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Induction of apoptosis by a novel indirubin-5-nitro-3′-monoxime, a CDK inhibitor, in human lung cancer cells

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Abstract—A novel indirubin analog, indirubin-5-nitro-3′-monoxime, inhibited cell proliferation against various human cancer cells. Additional studies indicate that the mechanism of action of this analog against human lung cancer cells might be to arrest cell cycle progression at the G_2/M phase and induce apoptosis via p53- and mitochondria-dependent pathways. These data suggest that indirubin-5-nitro-3′-monoxime might be a novel candidate for development of anticancer agents.

Cyclin-dependent kinases (CDKs) are the major molecular players with cyclins in cell cycle progression. Inhibition of CDK activity has turned out to be the most productive strategy for the discovery and design of novel anticancer agents specifically targeting the cell cycle. A large number of CDK inhibitors have been reported previously, and these drugs generally bind to the ATP pocket in the CDK catalytic site. CDK inhibitors may inhibit the growth of cancer cells by causing cell cycle arrest and apoptosis. Several representative CDK inhibitors are flavopiridol, UCN-01, olomoucine, rocosvitine, butyrolactone I, indirubin-5-sulfonate, and indirubin-3′-monoxime.[1]

Indirubin, a red-colored 3,2′-bisindole isomer, is a minor component of Qing Dai (*Indigo naturalis*), but the biological activity of Qing Dai has been attributed to indirubin, including antitumor activity.[2] Recent studies revealed that indirubin and its derivatives are potent inhibitors of CDKs.[3] Indirubin-5-sulfonate and indirubin-3′-monoxime inhibit the activity of CDK and lead to G_1 and G_2/M phase cell cycle arrest in hematopoietic Jurkat cells and SV40-transformed human breast epithelial HBL-100 cells.[4] Additional studies show that indirubin-3′-monoxime induces G_2/M phase cell cycle arrest by inhibiting CDK1 and GSK3 in HBL-100 cells and induces G_2/M phase cell cycle arrest as well as G_1 phase cell cycle arrest in MCF-7 cells.[5,6] In addition, these studies also reveal that meisoindigo induces G_1 phase cell cycle arrest and apoptosis in NB4 and U937 leukemia cells.[7] On this line, we recently designed and synthesized indirubin analogs to

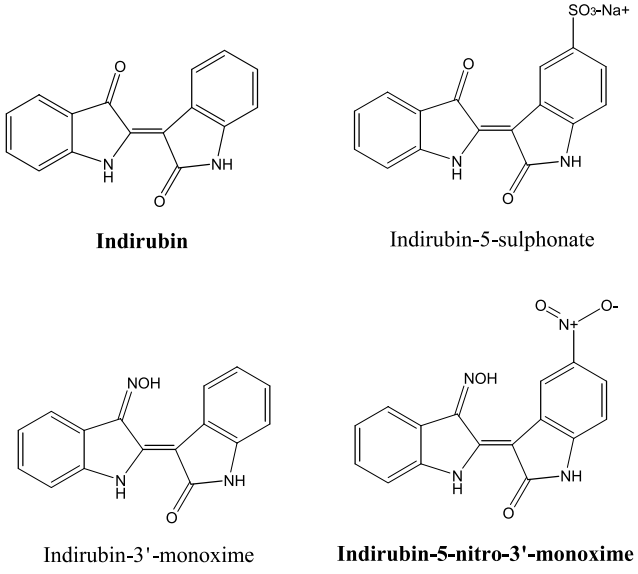

Figure 1. Chemical structure of indirubin and its derivatives.

Keywords: Indirubin-5-nitro-3′-monoxime; Apoptosis; A549 human lung cancer cells; p53; p21.
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develop potential antitumor agents (Fig. 1). We evaluated the growth-inhibitory potential of indirubin and its analogs against various human cancer cells. In addition, in this study, the mechanism of action study on the inhibitory effect of human cancer cell growth was performed with indirubin-5-nitro-3′-monoxime in human A549 lung cancer cells.

First, we evaluated inhibitory effects of indirubin and its analogs on the proliferation of various human cancer cells (A549, human lung carcinoma; Col2, human colon carcinoma; HT-1080, human fibrosarcoma; and HL-60, human myeloid leukemia) using sulforhodamine B (SRB) or MTT assays.[8] As summarized in Table 1, indirubin exhibited moderate inhibitory effects on the proliferation of A549 and HT-1080 cells. Indirubin-5-sulfonate did not inhibit the growth against tested human cancer cells. However, indirubin-5-nitro-3′-monoxime exhibited the most potent inhibitory effects against all tested human cancer cells with IC_{50} ranging from 5.4 to 25.5 μM (Table 1). In addition, this compound was most effective in A549 human lung cancer cells, which is approximately 12 times more potent than indirubin-3′-monoxime and six times than indirubin (Table 1 and Fig. 2). Therefore, prompted by the potency on A549 cells, we further investigated the growth-inhibitory mechanism of indirubin-5-nitro-3′-monoxime in A549 cells.

Because cancer cells are prone to inactivating cell cycle checkpoints and/or apoptotic progress, regulation of cell cycle progression and induction of apoptosis have been regarded as novel strategies for the control of proliferation of cancer cells. Generally, many anticancer agents, especially most CDK inhibitors, inhibited the growth of cancer cells through regulation of cell cycle progres-

Table 1. Effects of indirubin and its derivatives on cell proliferation in human cancer cells

Compounds	IC_{50}(μM)			
	A549	Co12	HT-1080	HL-60
Indirubin	31.0	>100	42.2	>100
Indirubin-5-sulfonate	>100	>100	>100	>100
Indirubin-3′-monoxime	62.0	>100	4.8	>100
Indirubin-5-nitro-3′-monoxime	5.4	25.5	5.9	9.2

Results are represented as IC_{50} values (μM).

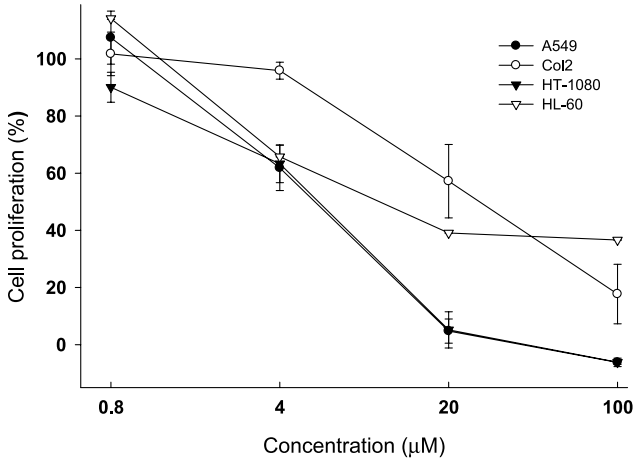

Figure 2. Inhibitory effects of indirubin-5-nitro-3′-monoxime against various human cancer cell proliferation. Human cancer cells were treated with various concentrations of indirubin-5-nitro-3′-monoxime for 72 h. The growth-inhibitory activities were evaluated using SRB or MTT assays. Data are represented as the mean ± SD ($n = 3$), and IC_{50} values were determined by nonlinear regression analysis.

Table 2. Effects of indirubin-5-nitro-3′-monoxime on cell cycle progression in A549 cells

Time (h)	Samples	Cell cycle distribution (%)			
		G_0/G_1	S	G_2/M	Sub-G_0
8	Control	59.6	24.3	16.0	0
	INM	46.1	26.8	27.1	0
16	Control	53.1	30.9	16.0	0
	INM	46.5	31.8	21.7	0
24	Control	54.2	19.7	23.2	2.9
	INM	39.9	16.1	22.0	22.0

INM: indirubin-5-nitro-3′-monoxime.

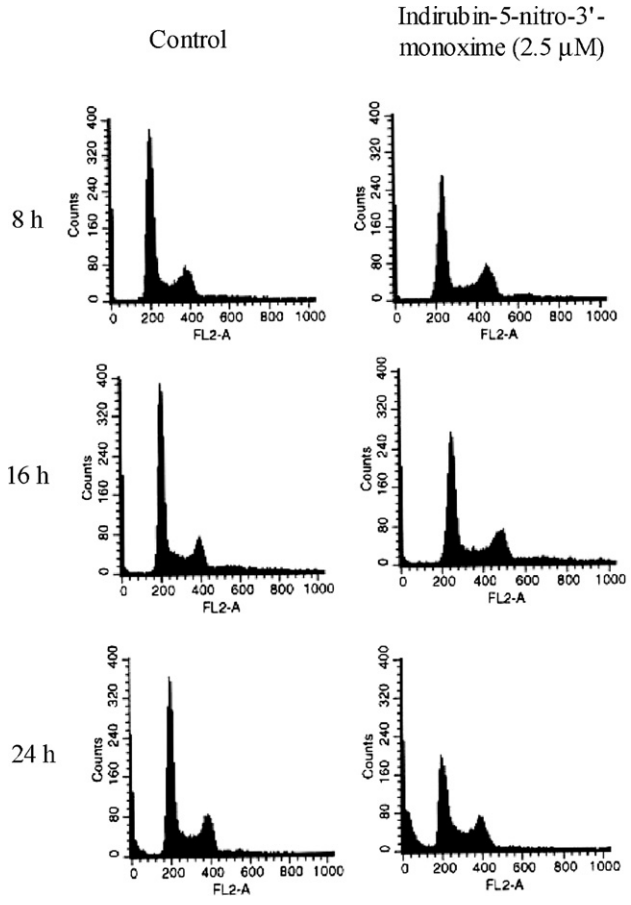

Figure 3. G_2/M phase cell cycle arrest by indirubin-5-nitro-3′-monoxime in A549 cells. Cells were treated with or without 2.5 μM indirubin-5-nitro-3′-monoxime for the indicated times. The distribution of cell cycle was analyzed by flow cytometry, and the percentage of distribution in distinct phases of the cell cycle was determined using ModFIT LT v.2.0 software.

sion.[4,9,10] Based on this line, we primarily evaluated the mechanism of growth inhibition by indirubin-5-nitro-3′-monoxime in relation to cell cycle regulation. A549 cells were incubated with 2.5 μM indirubin-5-nitro-3′-monoxime for 8, 16, and 24 h. The effect on cell cycle progression was analyzed by flow cytometry.[8] We found that, compared to control cells, cells exposed to indirubin-5-nitro-3′-monoxime slightly arrested at the G_2/M phase of the cell cycle up to 16 h, and subsequently accumulated in the sub-G_0 phase at 24 h (Table 2 and Fig. 3). In addition, when cells were treated with 2.5, 5, and 10 μM indirubin-5-nitro-3′-monoxime for 24 h, cell population in the sub-G_0 phase was drastically increased, and the percentage of cells was 22.0%, 36.8%, and 71.1% by the treatment with 2.5, 5, and 10 μM indirubin-5-nitro-3′-monoxime, respectively (Fig. 4). Because the increase of cells in the sub-G_0 phase generally indicate the increase of apoptotic cell death,[10–12] indirubin-5-nitro-3′-monoxime may induce apoptosis in A549 cells. These results suggest that indirubin-5-nitro-3′-monoxime induced the G_2/M phase cell cycle arrest and apoptosis in a time- and dose-dependent manner.

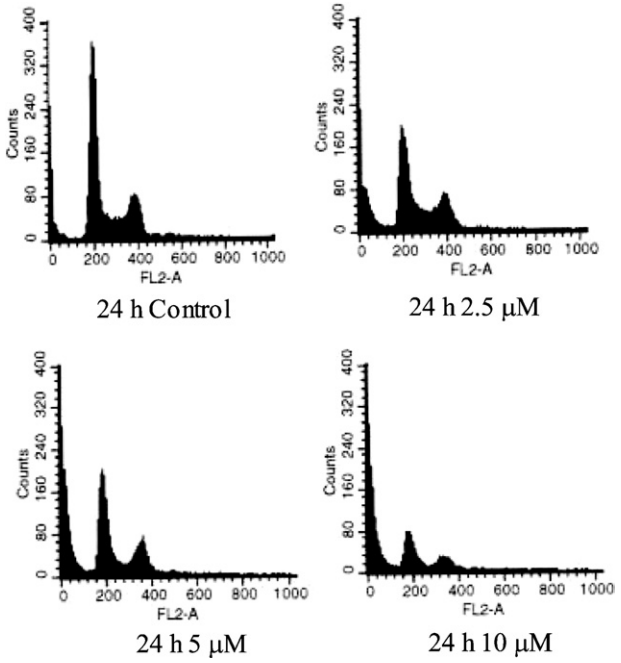

Figure 4. Increase of sub-G_0 DNA content by indirubin-5-nitro-3′-monoxime in A549 cells. Cells were treated with various concentrations of indirubin-5-nitro-3′-monoxime for 24 h. Cell cycle analysis was performed as described in the text.

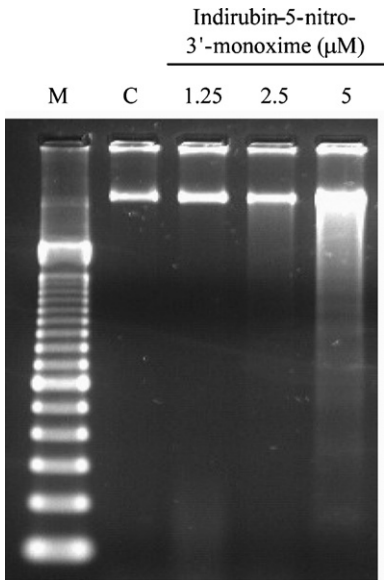

Figure 6. Induction of DNA fragmentation by indirubin-5-nitro-3′-monoxime in A549 cells. Cells were treated with various concentrations of indirubin-5-nitro-3′-monoxime for 24 h. Extracted DNA was separated by agarose gel electrophoresis. (M indicates 100-bp DNA ladder, and C indicates control cells.).

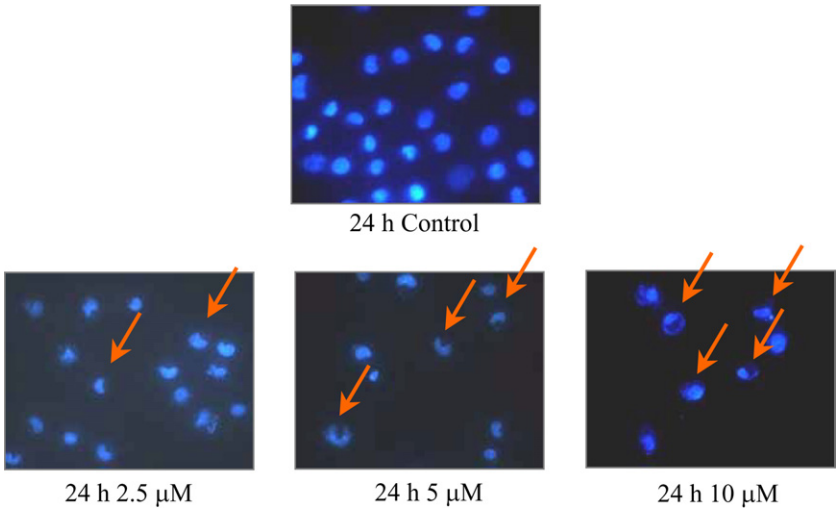

Figure 5. The increase of chromatin condensation by indirubin-5-nitro-3′-monoxime in A549 cells. Cells were treated with the indicated concentrations of test compound for 24 h. After treatment, the nuclei were stained with Hoechst 33258 fluorescent dye and observed under the fluorescence microscope (×100 magnification). Red arrows indicate chromatin condensation.

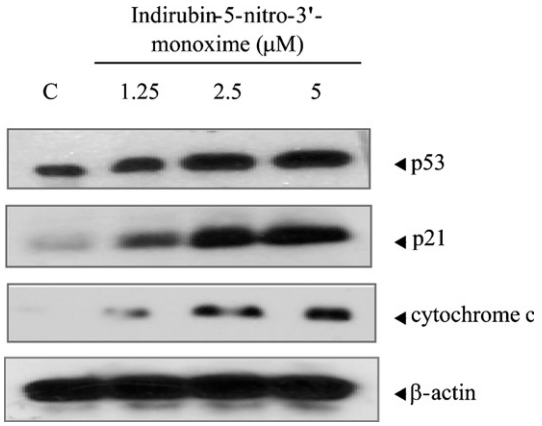

Figure 7. Effect of indirubin-5-nitro-3′-monoxime on the expression of the indicated proteins in A549 human lung cancer cells. Cells were exposed to various concentrations of indirubin-5-nitro-3′-monoxime for 24 h. The expression level was analyzed by Western blot analysis.

It is known that various characteristic responses occur during apoptosis, including loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation.[13] To confirm whether the induction of cell death by indirubin-5-nitro-3′-monoxime is related to apoptosis, we next examined cellular changes such as chromatin condensation and DNA fragmentation by indirubin-5-nitro-3′-monoxime using Hoechst 33258 staining method and agarose gel electrophoresis, respectively.[14,15] A549 cells were treated with various concentrations of indirubin-5-nitro-3′-monoxime for 24 h. As the concentration of indirubin-5-nitro-3′-monoxime was increased, the cell population of which chromatin was condensed in the nucleus was also gradually increased (Fig. 5). In addition, as illustrated in Figure 6, DNA fragmentation was observed at 5 μM of indirubin-5-nitro-3′-monoxime, the concentration of which about 40% of cells were accumulated in the sub-G_0 phase. Therefore, it is suggested that cell death induced by indirubin-5-nitro-3′-monoxime was closely related to apoptosis, which was revealed by the increase of chromatin condensation and DNA fragmentation, two hallmarks of apoptotic phenomena.[16]

Finally, to investigate the effect of indirubin-5-nitro-3′-monoxime on the expression of cell cycle and apoptosis-related proteins, Western blot analysis was performed.[8] Treatment of indirubin-5-nitro-3′-monoxime for 24 h was markedly induced p53 and p21 expression in a concentration-dependent manner (Fig. 7). p53 mediates cell cycle arrest and apoptosis by direct induction of various CDK inhibitors and apoptosis inducers, for example, CDK inhibitor p21.[17] In addition, several reports have suggested that p53-dependent p21 expression afforded cell cycle arrest and apoptosis.[18–20] Therefore, upregulation of p53 and p21 expression might contribute to cell cycle arrest and apoptosis mediated by indirubin-5-nitro-3′-monoxime. Moreover, the level of cytochrome *c*, which is released from mitochondria and mediates intrinsic apoptotic cascades, was also increased in a dose-dependent manner (Fig. 7), indicating apoptosis by indirubin-5-nitro-3′-monoxime might occur through mitochondria-mediated pathway as demonstrated in several reports published previously.[21]

In summary, the present study demonstrates that a novel CDK inhibitor, indirubin-5-nitro-3′-monoxime, inhibited cell proliferation in human lung cancer cells A549, and its mechanism might be to arrest cell cycle progression at G_2/M phase and induce apoptosis via p53- and mitochondria-dependent pathways. It suggests that indirubin-5-nitro-3′-monoxime might be a potential candidate for the development of anticancer agents.

Acknowledgments

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14. Hoechst staining: A549 cells (1×10^6 cells in a 100 mm dish) were seeded for 24 h and then incubated with test samples for additional 24 h. Floating and attached cells were combined, washed with ice-cold PBS, and then fixed in a 4% paraformaldehyde solution at 4 °C for 10 min. Fixed cells were placed on slides and stained with Hoechst 33258 (0.3 μg/ml) at room temperature for 10 min in the dark. Stained cells were mounted with 80% glycerol, and then visualized using fluorescence microscope.
15. DNA fragmentation: A549 cells were treated with test samples for 24 h. After incubation, floating and attached cells were combined by centrifugation and trypsinization. The combined cells were washed with ice-cold PBS and lysed in 100 μl of DNA isolation buffer [50 mM Tris–HCl (pH 7.5), 20 mM EDTA, 1% NP-40]. After centrifugation, 10 μl of 10% SDS solution (final 1%) and 10 μl of 50 mg/ml RNase A (final 5 μg/ml) were added to the supernatants, and incubated at 56 °C for 1 h. Subsequently, proteinase K (5 μg/ml) was added and then incubated at 37 °C for 2 h. DNA was precipitated with 0.5 volume of 10 M ammonium acetate and 2.5 volume of cold ethanol at −70 °C overnight. Precipitated DNA was dissolved in 50 μl of Tris–EDTA buffer (pH 8.0). The same concentration of DNA samples (5 μg) were resolved by electrophoresis in 2% agarose gel at 60 V for 2 h, stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) for 1 h in the dark, and visualized under the UV transilluminator.
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