Antitumor Activity of Novel Indirubin Derivatives in Rat Tumor Model

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Abstract

Purpose: The novel indirubin derivatives 5′-nitro-indirubinoxime, 5′-fluoro-indirubinoxime, and 5′-trimethylacetamino-indirubinoxime were designed and tested for antitumor activity both in vitro and in vivo using rat tumor model.

Experimental Design: Three-week-old male Sprague-Dawley rats were inoculated s.c. on the left flank with 107 RK3E-ras rat kidney epithelial cells harboring k-ras gene. Alternatively, 5 × 106 RK3E-ras cells were injected into the oral mucosa. Indirubin derivative treatment began on the 3rd or 6th day after oral or s.c. cell injection, respectively. Indirubin derivatives were directly injected into the tumor every other day for a total of five times. Animals were monitored daily and tumor volume was measured by caliper.

Results: Indirubin derivatives showed potent antiproliferative activity on various human cancer cells and oncogenic RK3E-ras rat kidney cells, with IC50 ranging from 1 to 12 μmol/L. Treatment with indirubin derivatives induced the activation of caspase-7 followed by apoptosis in RK3E-ras cells. Indirubin derivatives showed strong antitumor activity in rat solid and oral tumor models. Direct injection of indirubin derivatives every other day for 10 days induced significant inhibition of tumor growth in Sprague-Dawley rats bearing RK3E-ras-induced tumors. Histologically, treatment with indirubin derivatives caused significant inhibition of tumor formation with increased apoptosis and decreased tumor cell proliferation.

Conclusions: Our data showed that novel indirubin derivatives 5′-nitro-indirubinoxime, 5′-fluoro-indirubinoxime, and 5′-trimethylacetamino-indirubinoxime effectively arrested the tumor growth by inhibiting cell proliferation and inducing apoptosis. These findings provide the potential value of indirubin derivatives as novel candidates for antitumor agents.

Indirubin is an active component of Danggui Longhui Wan, a mixture of 11 herbal medicines that is used to treat chronic diseases, such as chronic myelogenous leukemia, in traditional Chinese medicine (1, 2). Clinical trials showed that indirubin has a definite efficiency against chronic myelogenous leukemia (1, 3). Over the past 20 years, numerous efforts were devoted to design and synthesize the indirubin derivatives to improve its pharmacologic effect because indirubin has poor solubility, has poor absorption, and causes irritation of the gastrointestinal tract.

Recent studies have revealed that indirubin and its derivatives are potent inhibitors of cyclin-dependent kinases (CDK-1, CDK-2, CDK-4, and CDK-5) and block the cell proliferation by arresting the cell cycle (4–6). The cell cycle of tumor cells treated with indirubin is arrested in the G1-S or G2-M phases, resulting in inhibition of cell proliferation followed by apoptosis (4, 6). The structure-activity studies suggested that indirubin has a strong affinity for CDKs and acts by competing with ATP for binding to the catalytic site of CDKs (6, 7). Furthermore, it has been reported that indirubin derivatives induce the apoptosis in several human cancer cells, such as lung, breast, and prostate cancer cells, suggesting a potential for application against human cancers (8–12).

Up to now, indirubin-3′-monoxime was the most potent indirubin derivative. Indirubin-3′-monoxime inhibits the tumor cell proliferation by arresting the cell cycle in the late G1 and G2-M phases and triggers apoptosis (11–13). Indirubin-3′-monoxime also blocks the abnormal hyperphosphorylation of τ that induces the formation of the typical neurofibrillary tangles in Alzheimer’s disease (14). Additionally, it has been reported that indirubin-3′-monoxime is a powerful inhibitor of c-Jun NH2-terminal kinase, an important regulator of neuronal cell death, and glycogen synthase kinase-3β, which is involved in neurodegenerative disorders (11, 12). Another indirubin derivative, meisoindigo, strongly inhibits DNA biosynthesis and microtubule assembly in tumor cells (15). Meisoindigo

References


also induces the leukemic cell differentiation by decreasing the c-myc expression (16). Previously, we reported that a novel indirubin derivative, 5'-nitro-indirubinoxime (5'-NIO), is a potent inhibitor of CDK-2 and induces apoptosis in human lung cancer cells (8). However, the precise mechanisms of action are not fully understood and preclinical trials are needed to approach the treatment of cancer. In an effort to develop the potential antimutant effect of indirubin, we designed and synthesized novel indirubin derivatives, 5'-fluoro-indirubinoxime (5'-FIO) and 5'-trimethylacetamino-indirubinoxime (5'-TAIO), along with 5'-NIO. These new indirubin derivatives significantly blocked the cancer cell proliferation in vitro. We also showed that these agents powerfully inhibited the tumor growth using our rat animal model.

Materials and Methods

Preparation of indirubin derivatives. The appropriate indirubin was dissolved in pyridine. With magnetic stirring, hydroxylamine hydroxide (1H NMR (300 MHz, DMSO-d6)) expression (16). c-myc also induces the leukemic cell differentiation by decreasing the CDK-2 and induces apoptosis in human lung cancer cells (8). However, the precise mechanisms of action are not fully understood and preclinical trials are needed to approach the treatment of cancer. In an effort to develop the potential antimutant effect of indirubin, we designed and synthesized novel indirubin derivatives, 5'-fluoro-indirubinoxime (5'-FIO) and 5'-trimethylacetamino-indirubinoxime (5'-TAIO), along with 5'-NIO. These new indirubin derivatives significantly blocked the cancer cell proliferation in vitro. We also showed that these agents powerfully inhibited the tumor growth using our rat animal model.

Fig. 1. Chemical structure of indirubin derivatives. The R-group on the indirubin structure was changed to nitro- (5'-nitro-indirubinoxime), fluoro- (5'-fluoro-indirubinoxime), or NHCOBu (-trimethylacetamino-indirubinoxime) group, respectively.

5'-NIO: -NO2 (5'-nitro-1H,1H-[2,3]biindolylidine-3,2'-dione 3-oxime)1H NMR (300 MHz, DMSO-d6) (ppm) 13.92 (1H, s, NOH) 11.90 (1H, s, N-H), 11.44 (1H, s, N-H), 9.47 (1H, s), 8.27 (1H, d, J = 7.5 Hz), 8.10 (1H, d, J = 2.3, 8.4 Hz), 7.48 (2H, m), 7.09 (2H, m), MS (MALDI-TOF) m/z: 321.0.

5'-FIO: -F (5'-fluoro-1H,1H-[2,3]biindolylidine-3,2'-dione 3-oxime)1H NMR (300 MHz, DMSO-d6) (ppm) 13.66 (1H, s, NOH) 11.80 (1H, s, N-H), 10.75 (1H, s, N-H), 8.48 (1H, d, J = 2.6, 11.3 Hz), 8.23 (1H, d, J = 7.5 Hz), 7.43 (2H, m), 7.00 (3H, m), MS (MALDI-TOF) m/z: 295.2.

5'-TAIO: -NHCOBu (-3-hydroxyimino-2'-oxo-1,3,1'-tetrahydro [2,3]biindolylidenyl-5'-yl)-2,2'-dimethyl-propionamide) NMR 300 MHz, DMSO-d6: (ppm) 13.40 (1H, s, NOH) 11.73 (1H, s, N-H), 10.67 (1H, s, N-H), 8.89 (1H, s, amide-NH), 8.46 (1H, s), 8.23 (1H, d, J = 7.5 Hz), 7.40 (2H, m), 7.24 (1H, d, J = 8.4 Hz), 7.03 (1H, m), 6.81 (1H, d, J = 8.4 Hz). 1.26 (9H, s, (CH3)3), MS (MALDI-TOF) m/z: 376.

Cell culture. Three human cancer cell lines (A549, lung carcinoma; SN5-538, stomach carcinoma; and HT-1080, fibrosarcoma) were maintained at 37 °C with 5% CO2 in RPMI 1640 containing 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. The rat kidney epithelial cell line RK3E and the k-ras–transformed RK3E cell line (RK3E-ras) were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. RK3E-ras cells were kindly provided by Dr. Eric Fearon (University of Michigan Medical School, Ann Arbor, MI) and have been described previously (17).

3-(4,5-dimethoxyazol-2-y1)-2,5-diphenylenetrazolium bromide assay. The antiproliferative effects of indirubin derivatives on various cancer cells were determined by the 3-(4,5-dimethoxyazol-2-y1)-2,5-diphenylenetrazolium bromide dye uptake method as described earlier (18). Briefly, cells were seeded on 24-well plates and treated with indirubin derivatives for 24 h. Each well was washed twice with PBS, and 0.25 ml of cell culture medium and 25 µl of 3-(4,5-dimethoxyazol-2-y1)-2,5-diphenylenetrazolium bromide solution (5 mg/ml in PBS) were added. After 3 h of incubation, medium was removed and 125 µl of acid-isopropanol (0.04 mol/L HCl in isopropanol) were added. The absorbance was measured at 570 nm using the Microplate Autoreader EL 311 (Bio-Tek Instruments, Inc., Winooski, VT).

Apoptosis measurement. RK3E-ras cells were treated with 10 µmol/L of indirubin derivatives for 24 h. The cells were labeled with Annexin

V-FITC and propidium iodide using the Annexin-V-FLUOS staining kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Apoptotic cells were visualized using a Nikon (Tokyo, Japan) Eclipse E800 automated fluorescent microscope equipped with a digital camera. For flow cytometric analysis, cells were measured with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using CellQuest software. Alternatively, apoptosis was measured using a cell death detection ELISA kit (Roche Molecular Biochemicals). Relative apoptosis that correlates with absorption at 405 nm with a reference wavelength of 490 nm was measured according to the manufacturer's instructions.

Immunoblotting. RK3E-ras cells were treated with indirubin derivatives for 24 h. Cells were washed twice with cold PBS and lysed in radioimmunoprecipitation assay buffer (PBS supplemented with 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 mmol/L sodium orthovanadate). The cell lysates were harvested and incubated at 4°C for 30 min and cleared by centrifugation at 10,000 x g for 10 min. For immunoblotting, proteins (10 µg/lane) were resolved by 12% SDS-PAGE and immunoblotted with anti-caspase-7 antibody (Cell Signaling Technology, Beverly, MA), anti-cleaved caspase-7 antibody (Cell Signaling Technology), anti-proliferating cell nuclear antigen (PCNA) antibody (Dako, Glostrup, Denmark), or anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of caspase-3 activity. RK3E-ras cells were treated with indirubin derivatives for 24 h, and the activity of caspase-3 was measured using ApoAlert Caspase Colorimetric Assay kit according to the manufacturer's instructions (Clontech Laboratories, Mountain View, CA). The effects of caspase inhibitor were examined by treating the cells with VAD-fmk (5 µmol/L) 3 h before the treatment of indirubin derivatives.

Construction of viral vector and generation of stable RK3E-ras-Fluc cell line. cDNAs for luciferase and green fluorescent protein were inserted into the p lentilM1.3 retroviral vector (VectorCore®, Daejeon, South Korea). Cloned pLentiM1.3-Fluc vector allows the expression of chimeric transcripts encoding luciferase fused to the green fluorescent protein.
protein gene. Pseudotyped lentiviruses were produced as described earlier (19). Briefly, three plasmids, including the transfer vector, VSV-G expression vector, and gag-pol expression vector, were cotransfected into 293T cells at a 1:1:1 molar ratio by using Lipofectamine Plus (Invitrogen, Carlsbad, CA). The culture supernatant containing viral vector particles was harvested 48 h later and clarified with a 0.45-μm membrane filter (Nalgene, Rochester, NY).

For generation of stable RK3E-ras-Fluc cell line, RK3E-ras cells were infected with supernatant containing viral vector particles in the presence of 4 μg/mL polybrene (Sigma, St. Louis, MO). The medium was replaced 24 h after infection. Forty-eight hours after infection, RK3E-ras cells were subjected to selection with hygromycin (200 μg/mL; Invitrogen). The isolated hygromycin-resistant clones were transferred to a 24-well plate. Before confluence, the cells were transferred into larger culture vessels and the expression of the green fluorescent protein gene was confirmed by immunoblot analysis.

In vivo tumor growth and measurement. Three-week-old male Sprague-Dawley rats (Samtaco, Osan, South Korea) were kept under standard housing conditions. RK3E-ras or RK3E-ras-Fluc cells were harvested by trypsinization and centrifuged and resuspended in DMEM containing 10% FCS and propidium iodide.

For bioluminescence imaging, rats received i.p. injection of luciferin (Molecular Probes, Palo Alto, CA) at a dose of 80 mg/kg body weight with xylazine/ketamine anesthesia. Rats were imaged with the Xenogen IVIS Imaging System (Xenogen Co., Alameda, CA) to record the bioluminescent signal emitted from the tumor. The IVIS-100 equipped with CCD camera system was used for emitted light acquisition, and Living Image software (Xenogen) was used for data analysis.

**Histopathology, immunohistochemistry, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay.** Excised solid tumors were fixed in 10% buffered formalin and embedded in paraffin. For light microscopic examination, 4-μm sectioned tissues were stained with H&E. Immunohistochemical staining was done with the avidin-biotin complex method using anti-PCNA antibody. Immune reactions were visualized with 3,3′-diaminobenzidine and counterstained with Mayer’s hematoxylin. The PCNA-positive cells were counted and represented as the average of the five highest areas within a single ×200 field.

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done using an ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Intergen, Purchase, NY) according to the manufacturer’s instructions. Briefly, slides were deparaffinized and treated with 20 μg/mL protease K at 37°C for 15 min to enhance the staining. After immersion in 3% hydrogen peroxide to block the endogenous peroxidase, slides were incubated with reaction buffer containing terminal deoxynucleotidyl transferase at 37°C for 1 h. Slides were then incubated with peroxidase-conjugated anti-digoxigenin antibody for 30 min, and the reaction products were visualized with 0.05% 3,3′-diaminobenzidine solution containing 2 mmol/L hydrogen peroxide. Counterstaining was achieved with 0.5% methyl green.

![Fig. 2. Indirubin derivatives induce the apoptosis in RK3E-ras cells. RK3E-ras cells were treated with 10 μmol/L of indirubin derivatives for 24 h. A. apoptosis was analyzed by the flow cytometric method by staining the cells with Annexin-V-FLUOS and propidium iodide. B. cell death was also detected by fluorescence microscopy by staining the cells with Annexin-V-FLUOS and propidium iodide. C. total cell lysates were prepared and proteins (10 μg/lane) were resolved by 12% SDS-PAGE. The expression level of caspase-7, cleaved caspase-7, PCNA, and actin was detected by Western blot analysis. D. caspase-3 activity was measured in RK3E-ras cells treated with indirubin derivatives for 24 h. Cells were lysed and caspase-3 activity was measured as described in Materials and Methods.](image)
TUNEL-positive cells were counted and represented as the average of the five highest areas within a single \( \times 200 \) field.

Statistical analysis. The differences in mean values among different groups were tested, and the values were expressed as mean \( \pm \) SD. All of the statistical calculations were carried out using Microsoft Excel.

**Results and Discussion**

Indirubin derivatives inhibit the proliferation of cancer cells. We designed and synthesized three novel indirubin derivatives (5’-NIO, 5’-FIO, and 5’-TAIO) to develop the potential antitumor agent (Fig. 1). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done to evaluate the effects of these indirubin derivatives on the proliferation of various human cancer cells (A549, lung carcinoma; SNU-638, stomach carcinoma; and HT-1080, fibrosarcoma) and rat kidney epithelial cells harboring k-ras gene (RK3E-ras). The IC\(_{50}\) values for each of the indirubin derivatives are shown in Table 1. All of the tested indirubin derivatives showed a more potent growth-inhibitory effect than indirubin and indirubin-3’-monoxime against human cancer cells with IC\(_{50}\) ranging from 1.2 to 12.2 \( \mu \)mol/L. RK3E-ras cells were also very susceptible to these compounds with IC\(_{50}\) ranging from 1.0 to 6.4 \( \mu \)mol/L. Notably, 5’-NIO showed the most potent growth-inhibitory effect in RK3E-ras cells.

Indirubin derivatives induce the apoptosis in RK3E-ras cells. We next examined whether the growth-inhibitory effect of indirubin derivatives was caused by apoptosis using the Annexin-V-FLUOS staining kit. RK3E-ras cells were treated with 10 \( \mu \)mol/L of indirubin derivatives for 24 h, and flow cytometric analysis was done to obtain quantitative results. As shown in Fig. 2A, the number of apoptotic cells was increased after treatment with indirubin derivatives. Annexin-V-FLUOS analysis also revealed an elevated number of apoptotic cells in

Fig. 3. Effect of caspase inhibitor on apoptosis induced by indirubin derivatives. A, RK3E-ras cells were treated with indirubin derivatives for 24 h. To examine the effect of caspase inhibitor, VAD-fmk (5 \( \mu \)mol/L) was treated 3 h before the treatment of indirubin derivatives. Relative apoptosis of cells was determined as described in Materials and Methods and plotted on the Y-axis as the relative apoptosis index. B, total cell lysates were prepared and proteins (10 \( \mu \)g/lane) were resolved by 12% SDS-PAGE. The expression level of caspase-7 and cleaved caspase-7 was detected by Western blot analysis.

Fig. 4. Antitumor effect of indirubin derivatives in rat tumor model. RK3E-ras cells (1 \( \times \) 10\(^7\)/rat) were injected i.c. into the left flank of Sprague-Dawley rats. Indirubin derivatives (10 \( \mu \)mol/L/100 \( \mu \)L) were directly injected into the tumor every other day beginning on day 6. A, serial tumor volumes were measured by caliper and calculated using the formula \( V = (ab^2)/2 \), in which \( a \) is the largest diameter and \( b \) is the shortest diameter of the tumor. Columns, mean; bars, SD. B, Softex X-ray imaging was obtained on day 15.

**Fig. 3.** Effect of caspase inhibitor on apoptosis induced by indirubin derivatives. A, RK3E-ras cells were treated with indirubin derivatives for 24 h. To examine the effect of caspase inhibitor, VAD-fmk (5 \( \mu \)mol/L) was treated 3 h before the treatment of indirubin derivatives. Relative apoptosis of cells was determined as described in Materials and Methods and plotted on the Y-axis as the relative apoptosis index. B, total cell lysates were prepared and proteins (10 \( \mu \)g/lane) were resolved by 12% SDS-PAGE. The expression level of caspase-7 and cleaved caspase-7 was detected by Western blot analysis.

**Fig. 4.** Antitumor effect of indirubin derivatives in rat tumor model. RK3E-ras cells (1 \( \times \) 10\(^7\)/rat) were injected i.c. into the left flank of Sprague-Dawley rats. Indirubin derivatives (10 \( \mu \)mol/L/100 \( \mu \)L) were directly injected into the tumor every other day beginning on day 6. A, serial tumor volumes were measured by caliper and calculated using the formula \( V = (ab^2)/2 \), in which \( a \) is the largest diameter and \( b \) is the shortest diameter of the tumor. Columns, mean; bars, SD. B, Softex X-ray imaging was obtained on day 15.
indirubin derivative–treated RK3E-ras cells compared with untreated control cells (Fig. 2B).

The level of caspase-7 and its cleaved form was examined by Western blot analysis because caspase-7 is an effector caspase of apoptotic cell death. Treatment with indirubin derivatives induced a dose-dependent increase of cleaved caspase-7 in RK3E-ras cells (Fig. 2C, top). Especially, 5'-NIO most strongly induced the activation of caspase-7. On the other hand, the expression level of PCNA, a marker for cell proliferation, was decreased by treatment with indirubin derivatives in a dose-dependent manner (Fig. 2C, middle). We also examined the caspase-3 activity using colorimetric assay. As shown in Fig. 2D, caspase-3 activity was increased in indirubin derivative–treated RK3E-ras cells depending on the concentration. Consistent with caspase-7 activation, 5'-NIO most strongly induced the activation of caspase-3.

Effect of pan-caspase inhibitor VAD-fmk in the indirubin derivative–induced apoptosis was examined. For the experiment, VAD-fmk was added 3 h before the treatment of indirubin derivatives. As shown in Fig. 3A, apoptotic cells were decreased by pretreatment with VAD-fmk, confirming that indirubin derivatives induced apoptotic mechanism. Additionally, we also showed that VAD-fmk completely inhibits the formation of cleaved caspase-7. These data suggest that indirubin derivatives 5'-NIO, 5'-FIO, and 5'-TAIO strongly inhibited cell proliferation and induced the apoptosis through the caspase-3 and caspase-7 activation.

**Indirubin derivatives inhibit the growth of solid tumors in vivo.** Recently, we developed a tumor animal model using RK3E-ras cells in Sprague-Dawley rats (21). This tumor animal model has the advantage of allowing short-term screening of antitumor agents. To examine the effect of indirubin derivatives on tumor growth, 20 Sprague-Dawley rats were injected with RK3E-ras cells in the left flank as described in Materials and Methods. Tumor masses were observed as early as 3 days after injection of RK3E-ras cells. Six days after cell injection, indirubin derivatives (10 μmol/L/100 μL) were directly injected into the solid tumor every other day for a total of five times (n = 5 for each group). Tumor growth was monitored every 3 days using a caliper. As shown in Fig. 4A, all of the tested indirubin derivatives effectively inhibited the growth of solid tumors in Sprague-Dawley rats. Especially, 5'-NIO showed the most potent growth-inhibitory effect in the RK3E-ras cell–induced tumor animal model. Treatment with 5'-NIO for 10 days (15 days after cell inoculation) completely blocked the tumor growth. 5'-FIO and 5'-TAIO also effectively inhibited tumor growth (Fig. 4A). We also tested the effect of indirubin and indirubin-3'-monoxime for the growth of solid tumor. Consistent with the cell proliferation assay data in Table 1, indirubin did not show any effect on tumor growth, whereas indirubin-3'-monoxime effectively inhibited the growth of solid tumor (data not shown). Softex X-ray images were obtained at 10 days after treatment with indirubin derivatives. As shown in Fig. 4B, Softex X-ray images showed arrested tumor size in the indirubin derivative–treated rats. These data show that indirubin derivatives effectively inhibited tumor growth in our rat animal model and are consistent with in vitro data, indicating that 5'-NIO has the most potent antitumor activity among the three tested indirubin derivatives. We did not give indirubin derivatives through orally or i.v. The local administration of the agent has an advantage of increased drug efficacy to the vicinity of tumors and allows for a longer duration of exposure of tumor cells to the drug, which also facilitates the binding and uptake of the drug by tumor cells (22). Indirubin derivatives showed a
strong growth-inhibitory effect on the tumor cells by direct injection. Moreover, treatment with indirubin derivatives did not lead to any side effects.

**Indirubin derivatives inhibit the growth of oral tumors in vivo.** We also developed an animal model for oral tumors using RK3E-ras cells. Ten Sprague-Dawley rats were injected with RK3E-ras cells on the oral mucosa as described in Materials and Methods. Three days after administration, the formation of an oral tumor could be observed. To examine the effect of indirubin derivatives on the progression of oral tumors, agents were directly injected into the oral tumor (10 μmol/L/100 μL) beginning 3 days after RK3E-ras cell injection (n = 5 for each group). Indirubin derivatives were given every other day for a total of five times. Ten days after treatment (12 days after cell inoculation), Softex X-ray images were obtained. Among the three indirubin derivatives, 5'-NIO profoundly inhibited the growth of oral tumors (Fig. 5A). Treatment with 5'-FIO or 5'-TAIO was also effective in reducing the tumor growth (Fig. 5A). Twelve days after cell injection, tumors were also detected in the bone, lymph nodes, and salivary glands in the indirubin untreated control group (data not shown).

For the purpose of analyzing the effect of indirubin derivatives in living rats, we also developed an optical reporter system using the pLentiM1.3-Fluc vector and obtained in vivo bioluminescence imaging. Like RK3E-ras cells, RK3E-ras-Fluc cells induced the formation of oral tumors as early as 3 days after cell injection (n = 5). On day 12, oral tumor-bearing rats were peritoneally injected with luciferin and bioluminescence imaging was obtained (n = 5). As shown in Fig. 5B, strong luminescence imaging could be obtained in luciferin-treated rats. However, 5'-NIO-treated rats did not show the luminescence imaging, showing that 5'-NIO strongly inhibits the oral tumor growth (Fig. 5C). These results showed that indirubin derivatives effectively inhibit the oral tumor growth. RK3E-ras cell–induced tumor animal models were highly effective for the screening of antitumor agents. Furthermore, bioluminescence imaging obtained from RK3E-ras-Fluc cell–induced tumors was very simple and rapid for assessing tumor growth and the antitumor effect of agents in living animals.

**Indirubin derivatives induce the apoptosis and decrease the proliferative activity of tumor cells in vivo.** Histologically, solid and oral tumors were anaplastic undifferentiated carcinoma showing many mitotic figures, multifocal necrosis, and hemorrhage (Fig. 6A, top). However, treatment with indirubin derivatives led to extensive cell death surrounded by granulomatous inflammation with calcification. Additionally, indirubin derivative–treated sections showed extensive loss of tumor cells, with some necrosis showing nuclear pyknosis and cytoplasmic eosinophilia (Fig. 6A, top).

Immunohistochemistry for PCNA showed that almost all of the tumor cells in the DMSO-treated control sections were positive, suggesting a high proliferative rate. In contrast, treatment with indirubin derivatives significantly decreased the amount of PCNA-positive cells (Figs. 6A, middle and 6B). Compared with untreated control, PCNA-positive cells were only 17%, 15%, and 11% for 5'-NIO, 5'-FIO, and 5'-TAIO, respectively.

A TUNEL assay was done to assess the amount of the apoptotic cells in vivo. As expected, the proportion of TUNEL-positive cells were strongly increased with indirubin derivative treatment compared with untreated control (Figs. 6A, bottom and 6C). Treatment with 5'-NIO, 5'-FIO, and 5'-TAIO induced the amount of TUNEL-positive cells for 11.1-, 7.1-, and 6.7-fold, respectively. These results confirm in vitro data that our
newly synthesized indirubin derivatives strongly inhibited the growth of RK3E-ras cell–derived tumors by inhibiting cell proliferation and activating apoptosis.

5'-NIO showed the most potent antitumor activity in RK3E-ras cell using in vitro and in vivo studies. However, we cannot rule out the possibility that 5'-FIO and 5'-TAIO may be a more effective antitumor agent than 5'-NIO in other cell types because the IC50 value was varied depending on the cell type.

In this study, we showed that novel indirubin derivatives 5'-NIO, 5'-FIO, and 5'-TAIO effectively arrested the tumor growth by inhibiting cell proliferation and inducing apoptosis. Indirubin induces many biochemical changes in tumor cells. Importantly, indirubin and its derivatives play a key role in the regulation of CDKs and lead to the inhibition of tumor cell growth (4–6). We showed that indirubin derivatives induced the apoptosis through the activation of caspase-3 and caspase-7. The mechanism of apoptosis induced by indirubin derivatives is not completely understood yet. Further biochemical and cellular studies will reveal the precise molecular mechanism of apoptosis induced by indirubin derivatives.

Acknowledgments

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References

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