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Synthesis of methoxy- and bromo-substituted indirubins and their activities on apoptosis induction in human neuroblastoma cells

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ABSTRACT

This paper reports the synthesis of methoxy- and bromo-indirubins, and their antiproliferative activities in human neuroblastoma. Among 20 compounds, 5′-methoxyindirubin induced cell death in human neuroblastoma cells (IMR-32, SK-N-SH and NB-39) without inhibiting normal cells (NHDF and HUVEC). Typical morphologic features of apoptosis were observed in 5′-methoxyindirubin-treated cells by Hoechst 33342 staining. Additional studies by flow cytometry support apoptosis induction. These data suggest that 5′-methoxyindirubin might be an effective drug for treatment of neuroblastoma.

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Neuroblastoma is the most common extracranial solid tumor in children and is derived from cells of the sympathetic nervous system.[1,2] Approximately 40% of patients with neuroblastoma are infants, and the incidence decreases with age. Most patients diagnosed with neuroblastoma are less than 10 years old. Its prognosis varies according to patient age at diagnosis. In patients more than 1 year old, the tumor is very aggressive and drug-resistant.[3] The 5-year survival rate of patients with such advanced tumors is only 25−30%, despite aggressive chemotherapy.[4] However, some pseudo-benign neuroblastomas, especially occurring in infants and children less than 1 year old, are known to regress spontaneously or to mature even if metastases to bone marrow, skin and/or liver (stage IVs) are present. Mechanisms of spontaneous regression still remain unclear, but delayed apoptosis or differentiation are suggested.[5] It is well established that apoptosis is an important mechanism in the normal development of nervous systems. However, neuroblastoma is derived from neural crest cells when apoptotic systems do not function properly. It is further suggested that resistance to apoptosis plays a contributory role in the mechanism of aggressive behavior shown by advanced neuroblastoma.[6] Accordingly, compounds exhibiting apoptosis-inducing activity on neuroblastoma cells specifically may contribute to a cure for advanced neuroblastoma.

Recently apoptosis-inducing activity of indirubin 3′-oxime has been revealed with human cell lines such as A498, CAKI-1 and CAKI-2 (renal cell cancer),[7] HeLa cell (cervical cancer),[8] HepG2 cell (hepatoma),[8] HCT116 cell (colon cancer),[8] and Hep-2 cell (laryngeal carcinoma).[9] Induction of caspase-independent cell death, possibly via necrosis, by its 7-bromo derivative has also been reported.[10] Since available indirubin derivatives have been limited, systematic screening to find lead compounds for novel cancer chemotherapy drugs has also been limited. In the present study, we aimed to identify compounds with potential to induce apoptosis in neuroblastoma cells, but not in normal cells, by systematic screening.

For systematic screening, we prepared indirubins with two different types of substituents on the aromatic rings (Fig. 1). One was a methoxy group, a typical electron-donating group, and another was bromine, a typical electron-withdrawing group. Some of these compounds were also converted into 3′-oxime derivatives (**14**–**20**) by reaction with hydroxylamine in pyridine.[11] The indirubins with a substituent at R^{2-4} (**2**–**4**, **9**–**11**) and 5′-bromoindirubin (**12**) were prepared by the usual manner: condensation of the corresponding isatins with indoxyl acetate (5-bromoindoxyl acetate for **12**) in the presence of Na_2CO_3 in methanol.[11] In contrast, indirubins with a methoxy group at 5′- or 6′-position (**5** and **6**) and bromine at 6′-position (**13**) were synthesized by the reaction between oxindole and 2-chloroindol-3-one derivatives prepared from the corresponding isatins (Scheme 1).[12–14]

We examined the effect of indirubins on cell viability with three human neuroblastoma cell lines (group I), IMR-32,[15–18] SK-N-SH[18] and NB-39[16–18], and with two normal cell lines (group II), NHDF (normal human dermal fibroblast)[19,20] and HUVEC (human

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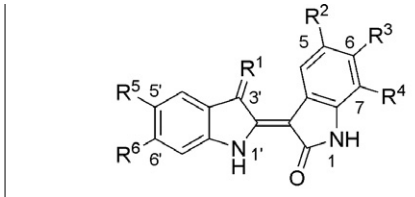
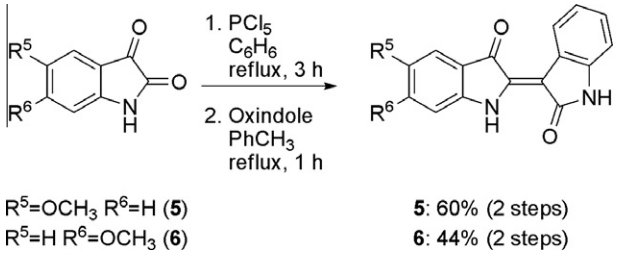


	R^1	R^2	R^3	R^4	R^5	R^6
1	O	H	H	H	H	H
2	O	OCH_3	H	H	H	H
3	O	H	OCH_3	H	H	H
4	O	H	H	OCH_3	H	H
5	O	H	H	H	OCH_3	H
6	O	H	H	H	H	OCH_3
7	O	H	H	H	CH_3	H
8	O	H	H	H	CH_2CH_3	H
9	O	Br	H	H	H	H
10	O	H	Br	H	H	H
11	O	H	H	Br	H	H
12	O	H	H	H	Br	H
13	O	H	H	H	H	Br
14	NOH	H	H	H	H	H
15	NOH	OCH_3	H	H	H	H
16	NOH	H	OCH_3	H	H	H
17	NOH	H	H	OCH_3	H	H
18	NOH	H	H	H	OCH_3	H
19	NOH	H	Br	H	H	H
20	NOH	H	H	H	Br	H

Figure 1. Structures of indirubins (**1**–**20**).



Scheme 1.

umbilical vein endothelial cell)[21,22] (Fig. 2). Our criteria for screening the potential of a compound as an anti-neuroblastoma drug is that cell viability of the cells in group I decreases to less than 50% while that of the cells in group II remain more than 90% at certain concentrations. Indirubin (**1**) and its 6- or 7-methoxylated derivatives (**3** and **4**) did not show any significant effect on cell viability of cells in both groups at the concentrations examined (up to 40 μM). 5-Methoxyindirubin (**2**) was cytotoxic, but no significant differences were observed between the cells in groups I and II. In contrast to the indirubins having methoxy group at R^{2-4}, 5′- and 6′-methoxyindirubins (**5**, **6**) exhibited selective toxicity to cells in group I. Although 5′-methoxyindirubin (**5**) satisfied the criteria described above, the 6′-isomer (**6**) had significant cytotoxicity to the cells in group II at 40 μM (82% in HNDF; 60% in HUVEC).

To determine the effectiveness of the methoxy moiety at position 5′, we prepared 5′-alkylindirubins as methoxy analogs. Interestingly, 5′-methylindirubin (**7**) and 5′-ethylindirubin (**8**) showed very weak cytotoxicities in both groups I and II up to 40 μM. Indirubins with bromine at $R^{5,6}$ (**12** and **13**) as well as R^{2-4} (**9**–**11**) did not show any significant effects on cell viability

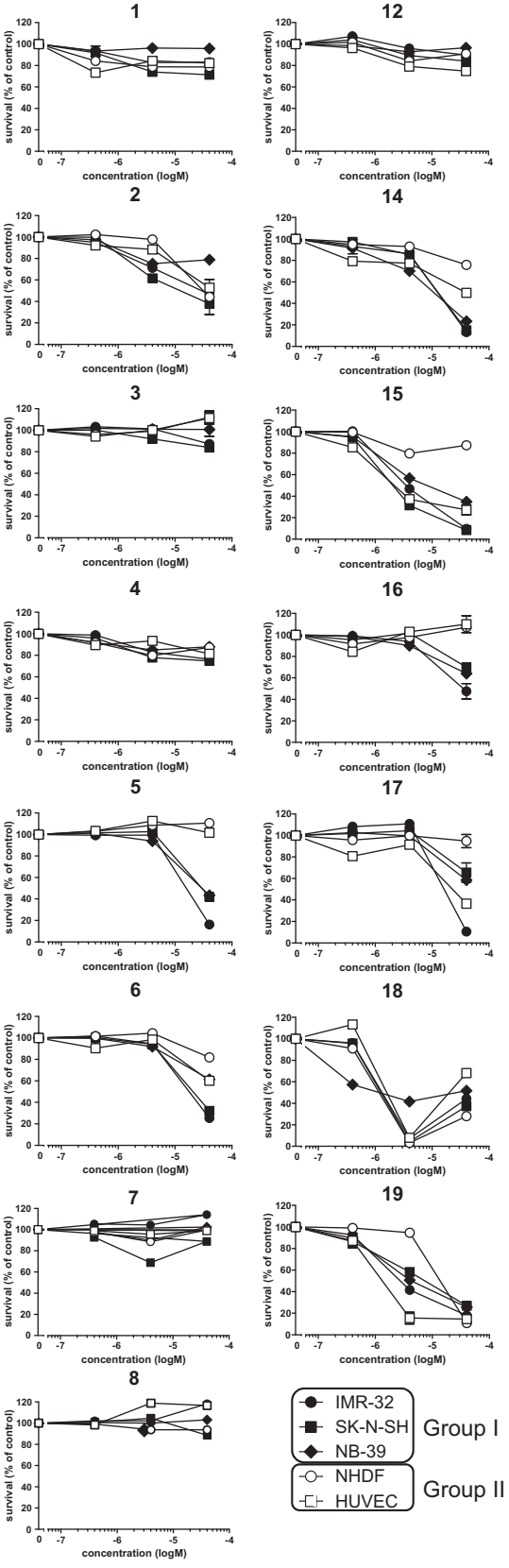


Figure 2. Cytotoxicity of indirubins on neuroblastoma cells and normal cells. Cell viability was measured using an MTT assay. IMR-32 (filled circles), SK-N-SH (filled squares), NB-39 (filled diamonds), NHDF (open circles), and HUVEC (open squares) cells were treated with the indicated concentrations of indirubins or DMSO (vehicle control) for 48 h. Each plot shows the survival rate relative to the vehicle control [mean ± standard error of the mean (SEM), $n = 3$].

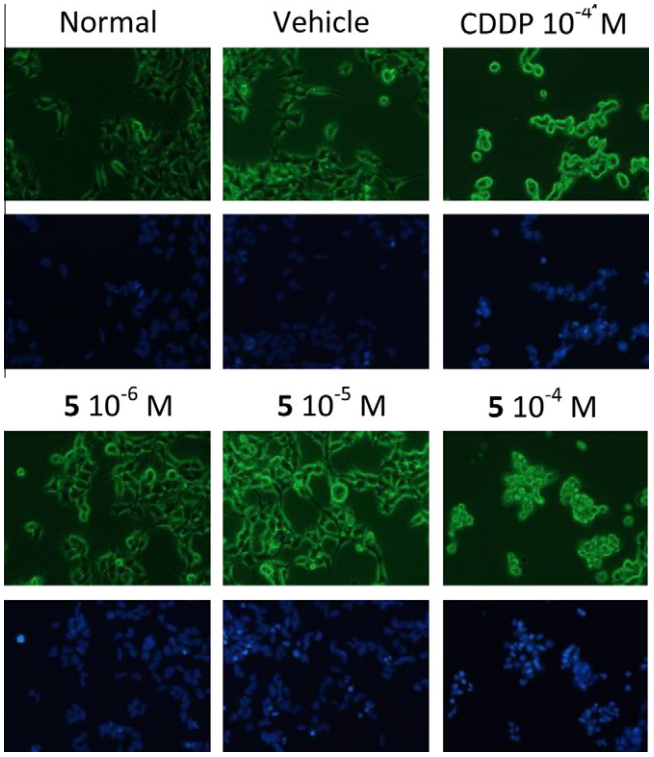


Figure 3. Typical morphologic features of apoptosis revealed using Hoechst 33342 staining. IMR-32 cells were exposed to **5** (1×10^{-6}–1×10^{-4} M) or CDDP (1×10^{-4} M) for 24 h and phase-contrast images (upper), and fluorescence images (lower) were obtained.

in either group (5′-bromoindirubin (**12**) is depicted as an example). Kim et al. recently mentioned an important effect between the bromine atom at position 5′ and cyclin-dependent kinase 2 (CDK2) based on a docking model simulation.[23] The electrostatic interaction between the lone pair electrons of the 5′-bromine group and the positively charged Lys89 residue may result in a higher binding affinity with CDK2. In our study, comparing 5′-methoxyindirubin (**5**) with 5′-alkylindirubins (**7**, **8**), much stronger cytotoxicities and selectivities against neuroblastoma were observed in 5′-methoxy analogs (**5**). These preferable effects might result from additional electrostatic effects of lone pair electrons and positively charged amino acids. However, bromine substitution at position 5′ does not exhibit positive results in this case.

We then focused on 3′-oxime derivatives because apoptosis-inducing potential of indirubin 3′-oxime (**14**) has been found in human cells.[7–9] Since this derivatization makes indirubin more soluble, we expected biological availability and efficacy to increase. All of the compounds gained additional cytotoxicity by this derivatization as presumed. Unexpectedly, 5′-methoxyindirubin (**5**) lost the selective cytotoxicity by conversion to 3′-oxime (**18**). On the other hand, indirubin 3′-oxime (**14**) and its 6-methoxylated derivative (**16**) gained cytotoxicity selective to the cells in group I, while the other 3′-oxime derivatives (**15**, **17**) including bromoindirubins (6-bromoindirubin 3′-oxime (**19**) is depicted as an example) exhibited cytotoxicity non-specifically. However, indirubin 3′-oxime (**14**) exhibited weak cytotoxicity to group II cells, too. 6-Methoxyindirubin 3′-oxime (**16**) was not cytotoxic to group II cells, but the cytotoxicity was not sufficient to some cells in group I. Accordingly, we chose 5′-methoxyindirubin (**5**) for further study.

To determine participation of apoptosis on cytotoxicity induced by 5′-methoxyindirubin (**5**), we subjected IMR-32 cells to two

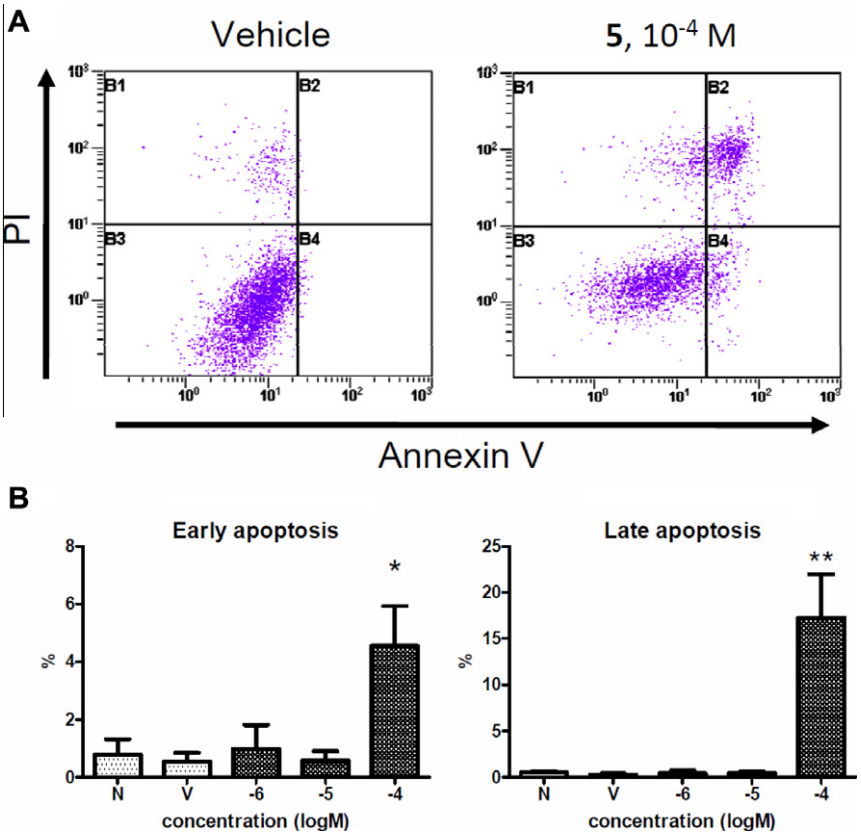


Figure 4. Analysis of apoptosis using flow cytometry. (A) IMR-32 cells were treated with **5** (1×10^{-4}) or DMSO (as a vehicle control (V)) for 48 h. Normal cells (N) were the untreated group. The vertical axis indicates PI (FL4 Log) and the horizontal axis indicates Annexin V-Alexa Fluor 488 (FL1 Log). **B.** Percentages of cell populations in each area (Early apoptosis: right lower, Late apoptosis: right upper). $^{*}p$ <0.05, $^{**}p$ <0.01 versus vehicle control as compared using a one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test.

different experiments. After 24-h incubation of the cells in the presence or absence of compound **5**, the cells were stained with Hoechst 33342, and morphological changes were observed by fluorescence microscopy (Fig. 3).[24] In the control experiment, the nuclei of the neuroblastoma cells were round in shape and stained homogenously. The cells treated with 5′-methoxyindirubin (**5**) showed typical morphological features of apoptosis such as cell shrinkage, chromatin condensation and fragmented fluorescent nuclei like that of authentic apoptosis inducer, cisplatin (CDDP).[25] Moreover, cells were subjected to flow cytometry after Annexin V and propidium iodide (PI) double staining.[26] Figure 4A shows the distribution of stained cells prepared from vehicle control and 5′-methoxyindirubin (**5**) (100 μM) as examples.[27] The percentages of apoptotic cells were estimated based on Figure 4A, and dramatically increased at 100 μM concentration of the indirubin **5** in both early and late apoptosis (Fig. 4B). These results lead us to conclude that the mechanism of cell death induced by 5′-methoxyindirubin (**5**) is apoptosis. Indirubins are known to inhibit cyclin-dependent kinases (CDKs) by binding to their ATP-binding site.[11] The inhibition of CDKs by indirubins arrests the cell cycle in the G_1 and/or G_2/M phases, prohibits cell growth, and induces apoptosis.[8,28–32] Since apoptotic mechanisms are involved in chemotherapeutic sensitivity and spontaneous regression of neuroblastoma, 5′-methoxyindirubin (**5**) may have comparatively high potential against neuroblastoma.

In summary, we prepared indirubins with two different types of substituents, methoxy group or bromine, on the aromatic rings for systematic screening. We found that 5′- and 6′-methoxy-indirubins (**5** and **6**) showed significant cytotoxicity to neuro-blastoma cells. When the carbonyl group at 3′-position was oxime, cytotoxicity appeared in every indirubin examined. In this case, indirubin 3′-oxime (**14**) and its 6-methoxylated derivative (**16**) showed selective cytotoxicity to neuroblastoma, while its 5′-methoxylated derivative (**18**) did not show any selectivity. Thus, it has been concluded that 5′-methoxyindirubin (**5**) is the most favorable indirubin examined for specific induction of cell death in neuroblastoma. Moreover, we concluded that the methoxy group is preferable to induce selective cytotoxicity against neuroblastoma rather than alkyl or bromine (**5** vs. **7**, **8**, and **12**). It was further clarified that 5′-methoxyindirubin (**5**) induced early and late apoptosis in neuroblastoma cells. Further studies are needed to elucidate the mechanism of apoptosis by biochemical approaches. Nonetheless, present data reinforce the anticancer potential of indirubins.

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