3′-Substituted 7-Halogenoindirubins, a New Class of Cell Death Inducing Agents

Yoan Ferandin,[†,‡] Karima Bettayeb,[†,‡] Marina Kritsanida,[§,‡] Olivier Lozach,[†] Panagiotis Polychronopoulos,[§] Prokopios Magiatis,[§] Alexios-Leandros Skaltsounis,*[,§] and Laurent Meijer*[,†]

Cell Cycle Group & UPS2682, C.N.R.S., Station Biologique, B.P. 74, 29682 Roscoff Cedex, Bretagne, France, and Department of Pharmacy, Division of Pharmacognosy and Natural Products Chemistry, University of Athens, Panepistimiopolis Zografou, GR-15771 Athens, Greece

Received March 19, 2006

Indirubins are kinase inhibitory bis-indoles that can be generated from various plant, mollusk, mammalian, and bacterial sources or chemically synthesized. We here report on the synthesis and biological evaluation of 3′-substituted 7-halogenoindirubins. Molecular modeling and kinase assays suggest that steric hindrance prevents 3′-substituted 7-halogenoindirubins from interacting with classical kinase targets of other indirubins such as cyclin-dependent kinases and glycogen synthase kinase-3. Surprisingly 3′-substituted 7-halogenoindirubins induce cell death in a diversity of human tumor cell lines. Although some 3′-substituted 7-halogenoindirubins appear to induce effector caspase-independent, nonapoptotic cell death, others trigger the landmarks of classical apoptosis. A structure−activity relationship study was performed to optimize 3′-substituted 7-halogenoindirubins with respect to solubility and cell death induction. Despite their unidentified targets, 3′-substituted 7-halogenoindirubins constitute a new promising family of antitumor agents.

Introduction

Indirubin **(1a)** is a dark-red isomer of the blue indigo. Both are bis-indoles derived from the spontaneous, nonenzymatic dimerization of isatin and indoxyl, two colorless precursors. Indirubin can be extracted from four different natural sources: indigo-producing plants, Tyrean purple-producing mollusks, various recombinant bacterial strains, and urine from various mammals including man.[1] Indirubin has been reported as the active ingredient of a traditional Chinese medicinal recipe, danggui longhui wan, used to treat chronic myelocytic leukemia.[2,3] Interest in indirubin and derived analogues (collectively referred to as indirubins) strongly increased when they were discovered to inhibit cyclin-dependent kinases (CDKs[a]),[4] glycogen synthase kinase-3 (GSK-3),[5] and glycogen phosphorylase *b*[6] and to bind and activate the aryl hydrocarbon receptor (AhR), known also as the dioxin receptor.[7−13] Indirubins have been cocrystallized with CDK2,[4] CDK2/cyclin A,[14] CDK5/p25,[15] PfPK5, the *Plasmodium falciparum* CDK1 homologue,[16] GSK-3β,[17−19] and glycogen phosphorylase *b*.[4]

Indirubins display clear antiproliferative and cell death inducing effects.[20−24] Although there is evidence suggesting that these effects originate from inhibition of CDKs, interaction with AhR and subsequent induction of $p27^{kip1}$ may also contribute to the cellular effects of indirubins.[12] Furthermore, some indirubins have recently been shown to prevent the activity of the transcription factor STAT3, probably by inhibition of its src-dependent tyrosine phosphorylation.[25] This leads to down-regulation of survival factors such as survivin and Mcl-1, followed by cell death induction.[25] While synthesizing and testing the biological activity of new indirubins, we fortuitously discovered that 7-bromoindirubin-3′-oxime (7BIO) displayed potent cell death inducing properties, despite a lack of activity toward CDKs and GSK-3.[26] In contrast to indirubin-3′-oxime (IO), 5-bromoindirubin-3′-oxime (5BIO), and 6-bromoindirubin-3′-oxime (6BIO) (Figure 1), which at least in part induce classical apoptosis, 7BIO induced caspase-independent, nonapoptotic cell death.[26]

In this article we report on the synthesis and biological properties of 3′-substituted 7-halogenoindirubins. Fifty-eight 3′-substituted 7-halogenoindirubins were synthesized and tested on various kinases as well as for their effects on neuroblastoma SH-SY5Y cell survival. We also characterized the effects of the most active compounds on a panel of 11 human tumor cell lines and investigated the intracellular mechanisms of cell death induction. Altogether, the results suggest that 3′-substituted 7-halogenoindirubins constitute a new family of potential antitumor agents, acting through both caspase-dependent and caspase-independent mechanisms. They also suggest that some indirubins may interact with new, yet unidentified, target proteins, distinct from their well characterized CDKs, GSK-3, and AhR targets.

Results

Despite considerable work on the bis-indole indirubins, substitutions on position 7 have never been investigated. In a previous report we have shown that, in contrast to other bromo-substituted indirubins, 7BIO had little effect on CDK1/cyclin B, CDK5/p25, and GSK-3α/β.[26] One reason might be that 3′-substituted 7-halogenoindirubins would be unable to bind to CDKs and GSK-3 because of steric hindrance. To investigate this hypothesis, we modeled 7BIO into GSK-3β, based on the cocrystal structures of 6BIO with this kinase, as described by Polychronopoulos et al.[19] Results show a potential steric clash between 7BIO's bromine and the side chain of Leu 132, the gatekeeper amino acid (Figure 2). This would most likely prevent interaction of 3′-substituted 7-halogenoindirubins with

* To whom correspondence should be addressed. For A.-L.S.: phone, +30 210 727 45 94; fax, 33 2 98 29 23 42; e-mail, skaltsounis@pharm.uoa.gr. For L.M.: phone, +33 (0)2 98 29 23 39; fax, 33 2 98 29 23 42; e-mail, meijer@sb-roscoff.fr.

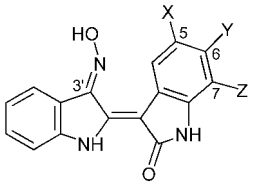
† Station Biologique.

‡ These authors contributed equally.

§ University of Athens.

[a] Abbreviations: AhR, aryl hydrocarbon receptor; 5BIO, 5-bromoindirubin-3′-oxime; 6BIO, 6-bromoindirubin-3′-oxime; 7BIO, 7-bromoindirubin-3′-oxime; CDK, cyclin-dependent kinase; FCS, fetal calf serum; GSK-3, glycogen synthase kinase-3; IO, indirubin-3′-oxime; LDH, lactate dehydrogenase; Me7BIO, 1-methyl-7-bromoindirubin-3′-oxime; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PBS, phosphate buffered saline.

10.1021/jm060314i CCC: $33.50
Published on Web 06/24/2006



	X	Y	Z
IO	H	H	H
5BIO	Br	H	H
6BIO	H	Br	H
7BIO	H	H	Br

Figure 1. Structure of indirubin-3′-oxime (IO), 5-bromoindirubin-3′-oxime (5BIO), 6-bromoindirubin-3′-oxime (6BIO), and 7-bromoindirubin-3′-oxime (7BIO).

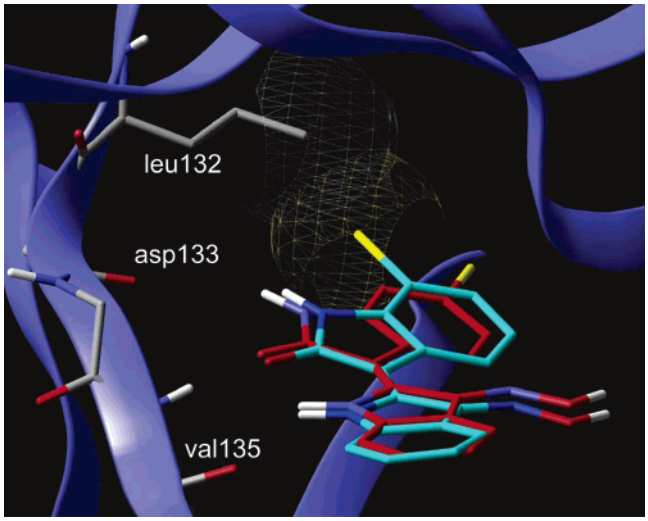


Figure 2. Superimposition of 7BIO (turquoise) and 6BIO (red) docked into the active site of GSK-3β. Only the backbone atoms of the residues from Asp133 to Val135 interacting with the ligands are visible, while the rest of the protein is presented as a ribbon. In the case of 7BIO a steric clash between the bromine atom at position 7 and the Leu132 residue occurs (visualized by overlap of the VdW spheres) resulting in a lower affinity of 7BIO for GSK-3β.

the ATP-binding site of these kinases. However, despite this lack of interaction with classical indirubin targets, 7BIO displays striking antiproliferative effects.[26]

Synthesis of 3′-Substituted 7-Halogenoindirubins. We thus started to investigate this subfamily of 7-substituted indirubins by designing a method to synthesize 7-halogenoindirubins with various substitutions on position 3′ (Schemes 1 and 2; Tables 1 and 2). The synthesis of 7-halogenoindirubins was mainly based on the dimerization reaction of an appropriately substituted isatin derivative with 3-acetoxyindole, as depicted in Scheme 1. The desired isatins were synthesized through a two-step procedure, using the corresponding commercial 2-halogenoanilines **2b**−**e** as starting material. In the first step, the appropriate aniline derivatives were reacted with chloral hydrate and hydroxylamine hydrochloride to give the corresponding isonitrosoacetanilides **3b**−**e**. In the second step, the intermediate isonitrosoacenilides were heated in concentrated sulfuric acid to give the 7-halogenoisatins (**4b**−**e**). 7-Halogeno-*N*-methylisatins (**5b**−**e**) were prepared from **4b**−**e**, respectively, by treatment with dimethyl sulfate and Na_2CO_3.

The substituted isatins, 7-halogenoisatins (**4b**−**e**) or 7-halogeno-*N*-methylisatins (**5b**−**e**), were reacted with 3-acetoxyindole in alkaline medium to give, generally in good yields, the corresponding bis-indoles **1b**−**e** and **6b**−**e** selectively in the *Z* form.

The oximes **7b**−**e** and **8b**−**e** were prepared selectively in a (2′*Z*,3′*E*) form following a typical procedure from the appropriate indirubin derivatives **1b**−**e** and **6b**−**e** with hydroxylamine hydrochloride in pyridine under reflux. A similar typical procedure was followed for the preparation of the methoximes **9b**−**e** and **10b**−**e** using methoxylamine hydrochloride. The acetoximes **11b**−**e** and **12b**−**e** were prepared from the oximes with acetic anhydride in pyridine. The temperature of the reaction was carefully kept at 0 °C to avoid bisacetylation.

In an effort to prepare derivatives with increased water solubility, we synthesized 3′-substituted oximes of 7BIO (**7d**) and Me7BIO (**8d**) bearing side chains with amine groups **13g**−**l** and **14g**−**l** (Scheme 2). The synthesis was based on the reaction of the 3′-[*O*-(2-bromoethyl)oxime] intermediate **13f** or **14f** with the appropriate amine: pyrrolidine, morpholine, piperazine, imidazole, dimethylamine, and diethylamine. The intermediates **13f** and **14f** were prepared by the reaction of 7BIO or Me7BIO with 1,2-dibromoethane in DMF and Et_3N at room temperature. In addition, two carbamates **15** and **16** were prepared by the reaction of 7BIO or Me7BIO with *N*,*N*-diethylcarbamyl chloride.

Indirubin (**1a**), nonhalogenated indirubin derivatives (**6a**−**9a** and **11a**), **5BIO**, and **6BIO** were synthesized as previously reported.[19]

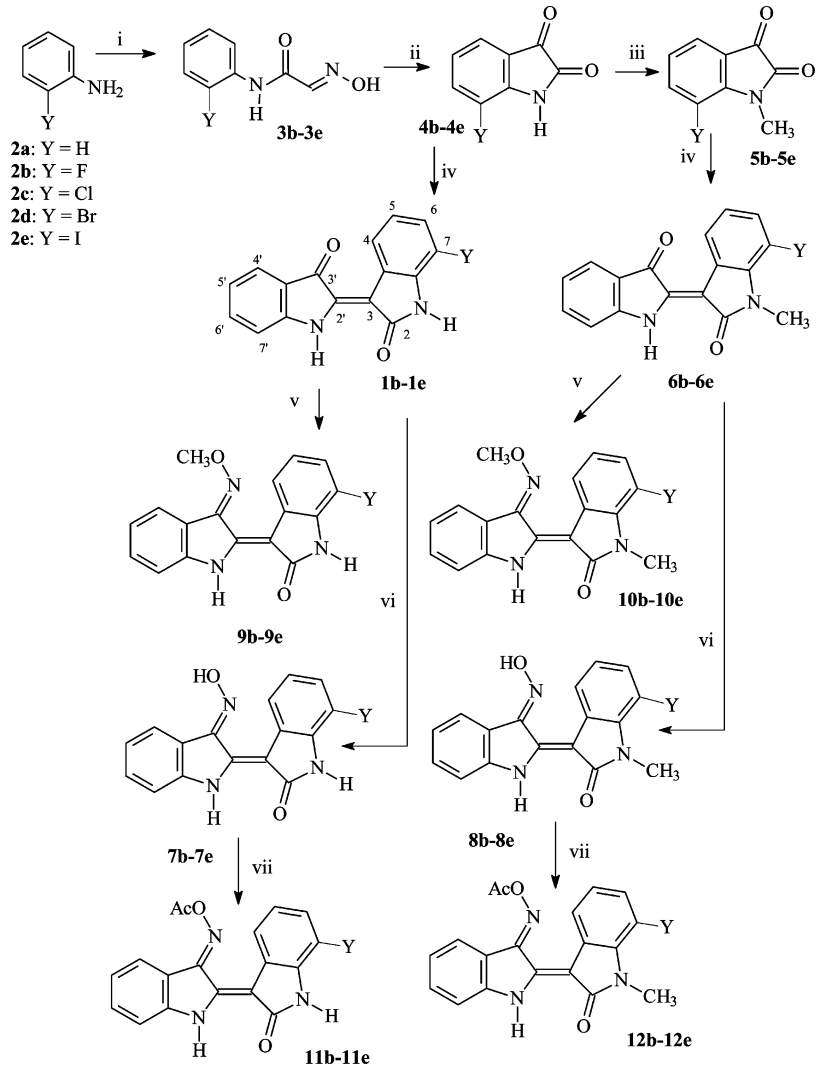
3′-Substituted 7-Halogenoindirubins Display Little Kinase Inhibitory Activity toward CDKs and GSK-3α/β. We first synthesized a variety of 7-halogenoindirubins with or without a methyl substitution at position N1 and with an oxime, methoxime, or acetoxime substitution at position 3′ (compounds **1b**−**e**, **6b**−**e**, **7b**−**e**, **8b**−**e**, **9b**−**e**, **10b**−**e**, **11b**−**e**, **12b**−**e**). In addition a few indirubins that are not substituted on position 7 were synthesized for comparison purposes (compounds **1a**, **6a**−**9a**, **11a**). Molecules were then tested on three kinases, CDK1/cyclin B, CDK5/p25, and GSK-3α/β (Table 1). A complete lack of activity was confirmed for all N1-methylated indirubins. A weak and gradually decreasing inhibitory activity was observed with 7-halogenoindirubin-3′-oxime when the size of the atom at position 7 increased (H > F > Cl > Br > I) (compare compounds **7a**−**e**), suggesting increased hindrance at this position 7.

In a second series of indirubins, the 3′ substituent was varied on a 7-bromoindirubin scaffold (± a methyl at N1) (compounds **13f**−**l**, **14f**−**l**, **15**, **16**) (Table 2). With the exception of compound **13h** on GSK-3, none of these compounds displayed any significant activity on any of the three kinases tested (Table 2).

Several 3′-Substituted 7-Halogenoindirubins Induce Cell Death in Culture. We next tested the effects of each indirubin, at a 25 μM final concentration, on the survival of the neuroblastoma SH-SY5Y cell line after 48 h of exposure (Tables 1 and 2). Cell survival was estimated by the MTS reduction assay. Several compounds showed clear effects on the SH-SY5Y cell survival rate. A complete dose response curve was performed for these active compounds after 24 and 48 h of exposure, and the IC_{50} values were calculated (Table 3). Because MTS reduction is occasionally observed under conditions different from the conditions of cell death, we used an independent cell death evaluation procedure, the lactate dehydrogenase (LDH) release assay. This assay confirmed the induction of cell death by our selection of indirubins, despite their overall lack of effects on CDKs and GSK-3.

We next tested the various 3′-substituted 7-bromoindirubins on 11 cell lines, namely, HT-29 and HCT116 (colon cancer), MDA-MB-231 (breast cancer), A549 (lung cancer), PC3 (prostate cancer), 5L and BP8 (hepatoma), F1 and Huh7

Scheme 1. General Synthesis of 7-Substituted Indirubins and Their Corresponding 3′-Oxime, Methoxime, and Acetoxime Derivatives[a]



[a] Reagents (i) chloral hydrate, Na_2SO_4, $H_2NOH{\cdot}HCl$, H_2O, HCl; (ii) H_2SO_4, 80 °C; (iii) $(CH_3)_2SO_4$, Na_2CO_3, DMF; (iv) 3-acetoxyindole, Na_2CO_3/MeOH, 25 °C; (v) $H_2NOCH_3{\cdot}HCl$, Py, 120 °C; (vi) $H_2NOH{\cdot}HCl$, Py, 120 °C; (vii) Ac_2O, Py, 0 °C.

(hepatoma), SH-SY5Y (neuroblastoma), and HEK293 (embryonic kidney). Like SH-SY5Y, these cell lines showed dose-dependent induction of cell death (Table 4), suggesting the generality of effect of these compounds on cell survival rather than a cell type or differentiation stage-specific effect. The similar sensitivity of 5L (AhR +/+) and BP8 (AhR −/−) suggests that AhR does not play a major role in 3′-substituted 7-bromoindirubins induced cell death.

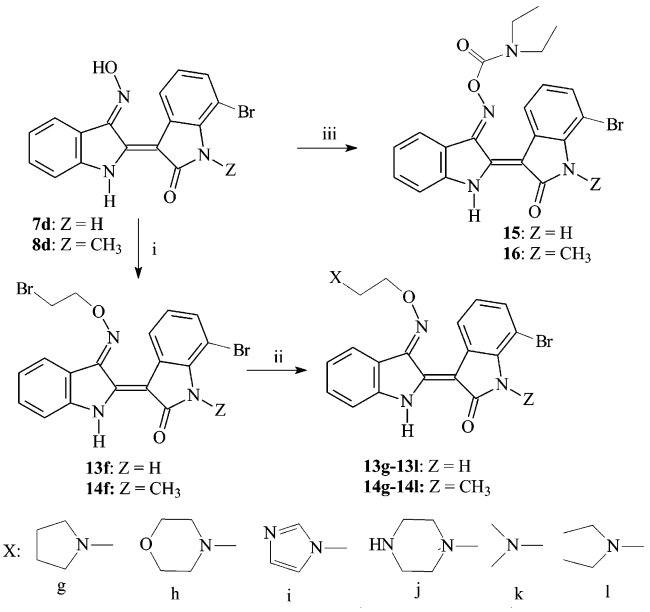
3′-Substituted 7-Halogenoindirubins Induce Apoptotic and Nonapoptotic Cell Death. In a previous work we showed that cell death induced by 7BIO is primarily different from apoptosis because it does not induce or require caspase activation.[26] We thus investigated whether this was also the case with the new indirubins presented here. To this aim, we tested the effects of the effector caspase (3, 8, 9, 10, 12) inhibitor Q-VD-OPh[27] (20 μM final concentration) on cell death induced by the selection of indirubins. Results show that 3′-substituted 7-bromoindirubins fall into three categories (Table 5, Figure 3). In the first category, some indirubins are completely insensitive to the presence of the caspase inhibitor, suggesting a caspase-independent mechanism. 7BIO (**7d**) falls into this category (Figure 3, upper left panel). In the second category, Q-VD-OPh shifts the dose response curves to the right and thus shifts the IC_{50} values toward higher values, suggesting a mixed, caspase-dependent and caspase-independent mechanism of action (compound **14gs**; Figure 3, upper central panel). In the third category, the presence of Q-VD-OPh essentially protects cells from cell death, suggesting that these indirubins act mostly through a classical, caspase-dependent mechanism (compound **13k**; Figure 3, upper right panel). Interestingly this is observed with the most active indirubins. In this last category, a small fraction of cells (20%) die despite the presence of Q-VD-OPh.

The activity of caspases was next assayed over a time-course in SH-SY5Y cells exposed to the three types of 3′-substituted 7-bromoindirubins (Figure 3, lower panel). As previously reported[26] 7BIO did not trigger any caspase activation. In contrast, compound **13k** induced strong activation of caspases, while compound **14gs** induced an intermediate activation. The level of caspase activation induced by the indirubins thus correlated very well with the sensitivity of their cellular effects to Q-VD-OPh.

Discussion

We here report on the synthesis and biological evaluation of a new subfamily of indirubins substituted on positions 3′ and 7. Despite a lack of inhibitory activity toward classical targets of indirubins such as CDKs and GSK-3, 3′-substituted 7-halogenoindirubins display potent cell death inducing activity. This

Scheme 2. General Synthesis Scheme of 3'-Substituted 7-Bromoindirubins[a]

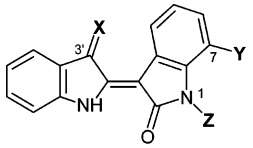


[a] Reagents: (i) dibromoethane, triethylamine, anhydrous DMF, 25 °C; (ii) anhydrous DMF, 25 °C, amines **g**−**l**; (iii) *N*,*N*-diethylcarbamyl chloride, triethylamine, anhydrous DMF, 25 °C.

observation poses a series of questions on the nature of the molecular targets of these indirubins, their cellular mechanism of action, and the possibility of further optimization and their potential development as antitumor agents.

Three pieces of evidence suggest that 3′-substituted 7-halogenoindirubins target very specific proteins. First, substitutions on position 7 inactivate indirubins as CDK and GSK-3 inhibitors and, in the case of 7BIO, as an inhibitor of quite a few kinases.[26] A large substituent like bromine at position 7 indeed creates a molecular hindrance preventing the interaction with CDKs and GSK3 (Figure 2). Second, a substitution on position 1 with a methyl inactivates indirubins as CDK and GSK-3 inhibitors.[18] This is likely the consequence of the prevention of a hydrogen bond between the cyclic nitrogen of the lactam ring of indirubins and the carbonyl oxygen of Asp133 (in GSK-3), Glu81 (in CDK5),[15,18,19] or Glu81 (in CDK2).[4] This hydrogen bond constitutes a key feature in the interaction between indirubins and their kinase targets. Nevertheless some *N*1-methyl-, 3′-substituted 7-halogenoindirubins (**14g, 14gs**, **14ks**, **14ls**) display very clear cell death inducing activity, suggesting that their interaction with an unidentified target does not involve this key hydrogen bond (Table 2). Third, varying the substituent on position 3′ leads to a different range of biological activity. However at this point we are unable to correlate the cell death inducing activity with a specific substitution on 3′. The hydroxyl of indirubin-3′-oxime may provide an indirect link (through a water molecule) to CDKs and GSK-3.[15,18,19] In CDK/GSK-3 indirubins cocrystal structures, the 3′ position appears to face the outside of the ATP-binding pocket and therefore is less prone to steric hindrance.[4,15,18,19] Altogether, this information suggests that the binding mode of 3′-substituted 7-halogenoindirubins to its (their) unknown target(s) is rather different from that of previously described indirubins to CDK2, CDK5, or GSK-3. Another possibility is that 3′-substituted 7-halogenoindirubins specifically bind to non-protein kinase targets and that the biological effects of this indirubin subclass are due to this interaction. An example is AhR. [7−13] However, SH-SY5Y cells appear to be devoid of AhR (data not shown) and quite sensitive

Table 1. Effects of Indirubin Derivatives **1**, **6**, and **7**−**12** on Three Protein Kinases and on the Survival of Neuroblastoma SH-SY5Y Cells[a]



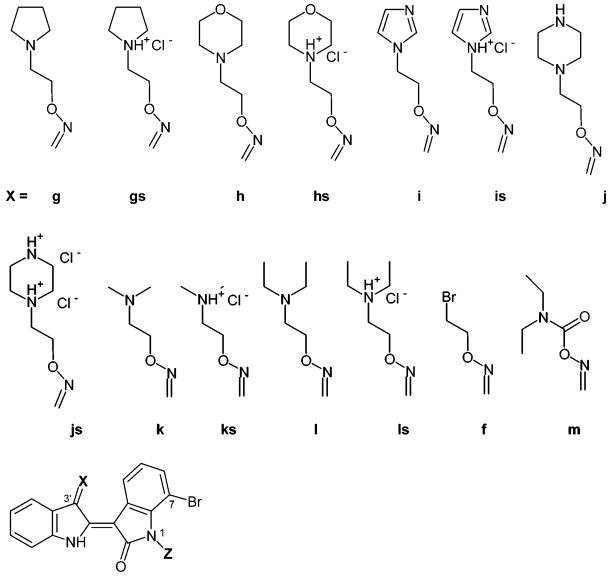
				IC$_{50}$ (μM)			SH-SY5Y
indirubin	X (3′)	Y (7)	Z (1)	CDK1	CDK5	GSK-3	% survival
1a	O	H	H	10	10	1.0	104 ± 4
7a	NOH	H	H	0.18	0.10	0.022	*27 ± 2*
9a	$NOCH_3$	H	H	1.4	0.4	0.3	86 ± 4
11a	$NOCOCH_3$	H	H	1.2	0.7	0.2	*36 ± 2*
6a	O	H	CH_3	100	>100	>100	98 ± 3
8a	NOH	H	CH_3	73	>100	>100	100 ± 5
1b	O	F	H	10	≥10	0.40	70 ± 5
7b	NOH	F	H	1.5	0.51	0.27	*46 ± 2*
9b	$NOCH_3$	F	H	>10	>100	0.44	105 ± 4
11b	$NOCOCH_3$	F	H	15	>100	0.33	*39 ± 4*
6b	O	F	CH_3	>10	>100	>100	101 ± 7
8b	NOH	F	CH_3	>100	>100	>100	99 ± 1
10b	$NOCH_3$	F	CH_3	>10	>100	>100	107 ± 3
12b	$NOCOCH_3$	F	CH_3	>10	>100	>100	105 ± 4
1c	O	Cl	H	>10	>100	>100	92 ± 1
7c	NOH	Cl	H	3.7	6	21	**7 ± 0**
9c	$NOCH_3$	Cl	H	>10	>100	>100	97 ± 2
11c	$NOCOCH_3$	Cl	H	>10	>100	>100	89 ± 3
6c	O	Cl	CH_3	>10	>100	>100	94 ± 5
8c	NOH	Cl	CH_3	>100	>100	>100	94 ± 2
10c	$NOCH_3$	Cl	CH_3	>10	>100	>100	99 ± 2
12c	$NOCOCH_3$	Cl	CH_3	>10	>100	>100	96 ± 1
1d	O	Br	H	>100	>100	>100	92 ± 1
7d	NOH	Br	H	22	33	32	**4 ± 0**
9d	$NOCH_3$	Br	H	>100	>100	>100	97 ± 3
11d	$NOCOCH_3$	Br	H	>100	>100	>100	61 ± 8
6d	O	Br	CH_3	100	>100	>100	98 ± 3
8d	NOH	Br	CH_3	>100	>100	>100	84 ± 2
10d	$NOCH_3$	Br	CH_3	>100	>100	>100	100 ± 2
12d	$NOCOCH_3$	Br	CH_3	70	>100	>100	95 ± 1
1e	O	I	H	>10	>100	>100	96 ± 2
7e	NOH	I	H	66	77	16	64 ± 3
9e	$NOCH_3$	I	H	>10	>100	>100	84 ± 3
11e	$NOCOCH_3$	I	H	>10	>100	>100	67 ± 2
6e	O	I	CH_3	>10	>100	>100	93 ± 2
8e	NOH	I	CH_3	>100	>100	30	74 ± 0
10e	$NOCH_3$	I	CH_3	>10	>100	>100	99 ± 2
12e	$NOCOCH_3$	I	CH_3	>10	>100	>100	82 ± 2

[a] A series of indirubin analogues were tested at various concentrations in three kinase assays, as described in the Experimental section. IC$_{50}$ values were calculated from the dose response curves. The compounds were also tested at 25 μM for their effects on SH-SY5Y cells. Cell survival was estimated by the MTS reduction assay and is expressed as percent of survival in untreated cells (average ± SE of three independent measurements; representative of two independent experiments): boldface numbers, ≤15% survival; italic numbers, ≤50% survival.

to 3′-substituted 7-halogenoindirubins. Furthermore, we were unable to see any significant difference in the biological responses of the hepatoma 5L (AhR +/+) and BP8 (AhR −/−) cell lines to these indirubins (Table 4).[26] Therefore, interaction with AhR is unlikely to be the major cause of cell death induced by 3′-substituted 7-halogenoindirubins, although we cannot exclude a secondary role. Identification of the molecular target-(s) of 3′-substituted 7-halogenoindirubins is one of our main priorities. First, we will explore the possible substitutions that can be added to these structures on additional positions (especially on 5′ and 5). Second, from this information, we will attempt to design an affinity reagent that should allow us to purify and identify some of the interacting proteins as successfully performed with other kinase inhibitors.[28,29]

3′-Substituted 7-halogenoindirubins induce cell death via at least three different mechanisms: some induce cell death in a caspase-independent way, some in a clearly caspase-dependent

Table 2. Effects of of Indirubin Derivatives **13**−**16** on Three Protein Kinases and on the Survival of Neuroblastoma SH-SY5Y Cells[a]



indirubin	X (3′)	Z (1)	IC$_{50}$ (μM) CDK1	CDK5	GSK3	SH- SY5Y % survival
7d	NOH	H	22	33	32	**4 ± 0**
13f	f	H	>100	25	100	87 ± 5
14f	f	CH_3	>10	>10	>10	99 ± 4
13g	g	H	>100	>10	7	**13 ± 0**
13gs	gs	H	>100	>10	3	**12 ± 0**
14g	g	CH_3	>10	>10	>10	**15 ± 1**
14gs	gs	CH_3	>10	>10	>10	**1 ± 0**
13h	h	H	>100	>10	0.57	98 ± 1
13hs	hs	H	>100	>10	>100	76 ± 1
14h	h	CH_3	>10	>10	>10	98 ± 2
14hs	hs	CH_3	>10	>10	>10	90 ± 2
13i	i	H	>10	>10	9	59 ± 1
13is	is	H	>10	>10	11	62 ± 1
14i	i	CH_3	>10	>10	>10	85 ± 1
14is	is	CH_3	>10	>10	>10	70 ± 1
13j	j	H	>10	>10	5	**3 ± 1**
13js	js	H	>10	>10	>10	**3 ± 1**
13k	k	H	>10	>10	8	**1 ± 0**
13ks	ks	H	>10	>10	>10	**5 ± 1**
14k	k	CH_3	>10	>10	>10	*49 ± 1*
14ks	ks	CH_3	>10	>10	>10	**4 ± 0**
13l	l	H	>10	>10	>10	*49 ± 3*
13ls	ls	H	>10	>10	>10	72 ± 3
14l	l	CH_3	>10	>10	>10	83 ± 2
14ls	ls	CH_3	>10	>10	>10	**15 ± 1**
15	m	H	>100	>100	>100	106 ± 5
16	m	CH_3	>10	>10	>10	105 ± 3
5BIO	NOH	H	0.045	0.028	0.016	**13 ± 0.4**
6BIO	NOH	H	0.320	0.083	0.005	**5 ± 0.6**

[a] A series of indirubin analogues were tested at various concentrations in three kinase assays, as described in the Experimental Section. IC$_{50}$ values were calculated from the dose response curves. Experiments were also performed with 5BIO and 6BIO for comparison. Kinase data for 6BIO is from Meijer.[18] The compounds were also tested at 25 μM for their effects on SH-SY5Y cells. Cell survival was estimated by the MTS reduction assay and is expressed as percent of survival in untreated cells (average ± SE of three independent measurements; representative of two independent experiments): boldface numbers, ≤15% survival; italic numbers, ≤50% survival.

manner, and some in a partially caspase-dependent manner. There are many possible reasons for this diversity of response: different targets, different intracellular distribution, different kinetics of two independent mechanisms, metabolism into different types of cell death inducers. At this point it will be most interesting to investigate whether cell death induced by the different 3′-substituted 7-halogenoindirubins requires gene expression and protein synthesis. If this is the case, analysis of the pattern of gene expression should provide clues on the mechanisms involved in cell death induction and how they impinge on classical apoptosis or on nonapoptotic cell death pathways. This work is currently underway.

Table 3. Effects of Indirubin Derivatives **7**, **13**, and **14** on the Survival and Death of Neuroblastoma SH-SY5Y Cells[a]

indirubin	cell survival IC$_{50}$ (μM), MTS reduction 24 h	48 h	% cell death, LDH release, 48 h
7a	>25	12	33
7c	14	12	**92**
7d (7BIO)	*8.0*	*7.1*	**94**
13g	*3.6*	*2.3*	14
13gs	*3.1*	*2.0*	*80*
14g	*7.8*	*5.0*	*78*
14gs	*7.3*	*4.0*	**94**
13j	10.5	*6.2*	**94**
13js	*6.0*	*6.0*	**100**
13k	*4.1*	*2.0*	**86**
13ks	*2.1*	*1.0*	*84*
14k	>25	*5.5*	33
14ks	21	*9.0*	*80*
14ls	23	18	*58*
5BIO	18	12	*59*
6BIO	18	*9.5*	*80*

[a] A series of indirubin analogues were tested at various concentrations for their effects on SH-SY5Y cells. Cell survival was estimated 24 or 48 h after the addition of each indirubin using the MTS reduction assay. Experiments were also performed with 5BIO and 6BIO for comparison. IC$_{50}$ values were calculated from the dose response curves (average ± SE of two independent measurements performed in triplicate): italic numbers, IC$_{50}$ < 10 μM. In addition, cell death was estimated 48 h after the addition of each indirubin (25 μM) using the LDH release assay. Results are expressed as percent cell death: boldface numbers, >85% cell death; italic numbers, >50% cell death.

We shall continue to explore the structure−activity relationship of these 3′-substituted 7-halogenoindirubins in order to improve their efficacy. This work may lead to the development of potential antitumor agents acting through a caspase-dependent or a caspase-independent pathway. Besides these cell efficacy improvements, we have identified compounds with much better water solubility (such as the **13gs**−**ls**, **14gs**−**ls** in Table 2). This is an important progress because insolubility in biologically compatible media has been a major issue in the development of indirubins.[22]

Experimental Section

Chemistry. General Chemistry 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 a Bruker DRX 400. Chemical shifts are expressed in ppm downfield from TMS. CI-MS spectra were determined on a Finnigan GCQ Plus ion-trap mass spectrometer using CH_4 as the chemical ionization reagent. Column chomatographies were conducted using flash silica gel 60 (40−63 μm) from Merck, with an overpressure of 300 mbars. All the compounds gave satisfactory combustion analysis results (C, H, N within ±0.4% of calculated values).

General Procedure for the Preparation of Isatins 4b−**e and 5b**−**e.** Chloral hydrate (5.0 g) and Na_2SO_4 (35.0 g) were dissolved in water (70 mL) in a 300 mL beaker and warmed to 35 °C. A warm solution of the appropriate commercial aniline derivative **2b**−**e** (27.6 mmol) in water (20 mL) and an aqueous solution of concentrated HCl (3 mL) was added (a white precipitate of the amine sulfate was formed), followed by a warm solution of hydroxylamine hydrochloride (6.1 g) in water (27.5 mL). The mixture was stirred by hand and heated on a hot plate (a thick paste

Table 4. Effects of Indirubin Derivatives **7**, **13**, and **14** on the Survival of Various Cell Lines[a]

	cell survival IC_{50} (μM), MTS reduction assay								
cell line	**7d**	**13gs**	**14g**	**14gs**	**13j**	**13js**	**13k**	**13ks**	**14ks**
HT-29 (colon)	21.2	10.4	19.8	*5.4*	*6.3*	*1.9*	21.8	*2.4*	20.0
HCT116 (colon)	*6.2*	*3.8*	13.6	*5.1*	*5.4*	*5.1*	*6.5*	*2.0*	15.7
MDA-MB-231 (breast)	10.5	>30.0	26.8	12.0	*5.3*	*5.1*	26.4	18.8	17.8
A549 (lung)	21.8	16.0	21.0	14.6	*5.9*	*6.0*	21.8	*5.8*	20.8
PC3 (prostate)	19.8	30.0	21.6	19.0	*6.1*	*6.0*	27.2	15.0	25.0
5L (hepatoma)	20.0	>30.0	18.8	13.0	*5.2*	*5.4*	25.0	>30.0	24.0
BP8 (hepatoma)	19.4	>30.0	18.8	*7.5*	*5.6*	*5.4*	19.5	>30.0	19.0
F1 (hepatoma)	*8.0*	*3.1*	*5.6*	*2.9*	*1.7*	*1.4*	*5.5*	*1.7*	*5.4*
Huh7 (hepatoma)	*9.0*	13.6	18.8	*5.6*	*5.3*	*2.0*	19.6	11.2	18.0
SH-SY5Y (neuroblastoma)	*7.4*	*2.1*	10.6	*5.0*	*5.4*	*5.1*	*5.4*	**0.8**	*8.0*
HEK293 (embryo kidney)	12.3	>30.0	21.4	*8.0*	*5.0*	*1.9*	14.4	*3.0*	18.8

[a] A series of indirubin analogues were tested at various concentrations for their effects on 11 different cell lines. Cell survival was estimated 48 h after the addition of each indirubin using the MTS reduction assay. IC_{50} values were calculated from the dose response curves (average ± SE of measurements performed in triplicate): italic numbers, IC_{50} < 10 μM; boldface numbers, IC_{50} < 1 μM.

Table 5. Cell Death Induced by Indirubin Derivatives **7**, **13**, and **14** Is Either Caspase-Independent or Caspase-Dependent[a]

	IC_{50} (μM)	
indirubin	−Q-VD-OPh (MTS)	+Q-VD-OPh (MTS)
7a	*13.0*	*>25.0*
7c	10.0	11.0
7d (7BIO)	7.0	7.0
13g	**2.0**	**− (>25.0)**
13gs	**2.8**	**− (>25.0)**
14g	*6.1*	*>25.0*
14gs	*5.0*	*13.0*
13j	10.0	10.3
13js	6.2	6.8
13k	**1.5**	**− (>25.0)**
13ks	**1.1**	**− (>25.0)**
14k	**6.4**	**− (>25.0)**
14ks	*11.0*	*>25.0*
14ls	*18.0*	*>25.0*
5BIO	*13.0*	*23.0*
6BIO	*10.0*	*13.0*

[a] SH-SY5Y cells were treated with various concentrations of indirubin analogues in the presence or absence of 20 μM Q-VD-OPh, a broad spectrum inhibitor of caspases. Cell survival was estimated 48 h after the addition of each indirubin using the MTS reduction assay. IC_{50} values were calculated from the dose response curves (average ± SE of two independent measurements): −, no cell death at highest concentration tested. Results are not emphasized when Q-VD-Oph has no effect on the dose response curve. Italic numbers: when Q-VD-Oph partially protects from cell death. Boldface numbers: when Q-VD-Oph provides complete protection.

formed at 75−70 °C) at 80−90 °C for 2 h, then allowed to cool for 1 h, 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 **3b**−**e**.

Sulfuric acid (100 mL) was heated in a 3 L beaker on a hot plate to 60 °C and then removed. The dry isonitrosoacetanilide **3b**−**e** was added in portions with stirring over 30 min so that the temperature did not exceed 65 °C. The mixture was then heated to 80 °C for 15 min, allowed to cool to 70 °C, and cooled on ice. The solution was poured onto crushed ice (500 mL) and left to stand for 1 h before the orange-red precipitate was filtered. The product was washed by stirring with water (100 mL) and filtered to give the corresponding isatins. Yields: **4b**, 57%; **4c**, 50%; **4d**, 65%; **4e**, 50%.

7-Fluoro-*N*-methylisatin (5b). To a solution of **4b** (380 mg, 2.30 mmol) in dry acetone (60 mL) was added Na_2CO_3 (anhydrous) (3.5 g) and dimethyl sulfate (0.4 mL) under Ar, and the reaction mixture was heated at 60 °C for 20 h. Then the mixture was filtered, and the filtrate was carefully evaporated using a high vacuum pump (under 40 °C). The solid residue was submitted to flash chromatography with CH_2Cl_2 to afford **5b** (288 mg, 1.61 mmol, 70%).

7-Chloro-*N*-methylisatin (5c). This compound was prepared from 7-chloroisatin (**4c**) by a procedure analogous to that of **5b**: yield 76%.

7-Bromo-*N*-methylisatin (5d). This compound was prepared from 7-bromoisatin (**4d**) by a procedure analogous to that of **5b**: yield 90%.

7-Iodo-*N*-methylisatin (5e). This compound was prepared from 7-iodoisatin (**4e**) by a procedure analogous to that of **5b**: yield 85%.

(2′*Z*)-7-Fluoroindirubin (1b). Methanol (25 mL) was vigorously stirred under nitrogen for 20 min, and then 7-fluoroisatin (**4b**) (150 mg, 0.91 mmol) and 3-acetoxyindole (106 mg, 0.61 mmol) were added and stirring was continued for 5 min. Anhydrous Na_2CO_3 (155 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) to give **7** (130 mg, 0.46 mmol, 77%) selectively in the *Z* form. Mp >300 °C; ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.37 (1H, s, N′−H), 11.12 (1H, s, N−H), 8.58 (1H, d, J = 7.7 Hz, H-4), 7.64 (1H, d, J = 7.5 Hz, H-4′), 7.57 (1H, t, J = 7.5 Hz H-6′), 7.42 (1H, d, J = 7.5 Hz, H-7′), 7.15 (1H, t, J = 8.0 Hz, H-6), 7.02 (2H, m, H-5′, 5). CI-MS m/z 281 $(M + H)^+$. Anal. ($C_{16}H_9N_2O_2F$) C, H, N.

(2′*Z*)-7-Chloroindirubin (1c). This compound was prepared from 7-chloroisatin (**4c**) by a procedure analogous to that of **7**: yield 80%. Mp >300 °C.^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.29 (1H, s, N′−H), 11.16 (1H, s, N−H), 8.72 (1H, d, J = 7.8 Hz, H-4), 7.66 (1H, d, J = 7.5 Hz, H-4′), 7.59 (1H, t, J = 7.8 Hz H-6′), 7.43 (1H, d, J = 7.8 Hz, H-7′), 7.30 (1H, d, J = 7.8 Hz, H-6), 7.05 (2H, m, H-5,5′). CI-MS m/z 297, 299 $(M + H)^+$. Anal. ($C_{16}H_9N_2O_2$-Cl) C, H, N.

(2′*Z*)-7-Bromoindirubin (1d). This compound was prepared from 7-bromoisatin (**4d**) by a procedure analogous to that of **7**: yield 85%. Mp >300 °C. IR 3350 (br), 1674, 1612, 1593, 1464, 1306, 1216 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.18 (2H, br s, N−H), 8.77 (1H, d, J = 7.9 Hz, H-4), 7.67 (1H, d, J = 7.5 Hz, H-4′), 7.59 (1H, t, J = 7.5 Hz, H-6′), 7.44 (1H, d, J = 7.9 Hz, H-6), 7.43 (1H, d, J = 7.5 Hz, H-7′), 7.04 (1H, t, J = 7.5 Hz, H-5′), 6.98 (1H, t, J = 7.9 Hz, H-5). CI-MS m/z 341, 343 $(M + H)^+$. Anal. ($C_{16}H_9N_2O_2Br$) C, H, N.

(2′*Z*)-7-Iodoindirubin (1e). This compound was prepared from 7-iodoisatin (**4e**) by a procedure analogous to that of **7**: yield 90%. Mp >300 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 8.77 (1H, d, J = 7.5 Hz, H-4), 7.64 (1H, d, J = 7.5 Hz, H-4′), 7.59 (2H, m, H-6,6′), 7.41 (1H, d, J = 7.5 Hz, H-7′), 7.04 (1H, t, J = 7.5 Hz, H-5′), 6.84 (1H, t, J = 7.5 Hz, H-5). CI-MS m/z 389 $(M + H)^+$. Anal. ($C_{16}H_9N_2O_2I$) C, H, N.

(2′*Z*)-7-Fluoro-1-methylindirubin (6b). This compound was prepared from 7-fluoro-*N*-methylisatin (**5b**) and 3-acetoxyindole by a procedure analogous to that of **7**: yield 78%. Mp 280 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.22 (1H, s, N′−H), 8.66 (1H, d, J = 8.0 Hz, H-4), 7.67 (1H, d, J = 7.7 Hz, H-4′), 7.60 (1H, t, J = 7.7 Hz, H-6′), 7.44 (1H, d, J = 7.7 Hz, H-7′), 7.22 (1H, t, J

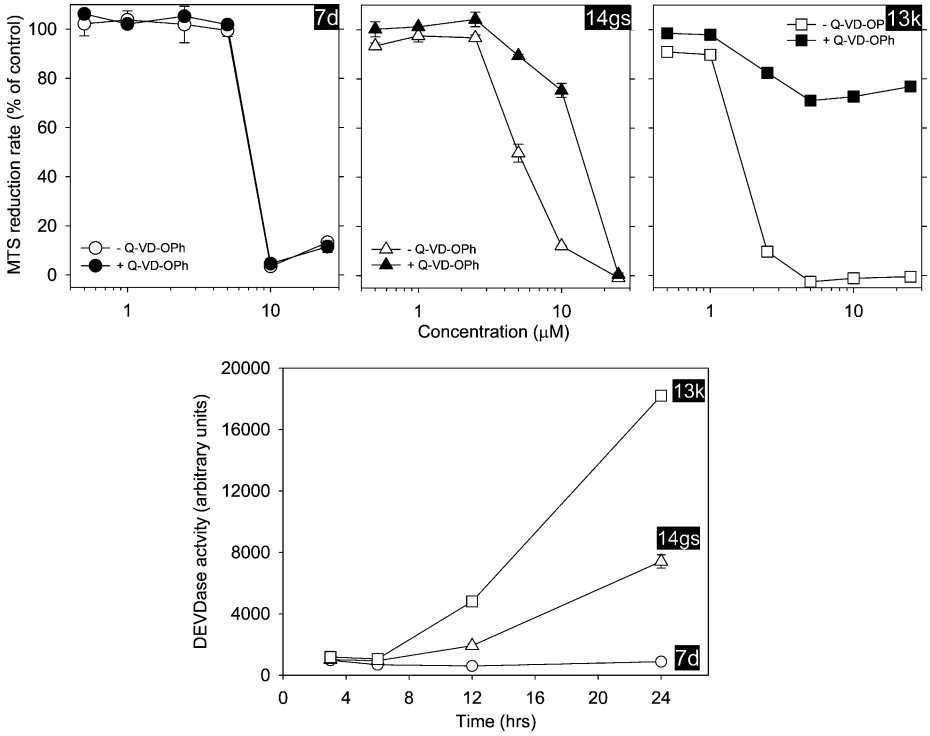


Figure 3. 7-Bromoindirubins induce caspase-independent or caspase-dependent cell death. (Upper panel) SH-SY5Ycells were exposed for 48 h to increasing concentrations of three 7-bromoindirubins (**7d**, **14gs**, **13k**) in the presence (filled symbols) or absence (open symbols) of 20 μM Q-VD-OPh. Cell survival was assessed by the MTS assay and is expressed as a percentage of untreated cells. Every point is the mean ± SE of two independent experiments with two independent measurements per experiment. (Lower panel) The time-course of effector caspase activity was determined in SH-SY5Y cells treated with 25 μM of three 7-bromoindirubins (**7d**, **14gs**, **13k**) for 24 h. DEVDase activity was measured as arbitrary fluorescence units. Every point is the mean ± SE of three independent determinations.

= 8.0 Hz, H-6), 7.07 (2H, m, H-5′, 5), 3.46 (3H, s, N−CH_3). CI-MS *m*/*z* 295 $(M + H)^+$. Anal. ($C_{17}H_{11}FN_2O_2$) C, H, N.

(2′*Z*)-7-Chloro-1-methylindirubin (6c). This compound was prepared from 7-chloro-*N*-methylisatin (**5c**) and 3-acetoxyindole by a procedure analogous to that of **7**: yield 95%. Mp 269 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 10.50 (1H, s, N′−H), 8.85 (1H, d, J = 7.5 Hz, H-4), 7.67 (1H, d, J = 7.4 Hz, H-4′), 7.60 (1H, t, J = 7.4 Hz, H-6′), 7.44 (1H, d, J = 7.4 Hz, H-7′), 7.32 (1H, d, J = 7.4 Hz, H-6), 7.08 (2H, m, H-5, 5′), 3.62 (3H, s, N−CH_3). CI-MS *m*/*z* 311, 313 $(M + H)^+$. Anal. ($C_{17}H_{11}ClN_2O_2$) C, H, N.

(2′*Z*)-7-Bromo-1-methylindirubin (6d). This compound was prepared from 7-bromo-*N*-methylisatin (**5d**) and 3-acetoxyindole by a procedure analogous to that of **7**: yield 83%. Mp 277 °C. IR 3312 (br), 1656, 1619, 1596, 1477, 1449, 1330, 1110 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 8.88 (1H, d, J = 7.9 Hz, H-4), 7.66 (1H, d, J = 7.5 Hz, H-4′), 7.60 (1H, t, J = 7.5 Hz, H-6′), 7.47 (1H, d, J = 7.5 Hz, H-7′), 7.38 (1H, d, J = 7.9 Hz, H-6), 7.04 (2H, m, H-5, 5′), 3.61 (3H, s, N−CH_3). CI-MS *m*/*z* 355, 357 $(M + H)^+$. Anal. ($C_{17}H_{11}BrN_2O_2$) C, H, N.

(2′*Z*)-7-Iodo-1-methylindirubin (6e). This compound was prepared from 7-iodo-*N*-methylisatin (**5e**) and 3-acetoxyindole by a procedure analogous to that of **7**: yield 87%. Mp 299 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.26 (1H, s, N′−H), 8.93 (1H, d, J = 7.7 Hz, H-4), 7.74 (1H, d, J = 7.4 Hz, H-4′), 7.66 (1H, d, J = 7.5 Hz, H-6), 7.60 (1H, t, J = 7.4 Hz, H-6′), 7.44 (1H, d, J = 7.4 Hz, H-7′), 7.05 (1H, t, J = 7.4 Hz, H-5′), 6.86 (1H, t, J = 7.7 Hz, H-5), 3.65 (3H, s, N−CH_3). CI-MS *m*/*z* 403 $(M + H)^+$. Anal. ($C_{17}H_{11}N_2O_2I$) C, H, N.

General Procedure for the Preparation of the Oximes 7b−e and 8b−e. The appropriate indirubin derivative **1b−e** and **6b−e** (1 mmol) was dissolved in pyridine (10 mL). With magnetic stirring, hydroxylamine hydrochloride (10 equiv) was added and the mixture was heated under reflux (120 °C) for 1.5 h. Then the solvent was evaporated under reduced pressure and the residue was washed with water to afford quantitatively the corresponding 3′-oxime selectively in the (2′*Z*,3′*E*) form.

Data for (2′*Z*,3′*E*)-7-Fluoroindirubin-3′-oxime (7b). Mp 253 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 13.61 (1H, brs, NOH), 11.85 (1H, s, N′−H), 11.19 (1H, s, N−H), 8.44 (1H, d, J = 7.8 Hz, H-4), 8.19 (1H, d, J = 7.5, H-4′), 7.39 (2H, m, H-6′, 7′), 7.00 (2H, m, H-5′, 6), 6.90 (1H, td, J = 7.8 Hz, 5.0, H-5). CI-MS *m*/*z* 296 $(M + H)^+$. Anal. ($C_{16}H_{10}N_3O_2F$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Chloroindirubin-3′-oxime (7c). Mp 263 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 13.70 (1H, brs, NOH), 11.86 (1H, s, N′−H), 11.09 (1H, s, N−H), 8.62 (1H, d, J = 7.9 Hz, H-4), 8.23 (1H, d, J = 7.6 Hz, H-4′), 7.44 (2H, m, H-6′, 7′), 7.17 (1H, d, J = 7.9 Hz, H-6), 7.06 (1H, t, J = 7.6 Hz, H-5′), 6.96 (1H, t, J = 7.8 Hz, H-5). CI-MS *m*/*z* 312, 314 $(M + H)^+$. Anal. ($C_{16}H_{10}N_3O_2Cl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-oxime (7d). Mp 276 °C. IR 3096 (br), 1674, 1613, 1563, 1463, 1440, 1330, 1314, 1230, 1186 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 13.68 (1H, brs, NOH) 11.90 (1H, s, N′−H), 10.91 (1H, s, N−H), 8.67 (1H, d, J = 7.8 Hz, H-4), 8.23 (1H, d, J = 7.8 Hz, H-4′), 7.42 (2H, m, H-6′, 7′), 7.29 (1H, d, J = 7.8 Hz, H-6), 7.06 (1H, t, J = 7.8 Hz, H-5′), 6.90 (1H, t, J = 7.8 Hz, H-5). CI-MS *m*/*z* 356, 358 $(M + H)^+$. Anal. ($C_{16}H_{10}N_3O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Iodoindirubin-3′-oxime (7e). Mp 284 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 13.65 (1H, brs, NOH), 11.87 (1H, s, N′−H), 10.63 (1H, s, N−H), 8.68 (1H, d, J = 7.8 Hz, H-4), 8.23 (1H, d, J = 7.2 Hz, H-4′), 7.47 (1H, d, J = 7.8 Hz, H-6), 7.43 (2H, m, H-6′, 7′), 7.06 (1H, t, J = 7.2 Hz, H-5′), 6.76 (1H, t, J = 7.8 Hz, H− 5). CI-MS *m*/*z* 404 $(M + H)^+$. Anal. ($C_{16}H_{10}N_3O_2I$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Fluoro-1-methylindirubin-3′-oxime (8b). Mp 264 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 13.72 (1H, brs, NOH), 11.90 (1H, s, N′−H), 8.56 (1H, d, J = 7.7 Hz, H-4), 8.23 (1H, d, J = 7.6 Hz, H-4′), 7.44 (2H, m, H-6′, 7′), 7.07 (1H, m, H-5′, 6), 6.97 (1H, td, J = 7.8, 5.0 Hz, H-5), 3.60 (3H, s, N−CH_3). CI-MS *m*/*z* 310 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2F$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Chloro-1-methylindirubin-3′-oxime (8c). Mp 276 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 13.79 (1H, brs,

NOH), 11.97 (1H, s, N′−H), 8.76 (1H, d, J = 7.8 Hz, H-4), 8.23 (1H, d, J = 7.3 Hz, H-4′), 7.45 (2H, m, H-6′, 7′), 7.18 (1H, d, J = 7.8 Hz, H-6), 7.07 (1H, t, J = 7.3 Hz, H-5′), 6.99 (1H, t, J = 7.8 Hz, H-5), 3.67 (3H, s, N−CH_3). CI-MS m/z 326, 328 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2Cl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromo-1-methylindirubin-3′-oxime (8d). Mp 280 °C. IR 3184 (br), 1652, 1613, 1565, 1455, 1344, 1240, 1138 cm^{-1}. ^1H NMR (DMSO, 400 MHz, δ ppm) 12.00 (1H, s, N′−H), 8.81 (1H, d, J = 7.9 Hz, H-4), 8.23 (1H, d, J = 7.9 Hz, H-4′), 7.43 (2H, m, H-6′, 7′), 7.34 (1H, d, J = 7.9 Hz, H-6), 7.07 (1H, t, J = 7.9 Hz, H-5′), 6.93 (1H, t, J = 7.9 Hz, H-5), 3.68 (3H, s, N−CH_3). CI-MS m/z 370, 372 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2$-Br) C, H, N.

Data for (2′*Z*,3′*E*)-7-Iodo-1-methylindirubin-3′-oxime (8e). Mp 280 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 13.70 (1H, brs, NOH), 12.00 (1H, s, N′−H), 8.85 (1H, d, J = 7.7 Hz, H-4), 8.24 (1H, d, J = 7.8 Hz, H-4′), 7.60 (1H, d, J = 7.7 Hz, H-6), 7.43 (2H, m, H-6′, 7′), 7.06 (1H, t, J = 7.8 Hz, H-5′), 6.77 (1H, t, J = 7.7 Hz, H-5), 3.70 (3H, s, N−CH_3). CI-MS m/z 418 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2I$) C, H, N.

General Procedure for the Preparation of the Methoximes 9b−e and 10b−e. The appropriate indirubin derivatives **1b−e** and **6b−e** (1 mmol) were dissolved in pyridine (10 mL). With magnetic stirring, methoxylamine hydrochloride (10 equiv) was added and the mixture was heated under reflux (120 °C) for 1.5 h. Then the solvent was evaporated under reduced pressure and the residue was washed with water to afford quantitatively the corresponding 3′-methoxime selectively in the (2′*Z*,3′*E*) form.

Data for (2′*Z*,3′*E*)-7-Fluoroindirubin-3′-methoxime (9b). Mp 277 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.79 (1H, s, N′−H), 11.24 (1H, s, N−H), 8.46 (1H, d, J = 7.5 Hz, H-4), 8.12 (1H, d, J = 7.6 Hz, H-4′), 7.44 (2H, m, H-6′, 7′), 7.05 (3H, m, H-5, 5′, 6), 4.39 (3H, s, OCH_3). CI-MS m/z 310 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2F$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Chloroindirubin-3′-methoxime (9c). Mp 272 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.24 (1H, s, N−H), 8.60 (1H, d, J = 7.9 Hz, H-4), 8.12 (1H, d, J = 7.9 Hz, H-4′), 7.46 (2H, m, H-6′, 7′), 7.20 (1H, d, J = 7.9 Hz, H-6), 7.05 (2H, m, H-5, 5′), 4.40 (3H, s, OCH_3). CI-MS m/z 326, 328 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2Cl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-methoxime (9d). Mp 280 °C. IR 3394 (br), 2982, 2872, 1672, 1640, 1610, 1560, 1459, 1325, 1307, 1229 cm^{-1}. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.84 (1H, s, N′−H), 11.02 (1H, s, N−H), 8.65 (1H, d, J = 7.9 Hz, H-4), 8.13 (1H, d, J = 7.9 Hz, H-4′), 7.46 (2H, m, H-6′, 7′), 7.34 (1H, d, J = 7.9 Hz, H-6), 7.06 (1H, m, H-5′), 6.97 (1H, t, J = 7.9 Hz, H-5), 4.41 (3H, s, OCH_3). CI-MS m/z 370, 372 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Iodoindirubin-3′-methoxime (9e). Mp 297 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 10.69 (1H, s, N−H), 8.66 (1H, d, J = 7.8 Hz, H-4), 8.12 (1H, d, J = 7.7 Hz, H-4′), 7.50 (1H, d, J = 7.8 Hz, H-6), 7.45 (2H, m, H-6′, 7′), 7.06 (1H, m, H-5′), 6.82 (1H, t, J = 7.8 Hz, H-5), 4.39 (3H, s, OCH_3). CI-MS m/z 418 $(M + H)^+$. Anal. ($C_{17}H_{12}N_3O_2I$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Fluoro-1-methylindirubin-3′-methoxime (10b). Mp 227 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.83 (1H, s, N′−H), 8.52 (1H, d, J = 7.4 Hz, H-4), 8.10 (1H, d, J = 7.6 Hz, H-4′), 7.44 (2H, m, H-6′, 7′), 7.06 (3H, m, H-5, 5′, 6), 4.39 (3H, s, OCH_3), 3.48 (3H, s, N−CH_3). CI-MS m/z 324 $(M + H)^+$. Anal. ($C_{18}H_{14}N_3O_2F$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Chloro-1-methylindirubin-3′-methoxime (10c). Mp 203 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.91 (1H, s, N′−H), 8.72 (1H, d, J = 7.8 Hz, H-4), 8.11 (1H, d, J = 7.8 Hz, H-4′), 7.46 (2H, m, H-6′, 7′), 7.21 (1H, d, J = 7.8 Hz, H-6), 7.05 (2H, m, H-5, 5′), 4.40 (3H, s, OCH_3), 3.66 (3H, s, N−CH_3). CI-MS m/z 340, 342 $(M + H)^+$. Anal. ($C_{18}H_{14}N_3O_2Cl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromo-1-methylindirubin-3′-methoxime (10d). Mp 212 °C. IR 2927 (br), 1646, 1613, 1555, 1463, 1332, 1234, 1161 cm^{-1}. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.94 (1H, s, N′−H), 8.80 (1H, d, J = 7.9 Hz, H-4), 8.13 (1H, d, J = 7.1 Hz, H-4′), 7.47 (2H, m, H-6′, 7′), 7.38 (1H, d, J = 7.9 Hz, H-6), 7.07 (1H, m, H-5′), 7.00 (1H, t, J = 7.9 Hz, H-5), 4.40 (3H, s, OCH_3), 3.68 (3H, s, N−CH_3). CI-MS m/z 384, 386 $(M + H)^+$. Anal. ($C_{18}H_{14}N_3O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Iodo-1-methylindirubin-3′-methoxime (10e). Mp 220 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.92 (1H, s, N′−H), 8.81 (1H, d, J = 7.7 Hz, H-4), 8.12 (1H, d, J = 7.7 Hz, H-4′), 7.64 (1H, d, J = 7.7 Hz, H-6), 7.50 (2H, m, H-6′, 7′), 7.06 (1H, m, H-5′), 6.83 (1H, t, J = 7.7 Hz, H-5), 4.39 (3H, s, OCH_3), 3.68 (3H, s, N−CH_3). CI-MS m/z 432 $(M + H)^+$. Anal. ($C_{18}H_{14}N_3O_2I$) C, H, N.

General Procedure for the Preparation of the Acetoximes 11b−e and 12b−e. The appropriate indirubin-3′-oxime derivatives **7b−e** and **8b−e** (0.2 mmol) were dissolved in pyridine (10 mL). Ac_2O was added (0.5 mL), and the mixture was stirred for 30 min at 0°C. Then water (1 mL) was added and the solvents were evaporated under reduced pressure. The residue was washed with water to afford quantitatively the corresponding 3′-acetoxime selectively in the (2′*Z*,3′*E*) form.

Data for (2′*Z*,3′*E*)-7-Fluoroindirubin-3′-acetoxime (11b). Mp 253 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.68 (1H, s, N′−H), 11.33 (1H, s, N−H), 8.92 (1H, d, J = 7.9 Hz, H-4), 8.25 (1H, d, J = 7.7 Hz, H-4′), 7.51 (2H, m, H-6′, 7′), 7.01 (2H, m, H-5′, 6), 6.96 (1H, td, J = 7.8, 4.5 Hz, H-5), 2.47 (3H, s, $OCOCH_3$). CI-MS m/z 338 $(M + H)^+$. Anal. ($C_{18}H_{12}N_3O_3F$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Chloroindirubin-3′-acetoxime (11c). Mp 251 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.70 (1H, s, N′−H), 11.23 (1H, s, N−H), 9.07 (1H, d, J = 8.0 Hz, H-4), 8.25 (1H, d, J = 7.6 Hz, H-4′), 7.52 (2H, m, H-6′, 7′), 7.24 (1H, d, J = 8.0 Hz, H-6), 7.11 (1H, t, J = 7.6 Hz, H-5′), 6.97 (1H, t, J = 8.0 Hz, H-5), 2.47 (3H, s, $OCOCH_3$). CI-MS m/z 354, 356 $(M + H)^+$. Anal. ($C_{18}H_{12}N_3O_3Cl$) C, H, N.

Data for (2′*Z*, 3′*E*)-7-bromoindirubin-3′-acetoxime (11d). Mp 250 °C. IR 3239 (br), 2926, 2853, 1778, 1672, 1613, 1564, 1462, 1321, 1174, 1122 cm^{-1}. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.73 (1H, s, N′−H), 11.11 (1H, s, N−H), 9.12 (1H, d, J = 7.5 Hz, H-4), 8.27 (1H, d, J = 7.9 Hz, H-4′), 7.53 (2H, m, H-6′, 7′), 7.37 (1H, d, J = 7.5 Hz, H-6), 7.11 (1H, t, J = 7.9 Hz, H-5′), 6.92 (1H, t, J = 7.5 Hz, H-5), 2.48 (3H, s, $OCOCH_3$). CI-MS m/z 398, 400 $(M + H)^+$. Anal. ($C_{18}H_{12}N_3O_3Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Iodoindirubin-3′-acetoxime (11e). Mp 263 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.71 (1H, s, N′−H), 10.78 (1H, s, N−H), 9.12 (1H, d, J = 7.9 Hz, H-4), 8.25 (1H, d, J = 7.5 Hz, H-4′), 7.52 (3H, m, H-6, 6′, 7′), 7.10 (1H, t, J = 7.5 Hz, H-5′), 6.77 (1H, t, J = 7.9 Hz, H-5), 2.47 (3H, s, $OCOCH_3$). CI-MS m/z 446 $(M + H)^+$. Anal. ($C_{18}H_{12}N_3O_3I$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Fluoro-1-methylindirubin-3′-acetoxime (12b). Mp 249 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.76 (1H, s, N′−H), 9.00 (1H, d, J = 8.0 Hz, H-4), 8.26 (1H, d, J = 7.4 Hz, H-4′), 7.53 (2H, m, H-6′, 7′), 7.12 (2H, m, H-5′, 6), 7.00 (1H, td, J = 7.8, 4.5 Hz, H-5), 3.50 (3H, s, N−CH_3), 2.47 (3H, s, $OCOCH_3$). CI-MS m/z 352 $(M + H)^+$. Anal. ($C_{19}H_{14}N_3O_3F$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Chloro-1-methylindirubin-3′-acetoxime (12c). Mp 240 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.83 (1H, s, N′−H), 9.20 (1H, d, J = 8.0 Hz, H-4), 8.27 (1H, d, J = 7.5 Hz, H-4′), 7.52 (2H, m, H-6′, 7′), 7.26 (1H, d, J = 8.0 Hz, H-6), 7.12 (1H, t, J = 7.5 Hz, H-5′), 7.01 (1H, t, J = 8.0 Hz, H-5), 3.66 (3H, s, N−CH_3), 2.47 (3H, s, $OCOCH_3$). CI-MS m/z 368, 370 $(M + H)^+$. Anal. ($C_{19}H_{14}N_3O_3Cl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromo-1-methylindirubin-3′-acetoxime (12d). Mp 237 °C. IR 3229, 2936, 2853, 1778, 1651, 1610, 1557, 1461, 1330, 1233, 1202 cm^{-1}. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.83 (1H, s, N′−H), 9.24 (1H, d, J = 7.9 Hz, H-4), 8.26 (1H, d, J = 7.5 Hz, H-4′), 7.54 (1H, d, J = 7.5 Hz, H-7′), 7.51 (1H, t, J = 7.5 Hz, H-6′), 7.41 (1H, d, J = 7.9 Hz, H-6), 7.12 (1H, t, J = 7.5 Hz, H-5′), 6.94 (1H, t, J = 7.9 Hz, H-5), 3.67 (3H, s, N−CH_3), 2.47 (3H, s, $OCOCH_3$). CI-MS m/z 412, 414 $(M + H)^+$. Anal. ($C_{19}H_{14}N_3O_3Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Iodo-1-methylindirubin-3′-acetoxime (12e). Mp 240 °C. ^1H NMR (DMSO, 400 MHz, δ ppm) 11.81 (1H, s, N′−H), 9.26 (1H, d, J = 7.8 Hz, H-4), 8.25 (1H, d, J = 7.5 Hz,

H-4′), 7.68 (1H, d, J = 7.8 Hz, H-6), 7.52 (2H, m, H-6′, 7′), 7.11 (1H, t, J = 7.5 Hz, H-5′), 6.78 (1H, t, J = 7.8 Hz, H-5), 3.68 (3H, s, N−CH_3), 2.47 (3H, s, OCOCH_3). CI-MS m/z 460 $(M + H)^+$. Anal. ($C_{19}H_{14}N_3O_3I$) C, H, N.

(2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-bromoethyl)oxime] (13f). To a solution of 7BIO (**7d**) (100 mg, 0.30 mmol) in 5 mL of anhydrous DMF, 120 μL of triethylamine (2.9 equiv) and 72 μL of 1,2-dibromoethane (2.8 equiv) were added, and the reaction mixture was stirred under Ar at room temperature for 48 h. Then the solvent was evaporated under reduced pressure and the residue was washed with water and dried at 50 °C to afford in 95% yield the corresponding 3′-substituted oxime **13f**. Mp 229 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.81 (1H, s, N′−H), 11.02 (1H, s, N−H), 8.59 (1H, d, J = 8.0 Hz, H-4), 8.22 (1H, d, J = 8.0 Hz, H-4′), 7.47 (2H, m, H-6′, 7′), 7.33 (1H, d, J = 8.0 Hz, H-6), 7.08 (1H, m, H-5′), 6.95 (1H, t, J = 8.0 Hz, H-5), 4.93 (2H, t, J = 5.4 Hz, H-1″), 3.98 (2H, t, J = 5.4 Hz, H-2″). CI-MS m/z 463, 465, 467 $(M + H)^+$. Anal. ($C_{18}H_{13}N_3O_2Br_2$) C, H, N.

(2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-bromoethyl)-oxime] (14f). This compound was prepared from Me7BIO (**8d**) by a procedure analogous to that of **13f**. Mp 218 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.92 (1H, s, N′−H), 8.74 (1H, d, J = 8.1 Hz, H-4), 8.22 (1H, d, J = 8.0 Hz, H-4′), 7.47 (2H, m, H-6′, 7′), 7.38 (1H, d, J = 8.1 Hz, H-6), 7.09 (1H, m, H-5′), 6.98 (1H, t, J = 8.1 Hz, H-5), 4.94 (2H, t, J = 5.3 Hz, H-1″), 3.98 (2H, t, J = 5.3 Hz, H-2″), 3.68 (3H, s, N−CH_3). CI-MS m/z 477, 479, 481 $(M + H)^+$. Anal. ($C_{19}H_{15}N_3O_2Br_2$) C, H, N.

General Procedure for the Preparation of 3′-Substituted Oximes of 7BIO (13g−l) or Me7BIO (14 g−l). 7-Bromoindirubin-3′-[*O*-(2-bromoethyl)oxime] (**13f**) or 1-methyl-7-bromoindirubin-3′-[*O*-(2-bromoethyl)oxime] (**14f**) (25 mg, 0.05 mmol) were dissolved in 3 mL of anhydrous DMF. The corresponding amine (pyrrolidine, morpholine, imidazole, piperazine, dimethylamine, and diethylamine) (30 equiv) was added, and the reaction mixture was stirred under Ar at room temperature for 48 h. Then the solvent was evaporated under reduced pressure and the residue was washed with water and dried at 50 °C to afford the corresponding 3′-substituted oximes **13g−l** or **14g−l** with 75−90% yield. For the preparation of the hydrochloric salts of the above compounds, 10 mg of each compound was dissolved in 5 mL of anhydrous tetrahydrofuran. Then a solution of hydrochloric acid in diethyl ether was added slowly and the formed precipitate was filtered, washed with dichloromethane, and dried at 50 °C to afford the corresponding hydrochloric salts **13gs−ls** and **14gs−ls**.

Data for (2′*Z*, 3′*E*)-7-Bromoindirubin-3′-[*O*-(2-pyrrolidin-1-ylethyl)oxime] (13g). Yield: 90%. Mp 198 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.00 (1H, s, N−H), 8.64 (1H, d, J = 8.0 Hz, H-4), 8.15 (1H, d, J = 7.7 Hz, H-4′), 7.45 (2H, m, H-6′, 7′), 7.33 (1H, d, J = 8.0 Hz, H-6), 7.07 (1H, ddd, J = 7.7, 5.5, 3.1 Hz, H-5′), 6.94 (1H, t, J = 8.0 Hz, H-5), 4.70 (2H, t, J = 5.9 Hz, H-1″), 2.98 (2H, t, J = 5.9 Hz, H-2″), 2.56 (4H, m, H-2‴, 5‴), 1.68 (4H, m, H-3‴, 4‴). CI-MS m/z 453, 455 $(M + H)^+$. Anal. ($C_{22}H_{21}N_4O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-pyrrolidin-1-ylethyl)oxime] Hydrochloride (13gs). Mp 210 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.84 (1H, s, N′−H), 11.06 (1H, s, N−H), 10.31 (1H, brs, N‴−H), 8.58 (1H, d, J = 7.9 Hz, H-4), 8.24 (1H, d, J = 8.3 Hz, H-4′), 7.49 (2H, m, H-6′, 7′), 7.37 (1H, d, J = 7.9 Hz, H-6), 7.09 (1H, ddd, J = 8.3, 4.4, 1.3 Hz, H-5′), 6.99 (1H, t, J = 7.9 Hz, H-5), 4.97 (2H, brs, H-1″), 3.77 (2H, brs, H-2″), 3.64 (2H, m, H-2‴a, 5‴a), 3.12 (2H, m, 2‴b, 5‴b), 2.00 (2H, m, H-3‴a, 4‴a), 1.86 (2H, m, H-3‴b, 4‴b). Anal. ($C_{22}H_{22}N_4O_2$-BrCl) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-pyrrolidin-1-ylethyl)oxime] (14g). Yield: 90%. Mp 169 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.93 (1H, s, N′−H), 8.80 (1H, d, J = 7.9 Hz, H-4), 8.16 (1H, d, J = 8.0 Hz, H-4′), 7.46 (2H, m, H-6′, 7′), 7.37 (1H, d, J = 7.9 Hz, H-6), 7.07 (1H, ddd, J = 8.0, 5.5, 3.1 Hz, H-5′), 6.97 (1H, t, J = 7.9 Hz, H-5), 4.71 (2H, t, J = 5.9 Hz, H-1″), 3.68 (3H, s, N−CH_3), 2.98 (2H, t, J = 5.9 Hz, H-2″), 2.56 (4H, m, H-2‴, 5‴), 1.68 (4H, m, H-3‴, 4‴). CI-MS m/z 467, 469 $(M + H)^+$. Anal. ($C_{23}H_{23}N_4O_2Br$) C, H, N.

Data for (2′*Z*,3′ *E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-pyrrolidin-1-ylethyl)oxime] Hydrochloride (14gs). Mp 214 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.94 (1H, s, N′−H), 10.05 (1H, brs, N‴−H), 8.73 (1H, d, J = 7.8 Hz, H-4), 8.24 (1H, d, J = 7.8 Hz, H-4′), 7.49 (2H, m, H-6′, 7′), 7.40 (1H, d, J = 7.8 Hz, H-6), 7.09 (1H, ddd, J = 7.8, 4.1, 1.7 Hz, H-5′), 7.01 (1H, t, J = 7.8 Hz, H-5), 4.96 (2H, m, H-1″), 3.68 (3H, s, N−CH_3), 3.64 (2H, m, H-2‴a, 5‴a), 3.14 (2H, m, 2‴b, 5‴b), 2.00 (2H, m, H-3‴a, 4‴a), 1.85 (2H, m, H-3‴b, 4‴b). Anal. ($C_{23}H_{24}N_4O_2BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-morpholin-1-ylethyl)oxime] (13h). Yield: 85%. Mp 223 °C. ^{1}H NMR (C_5D_5N, 400 MHz, δ ppm) 12.68 (1H, s, N′−H), 12.40 (1H, s, N−H), 9.02 (1H, d, J = 7.7 Hz, H-4), 8.42 (1H, d, J = 7.7 Hz, H-6), 7.54 (1H, d, J = 7.7 Hz, H-4′), 7.42 (1H, t, J = 7.7 Hz, H-6′), 7.18 (2H, overlapped, H-5′, H-7′), 7.10 (1H, t, J = 7.7 Hz, H-5), 4.80 (2H, t, J = 5.9 Hz, H-1″), 3.76 (4H, t, J = 4.2 Hz, H-3‴, 5‴), 2.94 (2H, t, J = 5.9 Hz, H-2″), 2.60 (4H, t, J = 4.2 Hz, H-2‴, 6‴). CI-MS m/z 469, 471 $(M + H)^+$. Anal. ($C_{22}H_{21}N_4O_3Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-morpholin-1-ylethyl)oxime] Hydrochloride (13hs). Mp 235 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.04 (1H, s, N−H), 10.71 (1H, brs, N‴−H), 8.58 (1H, d, J = 7.9 Hz, H-4), 8.23 (1H, d, J = 7.7 Hz, H-4′), 7.47 (2H, m, H-6′, 7′), 7.35 (1H, d, J = 7.9 Hz, H-6), 7.08 (1H, ddd, J = 7.7, 5.8, 2.3 Hz, H-5′), 6.99 (1H, t, J = 7.9 Hz, H-5), 5.02 (2H, m, H-1″), 3.95 (2H, m, H-3‴a, 5‴a), 3.74 (4H, overlapped, H-2”, 3‴b, 5‴b), 3.57 (2H, m, H-2‴a, 6‴a), 3.25 (2H, overlapped, 2‴b, 6‴b). Anal. ($C_{22}H_{22}N_4O_3BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-morpholin-1-ylethyl)oxime] (14h). Yield: 85%. Mp 169 °C. ^{1}H NMR (C_5D_5N, 400 MHz, δ ppm) 12.40 (1H, s, N′−H), 9.11 (1H, d, J = 7.8 Hz, H-4), 8.42 (1H, d, J = 7.7 Hz, H-6), 7.49 (1H, d, J = 7.7 Hz, H-4′), 7.40 (1H, m, H-6′, 7′), 7.10 (1H, m, H-5, 5′), 4.81 (2H, t, J = 5.9 Hz, H-1″), 3.76 (4H, t, J = 4.5 Hz, H-3‴, 5‴), 3.70 (3H, s, N−CH_3), 2.94 (2H, t, J = 5.9 Hz, H-2″), 2.60 (4H, t, J = 4.5 Hz, H-2‴, 6‴). CI-MS m/z 483, 485 $(M + H)^+$. Anal. ($C_{23}H_{23}N_4O_3Br$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-morpholin-1-ylethyl)oxime] Hydrochloride (14hs). Mp 214 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.94 (1H, s, N′−H), 10.52 (1H, brs, N‴−H), 8.73 (1H, d, J = 8.0 Hz, H-4), 8.23 (1H, d, J = 7.7 Hz, H-4′), 7.49 (2H, m, H-6′, 7′), 7.40 (1H, d, J = 8.0 Hz, H-6), 7.09 (1H, ddd, J = 7.7, 4.1, 1.0 Hz, H-5′), 7.01 (1H, t, J = 8.0 Hz, H-5), 5.02 (2H, m, H-1″), 3.98 (2H, m, H-3‴a, 5‴a), 3.72 (4H, overlapped, H-2″, 3‴b, 5‴b), 3.68 (3H, s, N−CH_3), 3.55 (2H, m, H-2‴a, 6‴a), 3.26 (2H, overlapped, 2‴b, 6‴b). Anal. ($C_{23}H_{24}N_4O_3$-BrCl) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-imidazol-1-ylethyl)oxime] (13i). Yield: 75%. Mp 211 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.79 (1H, s, N′−H), 10.99 (1H, s, N−H), 8.51 (1H, d, J = 8.0 Hz, H-4), 7.99 (1H, d, J = 7.4 Hz, H-4′), 7.67 (1H, s, H-2‴), 7.44 (2H, m, H-6′, 7′), 7.33 (1H, d, J = 8.0 Hz, H-6), 7.27 (1H, s, H-4‴), 7.02 (1H, ddd, J = 8.0, 5.5, 3.1 Hz, H-5′), 6.96 (1H, t, J = 8.0 Hz, H-5), 6.87 (1H, s, H-5‴), 4.90 (2H, t, J = 4.2 Hz, H-1″), 4.54 (2H, t, J = 4.2 Hz, H-2″). CI-MS m/z 450, 452 $(M + H)^+$. Anal. ($C_{21}H_{16}N_5O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-imidazol-1-ylethyl)oxime] Hydrochloride (13is). Mp 225 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.79 (1H, s, N′−H), 11.02 (1H, s, N−H), 9.19 (1H, s, H-2‴), 8.40 (1H, d, J = 7.9 Hz, H-4), 7.95 (1H, d, J = 7.5 Hz, H-4′), 7.86 (1H, s, H-5‴), 7.62 (1H, s, H-4‴), 7.44 (2H, m, H-6′, 7′), 7.35 (1H, d, J = 7.9 Hz, H-6), 6.94−7.04 (2H, overlapped, H-5, 5′), 5.04 (2H, t, J = 4.6 Hz, H-1″), 4.77 (2H, t, J = 4.6 Hz, H-2″). Anal. ($C_{21}H_{17}N_5O_2BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-imidazol-1-ylethyl)oxime] (14i). Yield: 76%. Mp 246 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.89 (1H, s, N′−H), 8.65 (1H, d, J = 8.0 Hz, H-4), 7.99 (1H, d, J = 8.1 Hz, H-4′), 7.69 (1H, s, H-2‴), 7.45 (2H, m, H-6′, 7′), 7.37 (1H, d, J = 8.0 Hz, H-6), 7.26 (1H, s,

H-4‴), 6.97−7.05 (2H, overlapped, H-5′, 5), 6.86 (1H, s, H-5‴), 4.90 (2H, t, J = 4.8 Hz, H-1″), 4.54 (2H, t, J = 4.8 Hz, H-2″), 3.67 (3H, s, N−CH_3). CI-MS m/z 464, 466 $(M + H)^+$. Anal. ($C_{22}H_{18}N_5O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-imidazol-1-ylethyl)oxime] Hydrochloride (14is). Mp 218 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.90 (1H, s, N′−H), 9.11 (1H, s, H-2‴), 8.55 (1H, d, J = 7.9 Hz, H-4), 7.96 (1H, d, J = 7.6 Hz, H-4′), 7.83 (1H, s, H-5‴), 7.58 (1H, s, H-4‴), 7.46 (2H, m, H-6′, 7′), 7.40 (1H, d, J = 7.9 Hz, H-6), 6.97−7.05 (2H, overlapped, H-5, 5′), 5.04 (2H, t, J = 4.6 Hz, H-1″), 4.75 (2H, t, J = 4.6 Hz, H-2″), 3.67 (3H, s, N−CH_3). Anal. ($C_{22}H_{19}N_5O_2BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-piperazin-1-ylethyl)oxime] (13j). Yield: 84%. Mp 150 °C. IR 3229 (br), 2946, 2853, 1665, 1611, 1587, 1562, 1463, 1308, 1221 cm^{-1}. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.00 (1H, s, N−H), 8.63 (1H, d, J = 8.0 Hz, H-4), 8.17 (1H, d, J = 7.8 Hz, H-4′), 7.45 (2H, m, H-6′, 7′), 7.33 (1H, d, J = 8.0 Hz, H-6), 7.06 (1H, ddd, J = 7.8, 5.1, 3.1 Hz, H-5′), 6.94 (1H, t, J = 8.0 Hz, H-5), 4.71 (2H, t, J = 5.6 Hz, H-1″), 2.87 (2H, t, J = 5.6 Hz, H-2″), 2.68 (4H, t, J = 4.6 Hz, H-2‴, 6‴), 2.44 (4H, brs, H-3‴, 5‴). CI-MS m/z 468, 470 $(M + H)^+$. Anal. ($C_{22}H_{22}N_5O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-piperazin-1-ylethyl)oxime] Dihydrochloride (13js). Mp >300 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.05 (1H, s, N−H), 9.32 (2H, br, piperazine N^+−H), 8.59 (1H, d, J = 8.0 Hz, H-4), 8.25 (1H, d, J = 7.5 Hz, H-4′), 7.48 (2H, m, H-6′, 7′), 7.35 (1H, d, J = 8.0 Hz, H-6), 7.06 (1H, ddd, J = 7.5, 4.1, 1.4 Hz, H-5′), 6.99 (1H, t, J = 8.0 Hz, H-5), 4.98 (2H, m, H-1″), 3.70 (2H, m, H-2″), 3.50 (8H, overlapped, H-2‴, 3‴, 5‴, 6‴). Anal. ($C_{22}H_{24}N_5O_2BrCl_2$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-dimethylaminoethyl)oxime] (13k). Yield: 90%. Mp 226 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.00 (1H, s, N−H), 8.65 (1H, d, J = 8.0 Hz, H-4), 8.15 (1H, d, J = 7.8 Hz, H-4′), 7.46 (2H, m, H-6′, 7′), 7.33 (1H, d, J = 8.0 Hz, H-6), 7.07 (1H, ddd, J = 7.8, 5.1, 3.4 Hz, H-5′), 6.94 (1H, t, J = 8.0 Hz, H-5), 4.70 (2H, t, J = 5.9 Hz, H-1″), 2.81 (2H, t, J = 5.9 Hz, H-2″), 2.26 (6H, s, N‴$(CH_3)_2$). CI-MS m/z 433, 435 $(M + H)^+$. Anal. ($C_{20}H_{25}N_4O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-dimethylaminoethyl)oxime] Hydrochloride (13ks). Mp 234 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.83 (1H, s, N′−H), 11.04 (1H, s, N−H), 9.74 (1H, brs, N‴−H), 8.58 (1H, d, J = 8.0 Hz, H-4), 8.23 (1H, d, J = 7.7 Hz, H-4′), 7.48 (2H, m, H-6′, 7′), 7.36 (1H, d, J = 8.0 Hz, H-6), 7.07 (1H, ddd, J = 7.7, 5.0, 3.3 Hz, H-5′), 6.97 (1H, t, J = 8.0 Hz, H-5), 4.94 (2H, t, J = 5.9 Hz, H-1″), 3.64 (2H, t, J = 5.9 Hz, H-2″), 2.85 (6H, s, N‴$(CH_3)_2$). Anal. ($C_{20}H_{26}N_4O_2BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-dimethylaminoethyl)oxime] (14k). Yield: 90%. Mp 164 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.94 (1H, s, N′−H), 8.80 (1H, d, J = 7.9 Hz, H-4), 8.16 (1H, d, J = 7.8 Hz, H-4′), 7.47 (2H, m, H-6′, 7′), 7.38 (1H, d, J = 7.9 Hz, H-6), 7.08 (1H, ddd, J = 7.8, 5.5, 2.6 Hz, H-5′), 6.97 (1H, t, J = 7.9 Hz, H-5), 4.70 (2H, t, J = 5.8 Hz, H-1″), 3.68 (3H, s, N−CH_3), 2.81 (2H, t, J = 5.8 Hz, H-2″), 2.26 (6H, s, N‴$(CH_3)_2$). CI-MS m/z 447, 449 $(M + H)^+$. Anal. ($C_{21}H_{27}N_4O_2Br$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-dimethylaminoethyl)oxime] Hydrochloride (14ks). Mp >300 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.94 (1H, s, N′−H), 10.07 (1H, brs, N‴−H), 8.73 (1H, d, J = 8.1 Hz, H-4), 8.25 (1H, d, J = 7.7 Hz, H-4′), 7.49 (2H, m, H-6′, 7′), 7.40 (1H, d, J = 8.1 Hz, H-6), 7.09 (1H, ddd, J = 7.7, 5.5, 3.5 Hz, H-5′), 7.02 (1H, t, J = 8.1 Hz, H-5), 5.00 (2H, t, J = 5.8 Hz, H-1″), 3.68 (3H, s, N−CH_3), 3.64 (2H, t, J = 5.8 Hz, H-2″), 2.85 (6H, s, N‴$(CH_3)_2$). Anal. ($C_{21}H_{28}N_4O_2BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-diethylaminoethyl)oxime] (13l). Yield: 89%. Mp 208 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.83 (1H, s, N′−H), 11.00 (1H, s, N−H), 8.66 (1H, d, J = 7.9 Hz, H-4), 8.17 (1H, d, J = 7.8 Hz, H-4′), 7.45 (2H, m, H-6′, 7′), 7.33 (1H, d, J = 7.9 Hz, H-6), 7.06 (1H, ddd, J = 7.8, 5.5, 3.4 Hz, H-5′), 6.93 (1H, t, J = 7.9 Hz, H-5), 4.66 (2H, t, J = 6.1 Hz, H-1″), 2.95 (2H, t, J = 6.1 Hz, H-2″), 2.59 (4H, q, J = 7.1 Hz, N‴$(CH_2CH_3)_2$), 0.98 (6H, t, J = 7.1 Hz, N‴-$(CH_2CH_3)_2$). CI-MS m/z 461, 463 $(M + H)^+$. Anal. ($C_{22}H_{29}N_4O_2$-Br) C, H, N.

Data for (2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(2-diethylaminoethyl)oxime] Hydrochloride (13ls). Mp >300 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.82 (1H, s, N′−H), 11.03 (1H, s, N−H), 10.52 (1H, brs, N‴−H), 8.58 (1H, d, J = 8.0 Hz, H-4), 8.23 (1H, d, J = 7.9 Hz, H-4′), 7.48 (2H, m, H-6′, 7′), 7.35 (1H, d, J = 8.0 Hz, H-6), 7.07 (1H, ddd, J = 7.9, 5.4, 2.9 Hz, H-5′), 7.00 (1H, t, J = 8.0 Hz, H-5), 5.03 (2H, t, J = 6.1 Hz, H-1″), 3.68 (2H, t, J = 6.1 Hz, H-2″), 3.25 (4H, m, N‴$(CH_2CH_3)_2$), 1.22 (6H, t, J = 7.1 Hz, N‴$(CH_2CH_3)_2$). Anal. ($C_{21}H_{28}N_4O_2BrCl$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-diethylaminoethyl)oxime] (14l). Yield: 88%. Mp 158 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.94 (1H, s, N′−H), 8.80 (1H, d, J = 7.9 Hz, H-4), 8.17 (1H, d, J = 7.8 Hz, H-4′), 7.46 (2H, m, H-6′, 7′), 7.37 (1H, d, J = 7.9 Hz, H-6), 7.07 (1H, ddd, J = 7.8, 5.1, 3.1 Hz, H-5′), 6.95 (1H, t, J = 7.9 Hz, H-5), 4.65 (2H, t, J = 6.1 Hz, H-1″), 3.67 (3H, s, N−CH_3), 2.94 (2H, t, J = 6.1 Hz, H-2″), 2.58 (4H, q, J = 7.1 Hz, N‴$(CH_2CH_3)_2$), 0.98 (6H, t, J = 7.1 Hz, N‴-$(CH_2CH_3)_2$). CI-MS m/z 475, 477 $(M + H)^+$. Anal. ($C_{23}H_{31}N_4O_2$-Br) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(2-diethylaminoethyl)oxime] Hydrochloride (14ls). Mp 199 °C. ^{1}H NMR (DMSO, 400 MHz, δ ppm) 11.93 (1H, s, N′−H), 9.95 (1H, brs, N‴−H), 8.72 (1H, d, J = 8.0 Hz, H-4), 8.22 (1H, d, J = 7.8 Hz, H-4′), 7.49 (2H, m, H-6′, 7′), 7.41 (1H, d, J = 8.0 Hz, H-6), 7.08 (1H, ddd, J = 7.8, 4.0, 1.5 Hz, H-5′), 7.02 (1H, t, J = 8.0 Hz, H-5), 5.00 (2H, t, J = 6.1 Hz, H-1″), 3.68 (5H, m, N−CH_3, H-2″), 3.26 (4H, m, N‴$(CH_2CH_3)_2$), 1.21 (6H, t, J = 7.3 Hz, N‴-$(CH_2CH_3)_2$). Anal. ($C_{22}H_{30}N_4O_2BrCl$) C, H, N.

(2′*Z*,3′*E*)-7-Bromoindirubin-3′-[*O*-(*N*,*N*-diethylcarbamyl)-oxime] (15). To a solution of 7BIO (25 mg, 0.07 mmol) in anhydrous DMF (3 mL), 14 μL of triethylamine (1.5 equiv) and 13 μL of *N*,*N*-diethylcarbamyl chloride (1.5 equiv) were added, and the reaction mixture was stirred under Ar at room temperature for 48 h. Then the solvent was evaporated under reduced pressure and the residue was washed with water and dried at 50°C to afford quantitatively the corresponding 3′-substituted oximes. Mp 227 °C. IR 3396 (br), 2954, 2917, 2853, 1734, 1661, 1610, 1555, 1459, 1413, 1261, 1216 cm^{-1}. ^{1}H NMR (C_5D_5N, 400 MHz, δ ppm) 12.70 (1H, s, N′−H), 12.29 (1H, s, N−H), 10.04 (1H, d, J = 7.6 Hz, H-4), 8.18 (1H, d, J = 7.6 Hz, H-6), 7.49 (2H, m, H-4′, 6′), 7.34 (1H, t, J = 7.9 Hz, H-5′), 7.22 (1H, overlapped, H-7′), 7.14 (1H, t, J = 7.6 Hz, H-5), 3.46 (4H, brs, N(CH_2CH_3)$_2$), 1.19 (6H, t, J = 6.5 Hz, N(CH_2CH_3)$_2$). CI-MS m/z 455, 457 $(M + H)^+$. Anal. ($C_{21}H_{19}N_4O_3Br$) C, H, N.

Data for (2′*Z*,3′*E*)-1-Methyl-7-bromoindirubin-3′-[*O*-(*N*,*N*-diethylcarbamyl)oxime] (16). This compound was prepared from Me7BIO (**27**) by a procedure analogous to that of **63**. Mp 229 °C. IR 3351 (br), 2972, 1738, 1642, 1610, 1555, 1463, 1417, 1330, 1229 cm^{-1}. ^{1}H NMR (C_5D_5N, 400 MHz, δ ppm) 12.32 (1H, s, N′−H), 10.10 (1H, d, J = 7.6 Hz, H-4), 8.18 (1H, d, J = 7.6 Hz, H-6), 7.46 (2H, m, H-4′, 6′), 7.30 (1H, t, J = 7.8 Hz, H-5′), 7.16 (2H, overlapped, H-5, 7′), 3.66 (3H, s, N−CH_3), 3.46 (4H, brs, N(CH_2CH_3)$_2$), 1.19 (6H, t, J = 6.8 Hz, N(CH_2CH_3)$_2$). CI-MS m/z 469, 471 $(M + H)^+$. Anal. ($C_{22}H_{21}N_4O_3Br$) C, H, N.

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

Buffers. Homogenization buffer: 60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenyl phosphate, 10 μg of leupeptin/mL, 10 μg of aprotinin/mL, 10 μg of soybean trypsin inhibitor/mL, and 100 μM benzamidine.

Buffer A: 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 25 mM Tris-HCl, pH 7.5, 50 μg of heparin/mL.

Buffer C: homogenization buffer but 5 mM EGTA, no NaF, and no protease inhibitors.

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

CDK1/cyclin B was extracted in homogenization buffer from M phase starfish (*Marthasterias glacialis*) oocytes and purified by affinity chromatography on $p9^{CKShs1}$-sepharose beads, from which it was eluted by free $p9^{CKShs1}$ as previously described.[30] The kinase activity was assayed in buffer C, with 1 mg of histone H1/mL, in the presence of 15 μM [γ-^{33}P]ATP (3000 Ci/mmol, 10 mCi/mL) in a final volume of 30 μL. After 30 min of incubation at 30 °C, 25 μL aliquots of supernatant were spotted onto 2.5 cm × 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 of phosphoric acid/L of water. The wet filters were counted in the presence of 1 mL of ACS (Amersham) scintillation fluid.

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 chomatography on glutathione agarose (vectors kindly provided by Dr. L. H. Tsai) (p25 is a truncated version of p35, the 35 kDa CDK5 activator). Its activity was assayed with histone H1 in buffer C as described for CDK1/cyclin B.

GSK-3α/β was purified from porcine brain by affinity chromatography on immobilized axin.[18] It was assayed, following a $^1/_{100}$ dilution in 1 mg of BSA/mL 10 mM DTT, with 5 μL of 4 μM GS-1 peptide substrate in buffer A in the presence of 15 μM [γ-^{33}P]-ATP (3000 Ci/mmol, 10 mCi/mL) in a final volume of 30 μL. After 30 min of incubation at 30 °C, 25 μL aliquots of supernatant were processed as described above.

Cell Biology. Antibodies and Chemicals. AcDEVDafc and Q-VD-OPh were purchased from MPbiomedicals (Vannes, France). Cell Titer 96 kit containing the MTS reagent was purchased from Promega (Madison, WI). The protease inhibitor cocktail was from Roche. Unless otherwise stated, the nonlisted reagents were also from Sigma.

Cell Lines and Culture Conditions. The mouse 5L hepatoma cell line (AhR +/+) and BP8 (an AhR −/− subclone) were kindly provided by Dr. M. Gottlicher (Forschungszentrum Karlsruhe, Institute of Genetics, 76021 Karlsruhe, Germany). They were cultured in Dulbecco's modified Eagle medium (DMEM) (Biowhittaker) supplemented with 2 mM L-glutamine (Eurobio), 10% fetal calf serum (FCS), and penicillin and streptomycin (Gibco BRL) at 37 °C in an atmosphere of 5% CO_2. Indirubin treatments were performed on cultures at the indicated times and concentrations. Control experiments were carried out using appropriate dilutions of DMSO.

SH-SY5Y human neuroblastoma cell line was grown in DMEM medium (Biowhittaker) supplemented with 2 mM L-glutamine from Eurobio (Courtaboeuf, France), antibiotics, and 10% volume of FCS from Invitrogen (Cergy Pontoise, France). HCT116 human adenocarcinoma cell line was kindly provided by Dr. B. Vogelstein (Howard Hughes Medical Institute, Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins School of Medicine, Baltimore, MD 21231). HCT116 cells were cultured in McCoy's 5A (Biowhittaker) supplemented with antibiotics and 10% FCS. General culture conditions were an atmosphere of 5% CO_2 and a temperature of 37 °C. Culture dishes and other plastic disposable tools were supplied by Corning (Corning, NY). Indirubin treatments were performed on exponentially growing cultures at the indicated times and concentrations. Control experiments were also carried out using appropriate dilutions of DMSO.

MDA-MB-231 cells (derived from hormone-independent breast cancer) were obtained from Dr. J. Mester and cultured in DMEM supplemented with 10% FCS. For experiments, these cells were seeded in 96-well plates (7500 cells/well) and exposed to indirubins as indicated.

Cell Death and Cell Viability Assessments. Cell viability was determined by measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) as previously described in detail.[31]

Caspase Assay. The measurement of caspase activity is based on determining the fluorescence released from the AcDEVDafc synthetic substrate after its direct addition to the culture medium, detergent lysis, and incubation at 37°C. This method is devised to be applied to 96 multiwell plates. It allows kinetic determinations of caspase activation and the characterization of multiple drugs simultaneously.[31]

Acknowledgment. We are grateful to our colleagues M. Gottlicher, J. Mester, L. H. Tsai, and B. Vogelstein for providing several reagents used in this study. This research was supported by a grant from the EEC (Grant FP6-2002, Life Sciences & Health, PRO-KINASE Research Project) (L. M.), a grant from the "Association pour la Recherche sur le Cancer" (Grant ARC3362) (L. M.) and a "Cancéropole Grand-Ouest" grant (L. M.). K.B. was supported by a fellowship from the "Ministère de la Recherche". A.L.S. and P.M. were supported by a "KAPODISTRIAS" grant of the University of Athens.

Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Meijer, L., Guyard, N., Skaltsounis, L. A., Eisenbrand, G. Eds. *Indirubin, the Red Shade of Indigo*; Life in Progress; Station Biologique: Roscoff, France, 2006; 27 chapters, 297 pp.

(2) Tang, W.; Eisenbrand, G. *Chinese Drugs of Plant Origin: Chemistry, Pharmacology, and Use in Traditional and Modern Medicine*. Springer-Verlag: Heidelberg, Germany, 1992.

(3) Xiao, Z.; Hao, Y.; Liu, B.; Qian, L. Indirubin and meisoindigo in the treatment of chronic myelogenous leukemia in China. *Leuk. Lymphoma* **2002**, *43*, 1763−1768.

(4) Hoessel, R.; Leclerc, S.; Endicott, J.; Noble, M.; Lawrie, A.; Tunnah, P.; Leost, M.; Damiens, E.; Marie, D.; Marko, D.; Niederberger, E.; Tang, W.; Eisenbrand, G.; Meijer, L. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat. Cell Biol.* **1999,** *1*, 60−67.

(5) Leclerc, S.; Garnier, M.; Hoessel, R.; Marko, D.; Bibb, J. A.; Snyder, G. L.; Greengard, P.; Biernat, J.; Mandelkow, E.-M.; Eisenbrand, G.; Meijer, L. Indirubins inhibit glycogen synthase kinase-3β and CDK5/p25, two kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most CDK inhibitors? *J. Biol. Chem.* **2001**, *276*, 251−260.

(6) Kosmopoulou, M. N.; Leonidas, D. D.; Chysina, E. D.; Bischler, N.; Eisenbrand, G.; Sakarellos, C. E.; Pauptit, R.; Oikonomakos, N. G. Binding of the potential antitumour agent indirubin-5-sulphonate at the inhibitor site of rabbit muscle glycogen phosphorylase b. Comparison with ligand binding to pCDK2-cyclin A complex. *Eur. J. Biochem.* **2004**, *271*, 2280−2290.

(7) Adachi, J.; Mori, Y.; Matsui S, Takigami, H.; Fujino, J.; Kitagawa, H.; Miller, C. A., 3rd; Kato, T., Saeki, K.; Matsuda, T. Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J. Biol. Chem.* **2001**, *276*, 31475−31478.

(8) Kawanishi, M.; Sakamoto, M.; Ito, A.; Kishi, K.; Yagi, T. Construction of reporter yeasts for mouse aryl hydrocarbon receptor ligand activity. *Mutat. Res.* **2003**, *540*, 99−105.

(9) Spink, B. C.; Hussain, M. M.; Katz, B. H.; Eisele, L.; Spink, D. C. Transient induction of cytochromes P450 1A1 and 1B1 in MCF-7 human breast cancer cells by indirubin. *Biochem. Pharmacol.* **2003**, *66*, 2313−2321.

(10) Adachi, J.; Mori, Y.; Matsui, S.; Matsuda, T. Comparison of gene expression patterns between 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and a natural arylhydrocarbon receptor ligand, indirubin. *Toxicol. Sci.* **2004**, *80*, 161−169.
(11) Guengerich, P. F.; Martin, M. V.; McCormick, W. A.; Nguyen, L. P.; Glover, E.; Bradfield, C. A. Aryl hydrocarbon receptor response to indigoids in vitro and in vivo. *Arch. Biochem. Biophys.* **2004**, *423*, 309−316.
(12) Knockaert, M.; Blondel, M.; Bach, S.; Leost, M.; Elbi, C.; Hager, G.; Naggy, S. R.; Han, D.; Denison, M.; Ffrench, M.; Ryan, X. P.; Magiatis, P.; Polychronopoulos, P.; Greengard, P.; Skaltsounis, L.; Meijer, L. Independent actions on cyclin-dependent kinases and aryl hydrocarbon receptor mediate the anti-proliferative effects of indirubins. *Oncogene* **2004**, *23*, 4400−4412.
(13) Sugihara, K.; Kitamura, S.; Yamada, T.; Okayama, T.; Ohta, S.; Yamashita, K.; Yasuda, M.; Fujii-Kuriyama, Y.; Saeki, K.; Matsui, S.; Matsuda, T. Aryl hydrocarbon receptor-mediated induction of microsomal drug-metabolizing enzyme activity by indirubin and indigo. *Biochem. Biophys. Res. Commun.* **2004**, *318*, 571−578.
(14) Davies, T. G.; Tunnah, P.; Meijer, L.; Marko, D.; Eisenbrand, G.; Endicott, J. A.; Noble, M. E. M. Inhibitor binding to active and inactive CDK2. The crystal structure of a CDK2-cyclin A/indirubin-5-sulphonate. *Structure* **2001**, *9*, 389−397.
(15) Mapelli, M.; Massimiliano, L.; Crovace, C.; Seeliger, M.; Tsai, L. H.; Meijer, L.; Musacchio, A. Mechanism of CDK5/p25 binding by CDK inhibitors. *J. Med. Chem.* **2005**, *48*, 671−679.
(16) Holton, S.; Merckx, A.; Burgess, D.; Doerig, C.; Noble, M.; Endicott, J. Structures of *P. falcip*arum PfPK5 test the CDK regulation paradigm and suggest mechanisms of small molecule inhibition. *Structure* **2003**, *11*, 1329−1337.
(17) Bertrand, J. A.; Thieffine, S.; Vulpetti, A.; Cristiani, C.; Valsasina, B.; Knapp, S.; Kalisz, H. M.; Flocco, M. Structural characterization of the GSK-3β active site using selective and non-selective ATP-mimetic inhibitors. *J. Mol. Biol.* **2003**, *33*, 393−407.
(18) Meijer, L.; Skaltsounis, AL.; Magiatis, P.; Polychonopoulos, P.; Knockaert, M.; Leost, M.; Ryan, XP.; Vonica, C. D.; Brivanlou, A.; Dajani, R.; Tarricone, A.; Musacchio, A.; Roe, S. M.; Pearl, L.; Greengard, P. GSK-3 selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* **2003**, *10*, 1255−1266.
(19) Polychronopoulos, P.; Magiatis, P.; Skaltsounis, L.; Myrianthopoulos, V.; Mikros, E.; Tarricone, A.; Musacchio, A.; Roe, S. M.; Pearl, L.; Leost, M.; Greengard, P.; Meijer, L. Structural basis for the synthesis of indirubins as potent and selective inhibitors of glycogen synthase kinase-3 and cyclin-dependent kinases. *J. Med. Chem.* **2004**, *47*, 935−946.
(20) Damiens, E.; Baratte, B.; Marie, D.; Eisenbrand, G.; Meijer, L. Anti-mitotic properties of indirubin-3′-monoxime, a CDK/GSK-3 inhibitor: induction of endoreplication following prophase arrest. *Oncogene* **2001**, *20*, 3786−3797.
(21) Marko, D.; Schätzle, S.; Friedel, A.; Genzlinger, A.; Zankl, H.; Meijer, L.; Eisenbrand, G. Inhibition of cyclin-dependent kinase 1 (CDK1) by indirubin derivatives in human tumor cells. *Br. J. Cancer* **2001**, *84*, 283−289.
(22) Jautelat, R.; Brumby, T.; Schafer, M.; Briem, H.; Eisenbrand, G.; Schwahn, S.; Kruger, M.; Lucking, U.; Prien, O.; Siemeister, G. From the insoluble dye indirubin towards highly active, soluble CDK2-inhibitors. *Chem. Biochem.* **2005**, *6*, 531−540.
(23) 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. Induction of apoptosis by a novel indirubin-5-3′-monoxime, a CDK inhibitor, in human lung cancer cells. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3948−3952.
(24) Moon, M. J.; Lee, S. K.; Lee, J. W.; Song, W. K.; Kim, S. W.; Kim, J. I.; Cho, C.; Choi, S. J.; Kim, Y. C. Synthesis and structure−activity relationships of novel indirubin derivatives as potent anti-proliferative agents with CDK2 inhibitory activities. *Bioorg. Med. Chem.* **2006**, *14*, 237−246.
(25) Nam, S.; Buettner, R.; Turkson, J.; Kim, D.; Cheng, J. Q.; Muehlbeyer, S.; Hippe, F.; Vatter, S.; Merz, K. H.; Eisenbrand, G.; Jove, R. Indirubin derivatives inhibit Stat3 signaling and induce apoptosis in human cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 5998−6003.
(26) Ribas, J.; Bettayeb, K.; Ferandin, Y.; Knockaert, M.; Totzke, F.; Schächtele, C.; Mester, J.; Polychronopoulos, P.; Magiatis, P.; Skaltsounis, A. L.; Boix, J.; Meijer, L. 7-Bromoindirubin-3′-oxime induces caspase-independent cell death. *Oncogene*, **2006**, in press.
(27) Caserta, T. M.; Smith, A. N.; Gultice, A. D.; Reedy, M. A.; Brown, T. L. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis* **2003**, *8*, 345−352.
(28) Knockaert, M.; Meijer, L. Identifying in vivo targets of cyclin-dependent kinase inhibitors by affinity chromatography. *Biochem. Pharmacol*. **2002**, *64*, 819.
(29) Daub, H.; Godl, K.; Brehmer, D.; Klebl, B.; Müller, G. Evaluation of kinase inhibitor selectivity by chemical proteomics. *Assay Drug Dev. Technol.* **2004**, *2*, 215−224.
(30) Meijer, L.; Borgne, A.; Mulner, O.; Chong, J. P. J.; Blow, J. J.; Inagaki, N.; Inagaki, M.; Delcros, J. G.; Moulinoux, J. P. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **1997**, *243*, 527−536.
(31) Ribas, J.; Boix, J. Cell differentiation, caspase inhibition, and macromolecular synthesis blockage, but not BCL-2 or BCL-XL proteins, protect SH-SY5Y cells from apoptosis triggered by two CDK inhibitory drugs. *Exp. Cell Res*. **2004**, *295*, 9−24.

JM060314I