activity of CDK2 was significantly blocked up to 0.1–1 μM concentration of the compounds. However, despite similar high potency of anti-proliferative activity with **4b** and **4c**, compounds **11** and **33** did not affect the CDK2 activity in the cancer cell line, even at 10 μM concentration, and only showed decreased CDK2 activity at 100 μM or higher concentrations of the compounds (Fig. 2B). This study demonstrated that compounds **11** and **33** might modulate other kinases or signaling molecules affecting cell proliferation, such as aryl hydrocarbon receptor (AhR),[23] JNK,[24] Src kinase[27], and Stat3.[27] N1-methylated indirubins were reported as potent AhR agonists by leading to a G1 arrest in a strictly AhR-dependent manner.[23] Also, indirubin-3′-alkyloxime derivatives were recently reported to directly block the Src-Stat3 signaling pathway, which has an important role in oncogenesis and is a promising anticancer target system, inducing apoptosis of human cancer cells.[27]

Further study to figure out the molecular mechanism of anti-proliferative activity of compound **11** or **33** is in progress.

3. Conclusion

Novel indirubin derivatives were developed with structure–activity relationship studies, determining anti-proliferative activity with a positive control, Ellipticine. 5-Substituted indirubin-3′-oxime analogs, **4b** and **4c**, as the potent cancer cell growth inhibitors displayed potent inhibition of CDK2, which should be one of the mechanisms of anti-proliferative activity. However, 5-acylamino analogs, **11** and **33**, which are equally potent

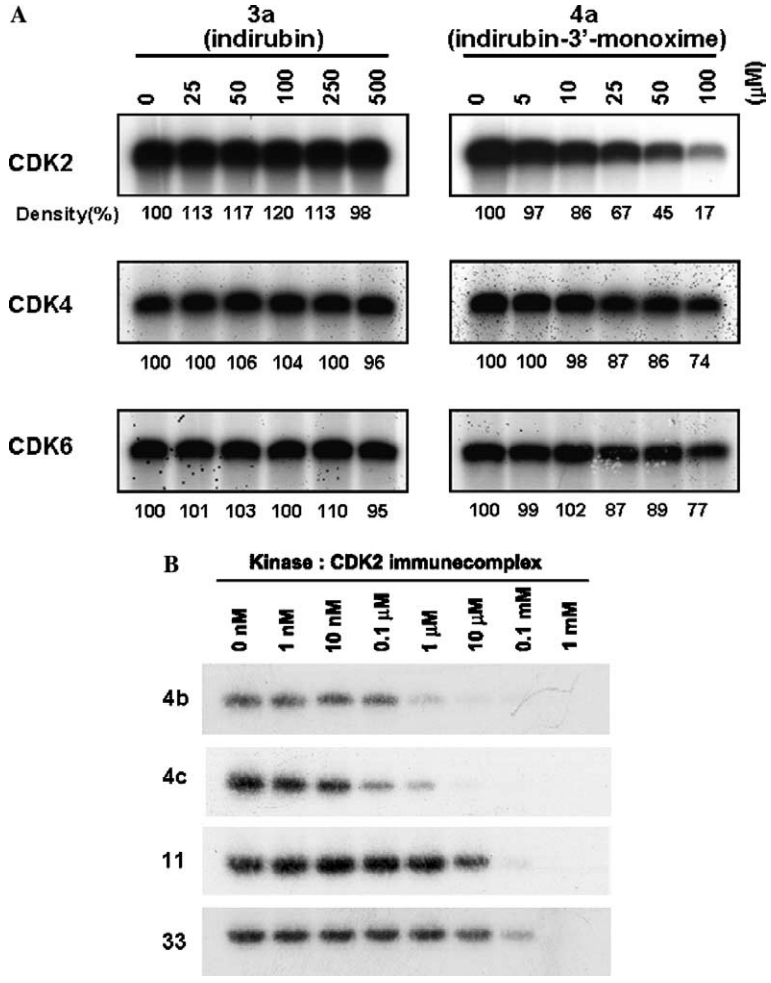


Figure 2. Inhibition of CDK catalyzed Rb protein phosphorylation by indirubin derivatives. Recombinant human Rb protein was phosphorylated in vitro with CDK2, 4, and 6 (A), CDK2 (B) in the presence of indirubin derivatives (**3a**, **4a**, **4b**, **4c**, **11**, and **33**) and resolved by SDS–PAGE, followed by autoradiography.

anti-proliferative derivatives did not inhibit CDK2 activity. The indirubin derivatives discovered in this study may provide valuable therapeutic intervention for the treatment of anti-proliferative diseases.

4. Experimental

4.1. Chemistry

All chemicals were purchased from Aldrich Chemical Co. Proton nuclear magnetic resonance spectroscopy was performed on Bruker AVANCE 600 and JEOL JNM-LA 300WB spectrometers, and spectra were taken in DMSO-d_6. Unless noted, chemical shifts are expressed in ppm downfield from TMS as the internal standard, and J values are given in Hz. Mass spectroscopy was carried out on a MALDI-TOF. Elemental and high-resolution mass analyses were performed in Seoul Branch Analytical Laboratory of Korea Basic Science Institute.

4.1.1. General procedure for the synthesis of indirubin derivatives (3a–l). Under nitrogen, 176 mg (1 mmol) of indoxyl acetate and 1 mmol of isatin analogs were dissolved in 5 ml methanol with 256 mg (2.5 mmol) Na_2CO_3 and stirring was continued for 2–3 h at room temperature. The dark violet precipitation was filtered, washed twice with methanol and several times with cold water, and dried under reduced pressure (yield 50–60%).

4.1.2. General procedure for the preparation of oxime derivatives (4a–l). One millimole of the appropriate indirubin analogs **3a–l** and **6** was added to 10 ml pyridine with 6 mmol of hydroxylamine hydrochloride. The mixture was refluxed for 2–3 h at 120 °C. After cooling, the product was neutralized with 1 N HCl. The precipitation was filtered and washed with water.

4.1.2.1. Indirubin-3′-oxime (4a). Yield: 44% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.49 (1H, s, NOH), 11.74 (1H, s, N′–H), 10.74 (1H, s, N–H), 8.65 (1H, d, J = 7.8 Hz), 8.23 (1H, d, J = 7.8 Hz), 7.20 (2H, m), 7.13 (1H, t, J = 7.5 Hz), 6.92 (4H, m); MS (MALDI-TOF) m/z: 276.

4.1.2.2. 5-Trifluoromethoxy-indirubin-3′-oxime (4b). Yield: 71% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.65 (1H, s, NOH), 11.85 (1H, s, N′–H), 10.91 (1H, s, N–H), 8.60 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 7.40 (2H, m), 7.01 (2H, m), 6.94 (1H, d, J = 8.4 Hz); MS (MALDI-TOF) m/z: 403.

4.1.2.3. 5-Nitro-indirubin-3′-oxime (4c). Yield: 94.1% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.92 (1H, s, NOH), 11.90 (1H, s, N′–H), 11.44 (1H, s, N–H), 9.47 (1H, s), 8.27 (1H, d, J = 7.5 Hz), 8.10 (1H, dd, J = 2.1, 8.7 Hz), 7.48 (2H, m), 7.09 (2H, m); MS (MALDI-TOF) m/z: 321.

4.1.2.4. 5-Fluoro-indirubin-3′-oxime (4d). Yield: 54.4% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.66 (1H, s, NOH), 11.80 (1H, s, N′–H), 10.75 (1H, s, N–H), 8.48 (1H, dd, J = 2.6, 11.1 Hz), 8.23 (1H, d, J = 7.5 Hz), 7.43 (2H, m), 7.00 (3H, m); MS (MALDI-TOF) m/z: 295.

4.1.2.5. 5-Methyl-indirubin-3′-oxime (4e). Yield: 78% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.40 (1H, s, NOH), 11.72 (1H, s, N′–H), 10.59 (1H, s, N–H), 8.48 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 7.39 (2H, m), 7.00 (2H, m), 6.77 (1H, d, J = 7.8 Hz), 2.34 (3H, s, CH_3); MS (MALDI-TOF) m/z: 291.

4.1.2.6. 5,7-Dimethyl-indirubin-3′-oxime (4f). Yield: 97% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.38 (1H, s, NOH), 11.77 (1H, s, N′–H), 10.61 (1H, s, N–H), 8.34 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 7.39 (2H, m), 7.02 (1H, s), 6.77 (1H, s), 2.30 (3H, s, CH_3), 2.22 (3H, s, CH_3); MS (MALDI-TOF) m/z: 305.

4.1.2.7. 5-Chloro-7-methyl-indirubin-3′-oxime (4g). Yield: 97.2% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.65 (1H, s, NOH), 11.88 (1H, s, N′–H), 10.88 (1H, s, N–H), 8.52 (1H, s), 8.24 (1H, d, J = 7.2 Hz), 7.43 (2H, m), 7.04 (2H, m), 2.22 (3H, s, CH_3); MS (MALDI-TOF) m/z: 325.

4.1.2.8. 5-Chloro-indirubin-3′-oxime (4h). Yield: 37% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.68 (1H, s, NOH), 11.83 (1H, s, N′–H), 10.85 (1H, s, N–H), 8.65 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 7.42 (2H, m), 7.10 (2H, m), 6.88 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 311.

4.1.2.9. 5-Iodo-indirubin-3′-oxime (4i). Yield: 90% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.67 (1H, s, NOH), 11.83 (1H, s, N′–H), 10.84 (1H, s, N–H), 8.90 (1H, s), 8.25 (1H, d, J = 7.8 Hz), 7.40 (3H, m), 7.06 (1H, s), 6.74 (1H, d, J = 7.8 Hz); MS (MALDI-TOF) m/z: 403.

4.1.2.10. Indirubin-3′-oxime-5-sulfonic acid sodium salt (4j). Yield: 30% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.70 (1H, s, NOH) 11.81 (1H, s, N–H), 10.79 (1H, s, N′–H), 8.87 (1H, s), 8.25 (1H, d, J = 7.5 Hz), 7.43 (3H, m), 7.03 (1H, m), 6.81 (1H, d, J = 7.8 Hz); MS (MALDI-TOF) m/z: 380.

4.1.2.11. 5′-Bromo-indirubin-3′-oxime (4k). Yield: 43% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.74 (1H, s, NOH), 11.75 (1H, s, N′–H), 10.75 (1H, s, N–H), 8.61 (1H, d, J = 10.2 Hz), 8.32 (1H, s), 7.57 (1H, d, J = 7.8 Hz), 7.41 (1H, d, J = 7.5 Hz), 7.14 (1H, t, J = 2.4 Hz), 6.92 (2H, m); MS (MALDI-TOF) m/z: 356.

4.1.2.12. 5′-Bromo-5-nitro-indirubin-3′-oxime (4l). Yield: 33% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 14.17 (1H, s, NOH), 11.90 (1H, s, N–H), 11.42 (1H, s, N′–H), 9.43 (1H, s), 8.36 (1H, s), 8.10 (1H, d, J = 8.4 Hz), 7.60 (1H, d, J = 7.8 Hz), 7.46 (1H, d, J = 8.4 Hz), 7.06 (1H, d, J = 8.4 Hz); MS (MALDI-TOF) m/z: 401.

4.1.2.13. Indirubin-3′-hydrazone (4m). Eighty milligrams (0.3 mmol) of indirubin (**3a**) was dissolved in 3 ml $H_2NNH_2 \cdot H_2O$ and heated under 70–80 °C for

24 h. The blue solid was filtered and washed with diethyl ether. The appropriate product was dissolved in *N*-methylpyrrolidone and purified by column chromatography on silica gel (*n*-hexanes/EtOAc, 1:1) to afford the product as a solid. The first time, NMP must be removed with constant hexane solvent. ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 11.86 (1H, s, NH), 11.86 (1H, s, N′–H), 11.37 (1H, s, N–H), 8.24 (1H, d, J = 7.8 Hz), 8.13 (1H, d, J = 7.8 Hz), 7.52 (2H, s, NH_2), 7.45 (2H, m), 7.38 (3H, m), 7.22 (1H, m); MS (MALDI-TOF) *m*/*z*: 276.

4.1.2.14. N1′-acetyl-indirubin (5a,b). 314.4 mg (1.2 mmol) of indirubin (**3a**,**b**) was added to 15 ml of acetic anhydride and refluxed for 10 h. The precipitate was filtered and washed with water several times. Then the residue was dried.

Compound **5a**: (yield 98%) ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 10.52 (1H, s, N–H), 9.00 (1H, dd, J = 7.8, 1.2 Hz), 8.29 (1H, d, J = 7.5 Hz), 7.73 (1H, d, J = 7.5 Hz), 7.52 (1H, t, J = 7.5 Hz), 7.31 (2H, m), 7.03 (2H, m), 2.76 (3H, s, CH_3); MS (MALDI-TOF) *m*/*z*: 304.2.

Compound **5b**: (yield 95%) ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 11.49 (1H, s, N–H), 8.98 (1H, s), 8.31 (1H, d, J = 9.3 Hz), 7.69 (1H, d, J = 7.2 Hz), 7.62 (1H, t, J = 7.2 Hz), 7.45 (1H, d, J = 8.1 Hz), 7.32 (1H, d, J = 9.3Hz), 7.09 (1H, t, J = 7.2 Hz); MS (MALDI-TOF) *m*/*z*: 388.

4.1.2.15. 5-Amino-indirubin (6). Five hundred milligrams (1.629 mmol) of indirubin-5-nitro (**3c**) and 1.8 g (8.145 mmol) of $SnCl_2 \cdot 2H_2O$ were dissolved in 5 ml DMF and heated under 70 °C for 2–4 h. The mixture was acidified with 1 N HCl and extracted with ethyl acetate. Then, the solvent was evaporated and the residue was dried under reduced pressure. Yield: 43% ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 10.95 (1H, s, N′–H), 10.45 (1H, s, N–H), 8.15 (1H, s), 7.63 (1H, d, J = 7.5 Hz), 7.55 (1H, t, J = 7.5 Hz), 7.39 (1H, d, J = 7.5 Hz), 6.99 (1H, t, J = 7.5 Hz), 6.59 (1H, d, J = 8.4 Hz), 6.53 (1H, d, J = 8.4 Hz), 4.75 (2H, s, NH_2); MS (MALDI-TOF) *m*/*z*: 277.

4.1.3. General procedure for the preparation of oxime derivatives (7–34). One millimole of 5-amino-indirubin (**6**) was dissolved in pyridine, 1.2 equiv of acyl chloride was dropwise added at 0 °C, and the mixture was stirred for 30 min. After workup, 5-NH acyl indirubins (**7–39**) were added to pyridine with 6 equiv of hydroxylamine hydrochloride. The mixture was refluxed for 2–3 h at 80–90 °C. After cooling, the product was neutralized with 1 N HCl. The precipitate was filtered and washed with water.

4.1.3.1. 5-Acetamido-indirubin-3′-oxime (7). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.33 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.63 (1H, s, N–H), 9.47 (1H, s, amide-NH), 8.52 (1H, d,J = 1.8 Hz), 8.24 (1H, d, J = 7.5 Hz), 7.42 (2H, m), 7.22 (1H, dd, J = 1.8, 1.8 Hz), 7.05 (1H, m), 6.82 (1H, d, J = 8.1 Hz), 1.23 (3H, s); MS (MALDI-TOF) *m*/*z*: 334.

4.1.3.2. 5-Propionamido-indirubin-3′-oxime (8). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.33 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.64 (1H, s, N–H), 9.48 (1H, s, amide-NH), 8.53 (1H, d, J = 1.8 Hz), 8.23 (1H, d, J = 7.8 Hz), 7.39 (2H, m), 7.31 (1H, dd, J = 8.1, 1.8 Hz), 7.03 (1H, m), 6.81 (1H, d, J = 8.1 Hz), 2.30 (2H, q, J = 7.5 Hz, CH_2), 1.11 (3H, t, J = 7.5 Hz, CH_3); MS (MALDI-TOF) *m*/*z*: 348.

4.1.3.3. 5-Butyramido-indirubin-3′-oxime (9). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.34 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.65 (1H, s, N–H), 9.49 (1H, s, amide-NH), 8.53 (1H, s), 8.23 (1H, d, J = 7.8 Hz), 7.33 (3H, m), 7.02 (1H, m), 6.81 (1H, d, J = 8.4 Hz), 2.27 (2H, t, J = 7.2 Hz, CH_2), 1.64 (2H, m, CH_2), 0.92 (2H, t, J = 7.2 Hz, CH_3); MS (MALDI-TOF) *m*/*z*: 377.

4.1.3.4. 5-Pantanamido-indirubin-3′-oxime (10). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.27 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.65 (1H, s, N–H), 9.49 (1H, s, amide-NH), 8.51 (1H, s), 8.23 (1H, s), 7.44 (3H, m), 7.02 (2H, m), 2.28 (2H, m, CH_2), 1.58 (2H, m, CH_2), 1.32 (2H, m, CH_2), 0.89 (3H, m, CH_3); MS (MALDI-TOF) *m*/*z*: 376.

4.1.3.5. 5-Trimethylacetamido-indirubin-3′-oxime (11). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.40 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.67 (1H, s, N–H), 8.89 (1H, s, amide-NH), 8.46 (1H, s), 8.25 (1H, d, J = 7.5 Hz), 7.40 (2H, m), 7.24 (1H, d, J = 8.4 Hz), 7.03 (1H, m), 6.81 (1H, d, J = 8.4 Hz), 1.26 (9H, s, $(CH_3)_3$); MS (MALDI-TOF) *m*/*z*: 376.

4.1.3.6. 5-(3,3-Dimethylbutanamido)-indirubin-3′-oxime (12). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.37 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.63 (1H, s, N–H), 9.43 (1H, s, amide-NH), 8.55 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.39 (2H, m), 7.31 (1H, d, J = 7.8 Hz), 7.03 (1H, m), 6.81 (1H, d, J = 7.8 Hz), 2.18 (2H, s, CH_2) 1.05 (9H, s, $(CH_3)_3$); MS (MALDI-TOF) *m*/*z*: 390.

4.1.3.7. 5-(3-Methylbut-2-enamido)-indirubin-3′-oxime (13). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.33 (1H, s, NOH), 11.72 (1H, s, N′–H), 10.64 (1H, s, N–H), 9.44 (1H, s, amide-NH), 8.53 (1H, d, J = 1.5 Hz), 8.23 (1H, d, J = 7.5 Hz), 7.39 (2H, m), 7.33 (1H, dd, J = 8.4, 1.5 Hz), 7.03 (1H, m), 6.81 (1H, d, J = 8.4 Hz), 5.88 (1H, s, CH) 2.15 (3H, s, CH_3), 1.86 (3H, s, CH_3); MS (MALDI-TOF) *m*/*z*: 374.

4.1.3.8. 5-(Cyclopropanecarboxamido)-indirubin-3′-oxime (14). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.37 (1H, s, NOH), 11.71 (1H, s, N′–H), 10.62 (1H, s, N–H), 9.85 (1H, s, amide-NH), 8.56 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.43 (2H, m), 7.33 (1H, d, J = 8.4 Hz), 7.03 (1H, m), 6.80 (1H, d, J = 8.4 Hz), 1.25 (1H, m, CH) 0.81 (4H, m, CH_2), MS (MALDI-TOF) *m*/*z*: 360.

4.1.3.9. 5-(Cyclohexanecarboxamido)-indirubin-3′-oxime (15). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.29 (1H, s, NOH), 11.71 (1H, s, N′–H), 10.62 (1H, s, N–H), 9.35 (1H, s, amide-NH), 8.50 (1H, d, J = 1.5 Hz), 8.23 (1H, d, J = 7.2 Hz), 7.39 (3H, m), 7.03 (1H, m), 6.79

(1H, d, J = 8.1Hz), 2.28 (H, m, CH) 1.80 (4H, m, CH_2), 1.26 (6H, m, CH_2); MS (MALDI-TOF) m/z: 402.

4.1.3.10. 5-(Furan-2-carboxamido)-indirubin-3′-oxime (16). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.37 (1H, s, NOH), 11.75 (1H, s, N′–H), 10.71 (1H, s, N–H), 9.84 (1H, s, amide-NH), 8.63 (1H, d, J = 1.8 Hz), 8.23 (1H, d, J = 7.5 Hz), 7.92 (1H, s), 7.40 (2H, m), 7.32 (2H, m), 7.03 (1H, m), 6.87 (1H, d, J = 8.1 Hz), 6.70 (1H, m); MS (MALDI-TOF) m/z: 386.

4.1.3.11. 5-(Thiophene-2-carboxamido)-indirubin-3′-oxime (17). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.40 (1H, s, NOH), 11.77 (1H, s, N′–H), 10.74 (1H, s, N–H), 10.06 (1H, s, amide-NH), 8.61 (1H, d, J = 1.8 Hz), 8.23 (1H, d, J = 7.8 Hz), 8.02 (1H, d, J = 3.6 Hz), 7.83 (1H, d, J = 3.8 Hz), 7.40 (2H, m), 7.28 (1H, dd, J = 8.1, 1.8 Hz), 7.23 (1H, t, J = 3.8 Hz), 7.03 (1H, m), 6.89 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 402.

4.1.3.12. 5-Benzamido-indirubin-3′-oxime (18). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.42 (1H, s, NOH), 11.76 (1H, s, N′–H), 10.71 (1H, s, N–H), 10.06 (1H, s, amide-NH), 8.78 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.99 (2H, m), 7.56 (3H, m), 7.37 (3H, m), 7.03 (1H, t, J = 7.5 Hz), 6.91 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 396.

4.1.3.13. 5-(2-Chlorobenzamido)-indirubin-3′-oxime (19). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.25 (1H, s, NOH), 11.74 (1H, s, N′–H), 10.70 (1H, s, N–H), 10.04 (1H, s, amide-NH), 8.74 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 7.53 (7H, m), 7.03 (1H, m), 6.87 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 430.

4.1.3.14. 5-(4-Chlorobenzamido)-indirubin-3′-oxime (20). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.35 (1H, s, NOH), 11.75 (1H, s, N′–H), 10.71 (1H, s, N′–H), 10.09 (1H, s, amide-NH), 8.68 (1H, d, J = 1.5 Hz), 8.23 (1H, d, J = 7.8 Hz), 8.06 (2H, d, J = 8.4 Hz), 7.61 (2H, d, J = 8.4 Hz), 7.40 (2H, m), 7.3 (1H, dd, J = 8.4, 1.5 Hz), 7.03 (1H, m), 6.89 (1H, d, J = 8.4 Hz); MS (MALDI-TOF) m/z: 430.

4.1.4. 5-(2-Fluorobenzamido)-indirubin-3′-oxime (21). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.30 (1H, s, NOH), 11.75 (1H, s, N′–H), 10.72 (1H, s, N–H), 9.95 (1H, s, amide-NH), 8.72 (1H, s), 8.23 (1H, d, J = 7.8 Hz), 7.75 (1H, t, J = 7.8 Hz), 7.58 (1H, m), 7.37 (5H, m), 7.03 (1H, m), 6.88 (1H, d, J = 8.4 Hz); MS (MALDI-TOF) m/z: 414.

4.1.4.1. 5-(4-Fluorobenzamido)-indirubin-3′-oxime (22). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.36 (1H, s, NOH), 11.76 (1H, s, N′–H), 10.73 (1H, s, N–H), 10.04 (1H, s, amide-NH), 8.66 (1H, s), 8.23 (1H, d, J = 8.4 Hz), 8.10 (2H, m), 7.34 (5H, m), 7.03 (1H, m), 6.89 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 414.

4.1.4.2. 5-(2-Trifluoromethylbenzamido)-indirubin-3′-oxime (23). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.33 (1H, s, NOH), 11.74 (1H, s, N′–H), 10.71 (1H, s, N–H), 10.13 (1H, s, amide-NH), 8.70 (1H, s), 8.23 (1H, s), 7.78 (4H, m), 7.40 (3H, m), 7.03 (2H, m); MS (MALDI-TOF) m/z: 464.

4.1.4.3. 5-(3-Nitrobenzamido)-3′-oxime (24). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.42 (1H, s, NOH), 11.79 (1H, s, N′–H), 10.74 (1H, s, N–H), 10.44 (1H, s, amide-NH), 8.87 (1H, s), 8.72 (1H, s), 8.46 (2H, m), 8.24 (1H, d, J = 7.2 Hz), 7.85 (1H, t, J = 8.1 Hz), 7.41 (2H, m), 7.33 (1H, d, J = 8.1 Hz), 7.03 (1H, m), 6.91 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 442.

4.1.4.4. 5-(4-Nitrobenzamido)-indirubin-3′-oxime (25). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.35 (1H, s, NOH), 11.75 (1H, s, N′–H), 10.73 (1H, s, N–H), 10.36 (1H, s, amide-NH), 8.73 (1H, d, J = 1.8 Hz), 8.38 (2H, m), 8.24 (2H, m), 7.69 (1H, m), 7.40 (2H, m), 7.35 (1H, dd, J = 8.1, 1.8 Hz), 7.03 (1H, m), 6.90 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 441.

4.1.4.5. 5-(3,5-Dinitrobenzamido)-indirubin-3′-oxime (26). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.42 (1H, s, NOH), 11.77 (1H, s, N′–H), 10.77 (2H, s, N–H, amide-NH), 9.29 (2H, s), 9.01 (1H, s), 8.75 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.40 (3H, m), 7.03 (1H, m), 6.92 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 486.

4.1.4.6. 5-(4-Methoxybenzamido)-indirubin-3′-oxime (27). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.42 (1H, s, NOH), 11.74 (1H, s, N′–H), 10.70 (1H, s, N–H), 9.86 (1H, s, amide-NH), 8.68 (1H, s), 8.23 (1H, d, J = 7.8 Hz), 8.04 (2H, m), 7.41 (3H, m), 7.05 (3H, m), 6.87 (1H, d, J = 8.4 Hz), 3.85 (3H, s, CH_3); MS (MALDI-TOF) m/z: 426.

4.1.4.7. 5-(4-Methylbenzamido)-indirubin-3′-oxime (28). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.35 (1H, s, NOH), 11.76 (1H, s, N′–H), 10.69 (1H, s, N–H), 9.89 (1H, s, amide-NH), 8.66 (1H, d, J = 1.8 Hz), 8.24 (1H, d, J = 7.5 Hz), 7.94 (2H, m), 7.36 (5H, m), 7.03 (1H, m), 6.89 (1H, d, J = 8.1 Hz); MS (MALDI-TOF) m/z: 410.

4.1.4.8. 5-(2-(3-Chlorophenoxy)acetamido)-indirubin-3′-oxime (29). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.38 (1H, s, NOH), 11.76 (1H, s, N′–H), 10.70 (1H, s, N–H), 9.85 (1H, s, amide-NH), 8.65 (1H, s), 8.25 (1H, d, J = 7.5 Hz), 7.65 (1H, dd, J = 7.2, 7.5 Hz), 7.37 (5H, m), 7.02 (2H, m), 6.88 (1H, J = 7.2 Hz), 4.69 (2H, s, CH_2); MS (MALDI-TOF) m/z: 460.

4.1.4.9. 5-(2-(4-Chlorophenoxy)acetamido)-indirubin-3′-oxime (30). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.40 (1H, s, NOH), 11.75 (1H, s, N′–H), 10.71 (1H, s, N–H), 9.72 (1H, s, amide-NH), 8.60 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.36 (5H, m), 7.02 (4H, m), 4.68 (2H, s, CH_2); MS (MALDI-TOF) m/z: 460.

4.1.4.10. 5-(Cinnamamido)-indirubin-3′-oxime (31). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.36 (1H, s, NOH), 11.76 (1H, s, N′–H), 10.68 (1H, s, N–H), 9.89 (1H, s, amide-NH), 8.63 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 7.59 (3H, m), 7.44 (6H, m), 7.03 (1H, m), 6.87 (2H, m); MS (MALDI-TOF) m/z: 422.

4.1.4.11. 5-(3-Phenylpropanamido)-indirubin-3′-oxime (32). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.35 (1H, s, NOH), 11.73 (1H, s, N′–H), 10.65 (1H, s, N–H), 9.60 (1H, s, amide-NH), 8.56 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.39 (2H, m), 7.32 (5H, m), 7.21 (1H, m), 7.04 (1H, m), 6.81 (1H, d, J = 8.1 Hz), 2.94 (2H, t, J = 7.2 Hz, CH_2), 2.61 (2H, t, J = 7.2 Hz, CH_2); MS (MALDI-TOF) m/z: 424.

4.1.4.12. 5-Diphenylacetamido-indirubin-3′-oxime (33). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.33 (1H, s, NOH), 11.71 (1H, s, N′–H), 10.65 (1H, s, N–H), 10.13 (1H, s, amide-NH), 8.55 (1H, s), 8.23 (1H, d, J = 6.6 Hz), 7.34 (13H, m), 7.02 (1H, m), 6.81 (1H, d, J = 8.4 Hz), 5.21 (1H, s, CH); MS (MALDI-TOF) m/z: 486.

4.1.4.13. 5-(1-Naphthamido)-indirubin-3′-oxime (34). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm) 13.36 (1H, s, NOH), 11.76 (1H, s, N′–H), 10.72 (1H, s, N–H), 10.11 (1H, s, amide-NH), 8.79 (1H, s), 8.32 (1H, m), 8.23 (1H, d, J = 7.2 Hz), 8.04 (2H, m), (1H, d, J = 6.9 Hz), 7.62 (3H, m), 7.52 (1H, d, J = 8.4 Hz), 7.03 (1H, m), 6.91 (1H, d, J = 8.4 Hz); MS (MALDI-TOF) m/z: 446.

4.1.5. Anti-proliferative activity using sulforhodamine B assay. Cells (A549, Col2, HT1080, HL-60, MCF-7, and Snu-638) were counted, diluted to 5×10^4 cells/ml with fresh medium (MEME, DMEM, or RPMI containing 10% FBS), and 190 μl of cell suspension was added to 96-well plates containing various concentrations of test compounds (10 μl in 10% aqueous DMSO).[28] Test plates were incubated for 3 days at 37 °C in a CO_2 incubator. For zero day controls, cells were incubated for 30 min at 37 °C in a CO_2 incubator. All treatments were performed in triplicate. After the incubation periods, cells were fixed with 50 μl cold 50% TCA at 4 °C for 30 min, washed five times with tap water, and air-dried. The fixed cells were stained with 0.4% sulforhodamine B (SRB) solution in 1% aqueous acetic acid at room temperature for 1 h. Free SRB solution was then removed by rinsing five times with 1% acetic acid and air-dried. The bound dye was dissolved with 200 μl of 10 mM Tris base (pH 10.0), and absorbance was determined at 515 nm using an ELISA microplate reader. Finally, the absorbance values obtained with each of the treatment procedures were averaged, and the average values obtained with the zero day control were subtracted. These results were expressed as a percentage, relative to solvent treated control incubations, and IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration).

4.1.6. Assay for the phosphorylation of Rb protein in vitro by immunoprecipitated CDK. MCF-7 cell extracts were prepared by lysing the cells in a lysis buffer (20 mM HEPES, pH 7.5, 135 mM NaCl, 1% Nonidet P40, 50 mM EDTA, and protease and phosphatase inhibitors) for 1 h at 4 °C, followed by centrifugation at 12,000g. Protein determinations were carried out using a protein measurement kit (protein assay, no. 500-0006; Bio-Rad, Hercules, CA). The 500 μg of cell extracts was precleaned with protein G–Sepharose (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and then incubated with anti-CDK2 antibody (Santa Cruz, CA) at 4 °C for 2 h. After immune complexes were collected with protein G–Sepharose. The kinase reaction using CDK2 immune complexes was carried out for 30 min in 30 μl containing 20 mM HEPES, pH 7.5, 1 mM DTT, 20 mM $MgCl_2$, 2 mM EGTA, 0.1 mM vanadate, 0.2 μCi [γ-^{33}P]ATP (3000 Ci mmol^{-1}, 25 μM ATP, and 1 μg substrate (GST-Rb fusion protein: amino acids 761–928)) at 30 °C. The reactions were stopped by adding Laemmli buffer and heated to 100 °C for 5 min. The proteins were separated by SDS–PAGE on 10% gels, dried, and exposed to an X-ray-sensitive film.

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References and notes

1. Zheng, L.; Lee, W. H. *Exp. Cell Res.* **2001**, *264*, 2.
2. Morgan, D. O. *Annu. Rev. Cell Dev. Biol.* **1997**, *13*, 261.
3. Hunter, T.; Pines, J. *Cell* **1994**, *79*, 573.
4. Sherr, C. J. *Science* **1996**, *274*, 1672.
5. Pines, J. *Semin. Cancer Biol.* **1994**, *5*, 305.
6. Pines, J. *Trends Biochem. Sci.* **1993**, *18*, 195.
7. Sherr, C. J.; Roberts, J. M. *Genes Dev.* **1999**, *13*, 1501.
8. Pines, J. *Nat. Cell Biol.* **1999**, *1*, E73.
9. Ortega, S.; Prieto, I.; Odajima, J.; Martin, A.; Dubus, P.; Sotillo, R.; Barbero, J. L.; Malumbres, M.; Barbacid, M. *Nat. Genet.* **2003**, *35*, 25.
10. Meijer, L.; Leclerc, S.; Leost, M. *Pharmacol. Ther.* **1999**, *82*, 279.
11. Garrett, M. D.; Fattaey, A. *Curr. Opin. Genet. Dev.* **1999**, *9*, 104.
12. Webster, K. R. *Expert Opin. Invest. Drugs* **1998**, *7*, 865.
13. Davis, S. T.; Benson, B. G.; Bramson, H. N.; Chapman, D. E.; Dickerson, S. H.; Dold, K. M.; Eberwein, D. J.; Edelstein, M.; Frye, S. V.; Gampe, R. T., Jr.; Griffin, R. J.; Harris, P. A.; Hassell, A. M.; Holmes, W. D.; Hunter, R. N.; Knick, V. B.; Lackey, K.; Lovejoy, B.; Luzzio, M. J.; Murray, D.; Parker, P.; Rocque, W. J.; Shewchuk, L.; Veal, J. M.; Walker, D. H.; Kuyper, L. F. *Science* **2001**, *291*, 134.
14. De Azevedo, W. F.; Leclerc, S.; Meijer, L.; Havlicek, L.; Strnad, M.; Kim, S. H. *Eur. J. Biochem.* **1997**, *243*, 518.
15. Senderowicz, A. M.; Sausville, E. A. *J. Natl. Cancer Inst.* **2000**, *92*, 376.
16. Xiao, Z.; Hao, Y.; Liu, B.; Qian, L. *Leuk. Lymphoma* **2002**, *43*, 1763.
17. Hoessel, R.; Leclerc, S.; Endicott, J. A.; Nobel, M. E.; Lawrie, A.; Tunnah, P.; Leost, M.; Damiens, E.; Marie, D.; Marko, D.; Niederberger, E.; Tang, W.; Eisenbrand, G.; Meijer, L. *Nat. Cell Biol.* **1999**, *1*, 60.

18. Marko, D.; Schatzle, S.; Friedel, A.; Genzlinger, A.; Zankl, H.; Meijer, L.; Eisenbrand, G. *Br. J. Cancer* **2001**, *84*, 283.
19. Leclerc, S.; Garnier, M.; Hoessel, R.; Marko, D.; Bibb, J. A.; Snyder, G. L.; Greengard, P.; Biernat, J.; Wu, Y. Z.; Mandelkow, E. M.; Eisenbrand, G.; Meijer, L. *J. Biol. Chem.* **2001**, *276*, 251.
20. Polychronopoulos, P.; Magiatis, P.; Skaltsounis, A. L.; Myrianthopoulos, V.; Mikros, E.; Tarricone, A.; Musacchio, A.; Roe, S. M.; Pearl, L.; Leost, M.; Greengard, P.; Meijer, L. *J. Med. Chem.* **2004**, 47.
21. Adachi, J.; Mori, Y.; Matsui, S.; Takigami, H.; Fujino, J.; Kitagawa, H.; Miller, C. A., 3rd; Kato, T.; Saeki, K.; Matsuda, T. *J. Biol. Chem.* **2001**, *276*, 31475.
22. Kawanishi, M.; Sakamoto, M.; Ito, A.; Kishi, K.; Yagi, T. *Mutat. Res.* **2003**, *540*, 99.
23. Knockaert, M.; Blondel, M.; Bach, S.; Leost, M.; Elbi, C.; Hager, G. L.; Nagy, S. R.; Han, D.; Denison, M.; Ffrench, M.; Ryan, X. P.; Magiatis, P.; Polychronopoulos, P.; Greengard, P.; Skaltsounis, L.; Meijer, L. *Oncogene* **2004**, *23*, 4400.
24. Xie, Y.; Liu, Y.; Ma, C.; Yuan, Z.; Wang, W.; Zhu, Z.; Gao, G.; Liu, X.; Yuan, H.; Chen, R.; Huang, S.; Wang, X.; Zhu, X.; Mao, Z.; Li, M. *Neurosci. Lett.* **2004**, *367*, 355.
25. Duensing, S.; Duensing, A.; Lee, D. C.; Edwards, K. M.; Piboonniyom, S. O.; Manuel, E.; Skaltsounis, L.; Meijer, L.; Munger, K. *Oncogene* **2004**, *23*, 8206.
26. Lee, J.-W.; Moon, M.-J.; Min, H.-Y.; Chung, H.-J.; Park, E.-J.; Park, H. J.; Hong, J.-Y.; Kim, Y.-C.; Lee, S. K. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3948.
27. Nam, S.; Buettner, R.; Turkson, J.; Kim, D.; Cheng, J. Q.; Muehlbeyer, S.; Hippe, F.; Vatter, S.; Merz, K. H.; Eisenbrand, G.; Jove, R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5998–6003.
28. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.