

## Synthesis and Antiproliferative Activity of 7-Azaindirubin-3'-oxime, a 7-Aza Isostere of the Natural Indirubin Pharmacophore

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The bis-indole alkaloid indirubin and its analogues bear a very interesting natural pharmacophore. They are recognized mainly as kinase inhibitors, but several other activities make them possible candidates for preclinical studies. Based on the previously reported activity of 7-bromoindirubin-3'-oxime and its derivatives, the synthesis of indirubins bearing a heterocyclic nitrogen atom at position 7 was carried out. Herein, we report the first synthesis of 7-azaindirubin-3'-oxime (**12**) as well as its antiproliferative activity against 57 cancer cell lines and its inhibitory activity against a series of kinases. 7-Azaindirubin (**10**) and its 3'-oxime derivative (**12**) showed reduced activity as kinase inhibitors in comparison with other known indirubin derivatives, but antiproliferative activity with a best GI<sub>50</sub> value of 0.77 μM.

The bis-indole alkaloid indirubin and its analogues (collectively referred to as indirubins) were among the early cyclin-dependent kinase (CDK) inhibitors to be discovered.<sup>1</sup> The red-purple indirubin (Figure 1) is an isomer of the blue indigo. Indirubins can be found in various indigo dye-producing plants (more than 200 species).<sup>2</sup> They are also present in the historic "Tyrean purple" dye extracted from several Muricidae mollusks.<sup>3,4</sup> Indirubins have also been found in various wild-type and recombinant bacteria.<sup>5,6</sup> Finally, indirubin and indigo are occasionally present in human urine.<sup>7</sup> Moreover, indirubin is the active ingredient of a Traditional Chinese Medicine recipe, "Danggui Longhui Wan", used to treat various diseases including chronic myelocytic leukemia.<sup>8</sup> Besides CDKs, indirubins were found to target glycogen synthase kinase-3 (GSK-3),<sup>9</sup> aurora kinases,<sup>10</sup> and the aryl hydrocarbon receptor (AhR), also known as the dioxin receptor.<sup>7,11</sup> Experimental evidence suggests that the antiproliferative effects of indirubins derive from their ability to inhibit CDKs.<sup>12,13</sup> However, interaction with the AhR contributes to a marked arrest in the G1 phase of the cell cycle.<sup>14</sup> Finally, it was recently shown that some indirubins are able to prevent the phosphorylation and subsequent activation of the transcription factor STAT3, leading to a down-regulation of survival factors such as survivin and Mcl-1 and leading to the subsequent induction of cell death.<sup>15</sup>

During the past few years, we have isolated or synthesized more than 150 indirubin derivatives.<sup>3,14,16–19</sup> Among these derivatives, the most effective is 6-bromoindirubin-3'-oxime, showing potent inhibitory activity on GSK-3, CDK1, and CDK5 with IC<sub>50</sub> values of 0.005, 0.320, and 0.083 μM, respectively.<sup>3</sup> This agent has been used as a pharmacological tool in numerous studies in order to investigate the physiological role of GSK-3 in various cellular settings and control the fate of embryonic stem cells.<sup>20</sup> Very recently we developed a new series of highly potent, water-soluble derivatives of 6-bromoindirubin-3'-oxime with activity on circadian rhythms due to inhibition of GSK-3.<sup>19</sup>

Another group of indirubins is the 7-substituted family. While synthesizing and testing the biological activity of new indirubins, we discovered randomly that 7-bromoindirubin-3'-oxime displayed potent cell-death-inducing properties. Despite weak or insignificant inhibitory activity on various classical kinase targets of indirubins (IC<sub>50</sub> values for GSK-3, CDK1, and CDK5 32, 22, and 33 μM,

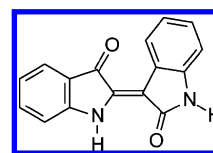


Figure 1. Structure of natural indirubin.

respectively), 7-bromoindirubin potently induced a rapid nonapoptotic, caspase-independent, cell death.<sup>18</sup>

On the basis of the activity of 7-bromoindirubin-3'-oxime and its derivatives<sup>17</sup> we envisaged the synthesis of new indirubins bearing a heterocyclic nitrogen atom at position 7. Herein, we report the first synthesis of 7-azaindirubin and its 3'-oxime as well as their antiproliferative activity and their inhibitory activity against a series of kinases. Recently, a series of *N*-substituted azaindirubins was synthesized and tested for their cytotoxicity<sup>21</sup> as well as a series of other indirubin derivatives.<sup>22</sup>

The synthesis of the aza-heterocyclic skeleton of indirubins **10** and **11** was achieved through a dimerization reaction of the appropriate 7-azaisatin (**2**) or *N*-Me-7-azaisatin (**6**) with 3-acetoxyindole (**9**) under conditions similar to those previously employed in the synthesis of indirubins.<sup>16</sup> The key nucleus of 7-azaisatin (**2**) was synthesized by treatment of commercial 7-azaindole with NBS/DMSO following a previously reported method for *N*-substituted azaindoles.<sup>23</sup> The yield of the above reaction was relatively low, but it increased greatly in the case of 1-methyl-7-azaindole (**4**), leading to 1-methyl-7-azaisatin (**6**) and subsequently to 1-methyl-7-azaindirubin (**11**). In the same reaction, two byproducts with an isoindigo skeleton (**7** and **8**) were obtained. The two synthesized azaindirubins were transformed into the corresponding 3'-oximes, **12** and **13**.

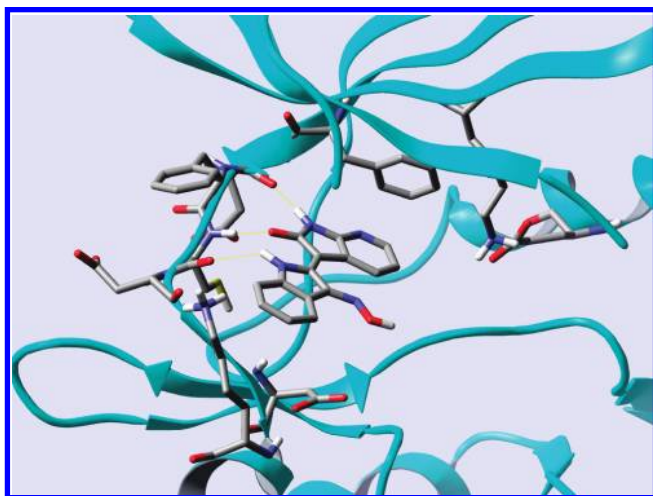
All test compounds showed IC<sub>50</sub> > 300 nM against the four kinases CDK1, CDK2, CDK5, and GSK3 beta (Table S1, Supporting Information). The activity was significantly weaker than that of indirubin-3'-oxime.<sup>3</sup> Although molecular modeling studies indicated that 7-azaindirubin and its 3'-oxime derivative show the same placement into the ATP pocket of the kinases investigated and the same number of hydrogen bonds as indirubin-3'-oxime (Figure 2), they exhibited reduced activity as kinase inhibitors, probably due to electrostatic reasons. In contrast, they showed interesting antiproliferative activity, probably via a mechanism not related to kinase inhibition.

Among the synthesized 7-azaindirubin derivatives, compound **12** gave 81% CDK5 inhibition at 3 μM and demonstrated slightly better selectivity than 6-bromoindirubin-3'-oxime. Compound **13**,

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**Figure 2.** Binding of 7-azaindirubin-3'-oxime (**12**) into the ATP pocket of GSK3 $\beta$ .

**Table 1.** *In Vitro* Antiproliferative Activity of 7-Azaindirubin-3'-oxime (**12**) against 10 Sensitive Cancer Cell Lines<sup>a</sup>

cell line	GI <sub>50</sub> ( $\mu$ M)
KM12 (colon)	0.77
NCI-H226 (non-small cell lung)	1.1
RXF393 (renal)	1.2
NCI-H322 M (non-small cell lung)	1.3
UO-31 (renal)	1.6
IGROV1 (ovarian)	1.6
SK-OV-3 (ovarian)	1.8
RPMI (leukemia)	1.9
A-498 (renal)	1.9
T-47D (breast)	2.0

<sup>a</sup> Tested by the Developmental Therapeutics Program, National Cancer Institute, Frederick, MD.

the N-1-methylated derivative of **12**, did not show any CDK inhibitory activity at the test concentrations used. According to our previous study, the N1-H group in 6-bromoindirubin-3'-oxime forms a hydrogen bond with the backbone carbonyl group of Glu81 in CDK5.<sup>3</sup> Capping the N1-H with a methyl group abolished this key H-bond and consequently diminished compound inhibitory activity.

7-Azaindirubin (**10**), 7-azaindirubin-3'-oxime (**12**), and diaza-isoidingo (**7**) were submitted for *in vitro* biological testing against the 57 cancer cell line screen provided by the U.S. National Cancer Institute, Developmental Therapeutics Program. The results obtained at a concentration of 10  $\mu$ M are given in Tables S2–S4, Supporting Information.

7-Azaindirubin-3'-oxime (**12**) presented the most potent antiproliferative activity and was further evaluated for growth inhibition (GI<sub>50</sub>). The GI<sub>50</sub> values (Table 1; Table S5, Supporting Information) ranged from 100 to 0.77  $\mu$ M, showing the most potent activity against a colon cancer cell line (KM12). Other cell lines showing GI<sub>50</sub> values of less than 2  $\mu$ M included three renal, two ovarian, and two non-small cell lung cancer cell lines. The fact that compound **10** had a similar antiproliferative profile (Table S2, Supporting Information), yet was weakly active versus CDK1, -2, and -5 and GSK3, suggests that inhibition of these kinases is not responsible for the observed antiproliferative activity of these compounds. The mechanism of action of indirubins with reduced kinase inhibitory activity like **10** and **12** may be attributed to their activity on the AhR receptor.<sup>14</sup> However, it is clear that the inhibition of the commonly screened kinases (CDK1, CDK2, CDK5, GSK3) is not enough to explain the antiproliferative activity of indirubins.

## Experimental Section

**General Experimental Procedures.** All chemicals were purchased from Aldrich Chemical Co. Melting points were determined with a Sanyo Gallenkamp apparatus. Infrared spectra (IR) were recorded on a Perkin-Elmer Paragon 500 FT-IR spectrometer using multiple internal reflectance (MIR) on a KRS-5 crystal at 45°. NMR spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers [<sup>1</sup>H (400 MHz) and <sup>13</sup>C (50 MHz)]. Chemical shifts are expressed in ppm downfield from TMS. CIMS spectra were determined on a Finnigan GCQ Plus mass spectrometer using CH<sub>4</sub> as the CI ionization reagent. Column chromatography was performed using Merck flash silica gel 60 (40–63  $\mu$ m), with an overpressure of 300 mbar. Elemental analysis was performed using a Perkin-Elmer 2400 CHN elemental analyzer. 7-Azaindole was purchased from AcrosOrganics.

**Preparation of 7-Azaisatin (**2**).** To a solution of 7-azaindole (**1**) (300 mg, 2.54 mmol) in anhydrous DMSO (21 mL) was added NBS (905 mg, 5.08 mmol), and the resulting solution was stirred at 60 °C for 6 h. Then, the mixture was stirred under vacuum in a water pump (in order to remove the HBr produced) at 80 °C for 20 h. Water (17 mL) was then added, and the product was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The CH<sub>2</sub>Cl<sub>2</sub> extract was washed with H<sub>2</sub>O, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and carefully evaporated using a high vacuum pump (N<sub>2</sub> trap, under 40 °C). The solid residue was submitted to flash chromatography with CH<sub>2</sub>Cl<sub>2</sub>–MeOH (100:0 to 90:10) to afford **2** (38 mg, 0.26 mmol, 10%) and **3** (90 mg, 0.32 mmol, 13%).

To a solution of **3** (16 mg, 0.058 mmol) in anhydrous DMSO (1.12 mL) was added NBS (10 mg, 0.058 mmol), and the mixture was stirred at 80 °C for 1.5 h under vacuum in a water pump. After purification following the procedure mentioned above, **2** was obtained quantitatively.

Storage of **2** under humidity and in the atmosphere caused degradation of the product.

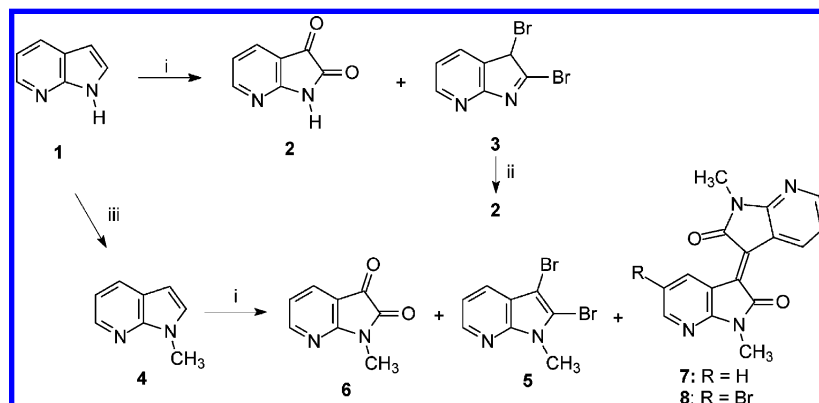
**7-Azaisatin (**2**):** yellowish, amorphous solid; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  9.34 (1H, brs, NH), 8.42 (1H, dd,  $J$  = 5.5, 1.5 Hz, H-6), 7.86 (1H, dd,  $J$  = 7.5, 1.5 Hz, H-4), 7.10 (1H, dd,  $J$  = 7.5, 5.5 Hz, H-5); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 50 MHz)  $\delta$  181.6 (C-3), 163.8 (C-7a), 158.1 (C-2), 155.3 (C-6), 133.8 (C-4), 119.7 (C-5), 112.2 (C-3a); CIMS  $m/z$  149 (M + H)<sup>+</sup>; *anal.* C 56.76%, H 2.72%, N 18.91%, calcd for C<sub>7</sub>H<sub>4</sub>N<sub>2</sub>O<sub>2</sub>, C 56.71%, H 2.73%, N 18.85%.

**2,3-Dibromo-3H-pyrrole-[2,3-*b*]pyridine (**3**):** orange-red, amorphous solid; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.23 (1H, dd,  $J$  = 5.3, 1.4 Hz, H-6), 7.85 (1H, dd,  $J$  = 7.6, 1.4 Hz, H-4), 7.13 (1H, dd,  $J$  = 7.6, 5.3 Hz, H-5), 2.70 (1H, s, H-3); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 50 MHz)  $\delta$  151.6 (C-7a), 148.9 (C-6), 133.9 (C-4), 126.3 (C-3a), 118.9 (C-5), 44.7 (C-2), 42.9 (C-3); CIMS  $m/z$  276, 278, 280 (M + H)<sup>+</sup>; *anal.* C 30.47%, H 1.46%, N 10.15%, calcd for C<sub>7</sub>H<sub>4</sub>Br<sub>2</sub>N<sub>2</sub>, C 30.41%, H 1.46%, N 10.13%.

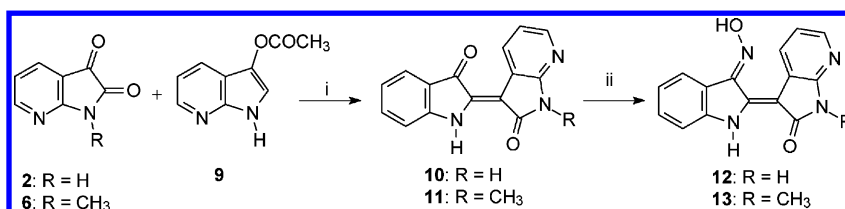
**Preparation of Isatins **6** and **9**.** In order to prepare the 1-methylated derivatives of 7-azaisatin, 1-methylation of 7-azaindole was carried out first. To a solution of **1** (350 mg, 2.96 mmol) in anhydrous DMF (10 mL) was added NaH (100 mg, 4.35 mmol), under N<sub>2</sub>, and the mixture was stirred for 30 min at room temperature. A solution of CH<sub>3</sub>I (218  $\mu$ L, 3.50 mmol) in anhydrous DMF (2 mL) was added slowly, and stirring was continued for 12 h at room temperature. Water (20 mL) was added, and the product was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The CH<sub>2</sub>Cl<sub>2</sub> extract was washed with H<sub>2</sub>O, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and carefully evaporated using a high vacuum pump (N<sub>2</sub> trap, under 40 °C). The solid residue was submitted to flash chromatography with CH<sub>2</sub>Cl<sub>2</sub> to give the yellowish oil **4** (377 mg, 2.86 mmol, 96%). Product **4** showed identical spectroscopic data with those described in the literature.<sup>23</sup>

Isatin **6** (74 mg, 0.46 mmol, 50%) was prepared by a procedure analogous to that of isatin **2**, and the spectroscopic data coincided with those described in the literature.<sup>23</sup> Purification by flash chromatography with CH<sub>2</sub>Cl<sub>2</sub>–MeOH (100:0 to 98:2) afforded also compounds **5** (15 mg, 0.052 mmol, 6%, spectroscopic data identical with those described in the literature),<sup>23</sup> **7** (12 mg, 0.041 mmol, 4.5%), and **8** (0.5 mg, 0.0013 mmol, 0.15%).

**(3E)-7,7'-Diaza-1,1'-dimethylisoidingo (**7**):** orange-red crystals; mp 278 °C; <sup>1</sup>H NMR (DMSO, 400 MHz)  $\delta$  9.39 (2H, dd,  $J$  = 8.0, 1.2 Hz, H-4,4'), 8.31 (2H, dd,  $J$  = 5.1, 1.2 Hz, H-6, 6'), 7.15 (1H, dd,  $J$  = 8.0, 5.1 Hz, H-5, 5'), 3.32 (6H, s, N-CH<sub>3</sub>, N'-CH<sub>3</sub>); <sup>13</sup>C NMR (DMSO, 50 MHz)  $\delta$  167.7 (C-2, 2'), 157.7 (C-7a, 7a'), 150.2 (C-6, 6'), 137.5 (C-4, 4'), 132.2 (C-3, 3'), 118.6 (C-5, 5'), 116.1 (C-3a, 3a'), 25.4 (2  $\times$  CH<sub>3</sub>);

Scheme 1. Oxidation of 7-Azaindole Reagents<sup>a</sup>

<sup>a</sup> Reagents and conditions: (i) NBS (2 equiv), anh. DMSO, H<sub>2</sub>O; (ii) NBS (1 equiv), anh. DMSO, H<sub>2</sub>O; (iii) NaH, CH<sub>3</sub>I.

Scheme 2. Synthesis of 7-Azairubins and Their Corresponding 3'-Oxime Derivatives<sup>a</sup>

<sup>a</sup> Reagents and conditions: (i) Na<sub>2</sub>CO<sub>3</sub>, MeOH, 25 °C; (ii) H<sub>2</sub>NOH·HCl, pyr, 120 °C.

CIMS *m/z* 293 (M + H)<sup>+</sup>; *anal.* C 65.75%, H 4.14%, N 19.17%, calcd for C<sub>16</sub>H<sub>12</sub>N<sub>4</sub>O<sub>2</sub>, C 65.62%, H 4.15%, N 19.21%.

**(3E)-5-Bromo-7,7'-diaza-1,1'-dimethylisoidingo (8):** orange-red, amorphous solid; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 9.67 (1H, d, *J* = 2.4 Hz, H-4), 9.46 (1H, dd, *J* = 8.0, 1.4 Hz, H-4'), 8.31 (1H, d, *J* = 2.4 Hz, H-6), 8.26 (1H, dd, *J* = 5.1, 1.4 Hz, H-6'), 7.03 (1H, dd, *J* = 8.0, 5.1 Hz, H-5'), 3.38 (3H, s, N-CH<sub>3</sub>), 3.35 (3H, s, N'-CH<sub>3</sub>); CIMS *m/z* 371, 373 (M + H)<sup>+</sup>; *anal.* C 51.77%, H 2.99%, N 15.09%, calcd for C<sub>16</sub>H<sub>11</sub>BrN<sub>4</sub>O<sub>2</sub>, C 51.82%, H 3.01%, N 15.06%.

**Preparation of Indirubins 10 and 11.** Methanol (10 mL) was stirred vigorously under N<sub>2</sub> for 20 min, and then 7-azaisatin (2) (50 mg, 0.34 mmol) and 3-acetoxyindole (42 mg, 0.24 mmol) were added; stirring was continued for 5 min. Anhydrous Na<sub>2</sub>CO<sub>3</sub> (76 mg) was added, and the stirring was continued for 3 h. The dark precipitate was filtered and washed with aqueous methanol (1:1, 20 mL). Purification was achieved by flash chromatography with EtOAc–THF (95:5) to afford **10** (146 mg, 0.17 mmol, 73%).

Indirubin **11** was prepared by a procedure analogous to that of **10**, but no further purification was necessary. After washing with aqueous methanol (1:1, 20 mL) and extensively with water, **11** was obtained quantitatively.

**(2Z)-7-Azairubirin (10):** purple crystals; mp >300 °C; IR 3355 (br), 1670, 1615, 1595, 1460, 1306, 1210 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO, 400 MHz) δ 11.43 (1H, s, N-H), 11.09 (1H, s, N'-H), 8.91 (1H, dd, *J* = 7.5, 1.0 Hz, H-4), 8.11 (1H, dd, *J* = 5.1, 1.0 Hz, H-6), 7.67 (1H, dd, *J* = 7.5, 1.0 Hz, H-4'), 7.60 (1H, t, *J* = 7.8 Hz, H-6'), 7.43 (1H, d, *J* = 7.8, Hz, H-7'), 7.05 (2H, overlapped, H-5, 5'); <sup>13</sup>C NMR (DMSO, 50 MHz) δ 188.4 (C-3'), 169.9 (C-2), 154.4 (C-7a), 152.3 (C-7a'), 146.3 (C-6), 138.8 (C-2'), 136.9 (C-6'), 130.6 (C-4), 124.1 (C-4'), 121.3 (C-5'), 118.4 (C-3a'), 117.1 (C-5), 115.5 (C-3a), 113.2 (C-7'), 103.4 (C-3); CIMS *m/z* 264 (M + H)<sup>+</sup>; *anal.* C 68.44%, H 3.45%, N 15.96%, calcd for C<sub>15</sub>H<sub>9</sub>N<sub>3</sub>O<sub>2</sub>, C 68.52%, H 3.45%, N 15.91%.

**(2Z)-7-Aza-1-methylindirubin (11):** purple crystals; mp 235 °C; IR 3320 (br), 1655, 1618, 1590, 1475, 1449, 1335, 1110 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO, 400 MHz) δ 11.13 (1H, s, N'-H), 8.92 (1H, dd, *J* = 7.5, 1.4 Hz, H-4), 8.19 (1H, dd, *J* = 5.1, 1.4 Hz, H-6), 7.67 (1H, d, *J* = 7.7 Hz, H-4'), 7.61 (1H, t, *J* = 7.7 Hz, H-6'), 7.43 (1H, d, *J* = 7.7, Hz, H-7'), 7.12 (1H, dd, *J* = 7.5, 5.1 Hz, H-5), 7.05 (1H, t, *J* = 7.7 Hz, H-5') 3.31 (3H, s, N-CH<sub>3</sub>); <sup>13</sup>C NMR (DMSO, 50 MHz) δ 188.7 (C-3'), 168.7 (C-2), 154.4 (C-7a), 152.7 (C-7a'), 146.5 (C-6), 139.5 (C-2'), 137.5 (C-6'), 130.9 (C-4), 124.6 (C-4'), 121.8 (C-5'), 119.0 (C-3a'), 118.0 (C-5), 115.6 (C-3a), 113.7 (C-7'), 102.7 (C-3), 24.9 (CH<sub>3</sub>);

CIMS *m/z* 281 (M + H)<sup>+</sup>; *anal.* C 69.31%, H 4.00%, N 15.15%, calcd for C<sub>16</sub>H<sub>11</sub>N<sub>3</sub>O<sub>2</sub>, C 69.37%, H 4.00%, N 15.17%.

**Preparation of Oximes 12 and 13.** Indirubin derivative **10** (26 mg, 0.099 mmol) was dissolved in pyridine (5 mL). With magnetic stirring, hydroxylamine hydrochloride (70 mg, 1 mmol) was added and the mixture was heated under N<sub>2</sub> and refluxed (120 °C) for 24 h. Water (20 mL) was added, and the red precipitate obtained was filtered and washed successively with H<sub>2</sub>O and cyclohexane. Purification was achieved by flash chromatography with CH<sub>2</sub>Cl<sub>2</sub>–THF (95:5) to afford **12** (26 mg, 0.09 mmol, 95%).

Oxime **13** was prepared by a procedure analogous to that used for oxime **12**, but no further purification was necessary. After washing extensively with H<sub>2</sub>O and cyclohexane, **13** was obtained quantitatively.

**(2Z,3'E)-7-Azairubirin-3'-oxime (12):** red crystals; mp >300 °C; IR 3090 (br), 1673, 1615, 1560, 1460, 1442, 1337, 1315, 1231, 1185 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO, 400 MHz) δ 13.64 (1H, brs, N-OH), 11.71 (1H, s, N'-H), 11.15 (1H, s, N-H), 8.78 (1H, dd, *J* = 6.3, 1.0 Hz, H-4), 8.23 (1H, d, *J* = 7.5 Hz, H-4'), 7.97 (1H, d, *J* = 6.3 Hz, H-6), 7.44 (1H, d, *J* = 7.5 Hz, H-7'), 7.38 (1H, t, *J* = 7.5 Hz, H-6'), 7.05 (1H, t, *J* = 7.5 Hz, H-5'), 6.94 (1H, t, *J* = 6.3 Hz, H-5); <sup>13</sup>C NMR (DMSO, 50 MHz) δ 169.9 (C-2), 152.0 (C-7a), 150.8 (C-3'), 146.0 (C-2'), 144.4 (C-7a'), 143.4 (C-6), 131.7 (C-6'), 128.4 (C-4'), 127.3 (C-4), 121.5 (C-5'), 116.7 (C-3a), 116.1 (C-5), 115.9 (C-3a'), 111.5 (C-7'), 95.9 (C-3); CIMS *m/z* 279 (M + H)<sup>+</sup>; *anal.* C 64.74%, H 3.62%, N 20.13%, calcd for C<sub>15</sub>H<sub>10</sub>N<sub>4</sub>O<sub>2</sub>, C 64.53%, H 3.63%, N 20.19%.

**(2Z,3'E)-7-Aza-1-methylindirubin-3'-oxime (13):** red crystals; mp 295 °C; IR 3183 (br), 1655, 1611, 1564, 1450, 1345, 1245, 1136 cm<sup>-1</sup>; <sup>1</sup>H NMR (DMSO, 400 MHz) δ 13.68 (1H, brs, N-OH), 11.67 (1H, s, N'-H), 8.81 (1H, dd, *J* = 7.5, 1.0 Hz, H-4), 8.22 (1H, d, *J* = 7.8 Hz, H-4'), 8.09 (1H, dd, *J* = 5.1, 1.0 Hz, H-6), 7.46 (1H, d, *J* = 7.8 Hz, H-7'), 7.42 (1H, t, *J* = 7.8 Hz, H-6'), 7.07 (1H, t, *J* = 7.8 Hz, H-5'), 7.02 (1H, dd, *J* = 7.5, 5.1 Hz, H-5), 3.31 (3H, s, N-CH<sub>3</sub>); <sup>13</sup>C NMR (DMSO, 50 MHz) δ 168.7 (C-2), 151.9 (C-7a), 151.3 (C-3'), 147.1 (C-2'), 144.6 (C-7a'), 143.4 (C-6), 132.0 (C-6'), 128.7 (C-4'), 127.5 (C-4), 122.2 (C-5'), 117.1 (C-3a), 116.9 (C-5), 116.4 (C-3a'), 112.0 (C-7'), 94.3 (C-3), 24.8 (CH<sub>3</sub>); CIMS *m/z* 293 (M + H)<sup>+</sup>; *anal.* C 65.75%, H 4.14%, N 19.17%, calcd for C<sub>16</sub>H<sub>12</sub>N<sub>4</sub>O<sub>2</sub>, C 65.72%, H 4.15%, N 19.23%.

**Kinase Inhibition Assays.** The kinase inhibition assays were performed using γ-<sup>33</sup>P-ATP as the radioligand. The targeted kinases were human GSK3β, CDK1, CDK2, and CDK5. The concentration of

ATP used in the assays was within 15  $\mu\text{M}$  of the apparent  $K_m$  for ATP on the corresponding kinase. All experiments were performed as duplicates.

**GSK3 $\beta$  Assay.** In a final reaction volume of 25  $\mu\text{L}$ , GSK3 $\alpha$  (h) (5–10 mU) was incubated with 8 mM 3-(*N*-morpholino)propane-sulfonic acid (MOPS) at pH 7.0, 0.2 mM EDTA, 20  $\mu\text{M}$  YR-RAAVPPSPSLSRHSSPHQS(p)EDEEE (phospho GS2 peptide), 10 mM Mg acetate, and [ $\gamma$ -<sup>33</sup>P-ATP] (specific activity, ca. 500 cpm/pmol, concentration as required). The reaction was initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction was stopped by the addition of 5  $\mu\text{L}$  of a 3% phosphoric acid solution. Then 10  $\mu\text{L}$  of the reaction was spotted onto a P30 filtermat and washed three times for 5 min in 50 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

**CDK1, CDK2, and CDK5 Assays.** In a final reaction volume of 25  $\mu\text{L}$ , CDK1/cyclinB (h) (5–10 mU), CDK2/cyclinA (h) (5–10 mU), or CDK5/p35 (h) (5–10 mU) was incubated with 8 mM MOPS pH 7.0, 0.2 mM EDTA, 0.1 mg/mL histone H1, 10 mM Mg acetate, and [ $\gamma$ -<sup>33</sup>P-ATP] (specific activity, ca. 500 cpm/pmol, concentration as required). The reaction was initiated by the addition of the MgATP mixture. After incubation for 40 min at room temperature, the reaction was stopped by the addition of 5  $\mu\text{L}$  of a 3% phosphoric acid solution. Then 10  $\mu\text{L}$  of the reaction was spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

**Antiproliferative Activity Test.** The Developmental Therapeutics Program—National Cancer Institute screening procedure is described in detail elsewhere.<sup>24,25</sup>

**Supporting Information Available:** Details about biological activity of tested compounds as well as NMR spectra of the new synthesized indirubins (10–13) are available free of charge via the Internet at <http://pubs.acs.org>.

## References and Notes

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