Cytometrical analysis of the adverse effects of indican, indoxyl, indigo, and indirubin on rat thymic lymphocytes

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Highlights

- Plants containing dyes are used in functional or health foods.
- Adverse effects of dyes, including indigo and indirubin, were examined.
- Among the dyes analyzed, indirubin exerted various cellular adverse effects.
- Indirubin should be removed from the products that contain dye plant extracts.

Abstract

Many businesses thrive by producing health supplements from agricultural products, as exemplified by the production of functional (or health) food using plants traditionally cultivated in the rural areas. Dyes, such as indican, indigo, indoxyl, and indirubin, present in dye plants, possess antibacterial, antifungal, and antiproliferative activities. However, these effects may also lead to cytotoxicity. Thus, studies in normal mammalian cells are necessary to identify cytotoxicity and prevent adverse effects of functional foods that contain these dyes. In this study, the effects of indican, indigo, indoxyl, and indirubin were evaluated by flow cytometry using appropriate fluorescent probes in rat thymic lymphocytes. Among the dyes analyzed, indirubin exerted distinct cellular activities. Treatment with indirubin (10–30 μM) increased the population of shrunken dead cells. The side scatter, but not forward scatter, increased in indirubin-treated living cells. It increased the population of annexin V-bound living and dead cells and that of dead cells without annexin V. Indirubin elevated intracellular Ca^{2+}, but not Zn^{2+} levels. The cellular content of superoxide anions and increased glutathione decreased. Indirubin depolarized the cellular plasma and mitochondrial membranes. It did not potentiate or attenuate the cytotoxicity of A23187 (Ca^{2+} overload) and H_2O_2 (oxidative stress). The results suggested that indirubin induces both apoptotic and non-apoptotic cell death. It may be difficult to predict and prevent adverse effects of indirubin due to its diverse activities on normal mammalian cells. Therefore, indirubin should be removed from products that contain dye plant extracts.

Keywords: Indican; Indigo; Indoxyl; Indirubin; Cytotoxicity; Lymphocytes.

1. Introduction

Indigo is one of the most famous ancient dyes (Splitstoser et al., 2016). It is the main component of plants, such as *Indigo naturalis, Polygonum tinctorium* Ait., *Isatis tinctoria* Ait., and *Baphicacanthus cusia* Brem. (for a review, Stasiak et al., 2014). Its precursor, indican, undergoes hydrolysis to indoxyl, which oxidizes to indigo and indirubin. These plants are used in Chinese medicine as coolants to treat various ailments (Stasiak et al., 2014). Particularly, formulations of *I. naturalis* are employed for the treatment of promyelocytic leukemia, psoriasis, and ulcerative colitis (Shilin et al., 1995; Lin et al., 2008; Sugimoto et al., 2016). Indican exhibits antibacterial and antifungal activities (da Silva et al., 2016). Indigo and indirubin are potent aryl hydrocarbon receptor ligands (Adachi et al., 2001). Indirubin exerts antiproliferative activity on leukocytes and keratinocytes (Hoessel et al., 1999; Lin et al., 2009). Although the bioactivity of indoxyl is not well elucidated, indoxyl sulfate has been demonstrated to be a uremic toxin (for a review, Vanholder, et al., 2014; Barreto et al., 2017). Thus, to evaluate whether constituents of these plants may cause adverse effects in normal cells, the effects of indican, indoxyl, indigo, and indirubin on rat thymic lymphocytes were studied using cytometric techniques with fluorescent probes. Another reason for conducting this study was that the Japanese central and local governments have recently started supporting rural agricultural industrialization. Under the banner of "sixth-sector industrialization," local production of agricultural commodities to generate employment in rural areas is encouraged (Nakano, 2014; Yu et al., 2015). Production of functional foods using plants traditionally cultivated in rural areas is exemplified using *Persicaria tinctoria* as a raw material for the “Japanese indigo.” The whole or isolated extract of this plant exhibits antitumor, antioxidant, and anti-inflammatory activities. (Kim et al., 2014; Kim et al., 2015). In addition, genotoxicity was observed at a high dose of the extract that contained 0.043 % indigo and 0.009 % indirubin (Lee et al., 2016). Therefore, this study was conducted to identify the adverse effects of edible products majorly

containing these dye-related constituents.

2. Materials and methods

2.1. Chemicals

Indican (97.5 %), indigo (97.8 %), and indirubin (99.9 %) were supplied from Tokyo Kasei TCI Co., Ltd. (Tokyo, Japan). Indoxyl sulfate (99.5 %) was obtained from Nacalai Tesque (Kyoto, Japan). Table 1 shows fluorescent probes in this study are listed in Table 1. Other chemicals, including dimethyl sulfoxide (DMSO), were also supplied from Wako Pure Chemicals (Osaka, Japan) unless specifically mentioned.

2.2. Animals and cell preparation

The Animal Experiment Committee of Tokushima University approved this study using experimental animals (No. 05279).

The procedure to prepare the cell suspension was described elsewhere (Chikahisa et al., 1996). In brief, the thymus glands were dissected from ether-anesthetized rats and their slices were triturated in Tyrode's solution (1–4°C) to dissociate single cells. The cells were incubated at 36–37°C for 1 h before the experiment. The cell suspension contains trace zinc (216.9 ± 14.4 nM) from the preparation (Sakanashi et al., 2009).

DMSO solution (2 μL) containing the test agents (1–30 mM) was added to the suspensions (1.998 mL) to achieve final concentrations (1–30 μM). Thereafter, the cells were treated with and without test agent(s) at 36–37°C for 1–3 h, depending on the experiment. Cellular and membrane changes were observed in a sample (100 μL) from each cell suspension. Data acquisition for 10–15 s allowed analysis of 2000–2500 cells.

2.3. Estimations of membrane and cellular parameters with fluorescent probes

Fluorescence analysis was done with flow cytometry (CytoACE-150; JASCO, Tokyo, Japan) and JASCO software (Version 3.06).

Cell lethality was assessed by adding 5 μM propidium iodide (PI). Measurement of PI fluorescence at 488 ± 20 nm / 600 ± 20 nm (excitation / emission wavelengths) from cells provided information on cell lethality. Exposed phosphatidylserine (PS) on the outer membrane surface of cells was detected after incubation with a 10 μL/mL of annexin V-fluorescein isothiocyanate (FITC) for 30 min (Koopman et al., 1994). FluoZin-3-AM and Fluo-3-AM were used to monitor changes in the intracellular Zn^{2+} and Ca^{2+} concentrations ($[Zn^{2+}]i$ and $[Ca^{2+}]i$), respectively (Gee et al., 2002; Kao et al., 1989). The cells were incubated with 1 μM FluoZin-3-AM and 1 μM Fluo-3-AM for 1 h prior to the fluorescence measurement. The superoxide-specific (BES-So-AM) and thiol-specific [5-chloromethylfluorescein diacetate (5-CMF-DA)] fluorescent probes were used to monitor cellular contents of the superoxide anion ($[O_2^-]i$) and glutathione ([GSH]i), respectively (Chikahisa et al., 1996; Maeda et al., 2005). The cells were incubated with 5 μM BES-So-AM for 1 h before the application of test agent. 5-CMF-DA (500 nM) was applied to the cells at 30 min before the measurement. Changes in the plasma and mitochondrial membrane potentials were estimated using 500 nM 3,3'-dipentyloxacarbocyanine iodide [DiOC5(3)] and 1 μM 5,6-dichloro-2-[(E)-3-(5,6-dichloro-1,3-diethylbenzimidazol-3-ium-2-yl)prop-2-enylidene]-1,3-diethylbenzimidazole;iodide (JC-1), respectively (Smiley et al., 1991; Labeed et al., 2003). The excitation and emission wavelengths for FITC, FluoZin-3, Fluo-3, BES-So, 5-CMF, DiCO5(3), and JC-1 were 488 ± 20 nm and 530 ± 20 nm, respectively. The fluorescence of JC-1 was also monitored at 600 ± 20 nm.

2.4. Statistical analysis and presentation

Statistical analyses were performed by ANOVA with Tukey's *post hoc* multivariate analysis and $P < 0.05$ was considered significant. All values are expressed as means ± SD of 4 samples. Each experiment was repeated 3 times unless noted otherwise.

3. Results

3.1. Increase in cell lethality by indican, indigo, indoxyl, and indirubin

Treatment of cells with 30 μM indirubin for 3 h significantly increased the population of cells exhibiting propidium fluorescence (dead cells) with decreased forward scatter intensity (Fig. 1A). However, treatment with indican, indigo, or indoxyl sulfate at 30 μM for 3 h did not increase the cell lethality (Fig. 1B). The threshold cytotoxic concentration of indirubin in rat thymocytes was 3–10 μM after treatment for 3 h.

(Figure 1)

3.2. Changes in forward and side scatter by indican, indigo, indoxyl, and indirubin

The forward scatter and side scatter profiles of the cells exhibited no propidium fluorescence (indicating no cell death) after treatment with indican, indigo, or indoxyl at 30 μM for 3 h. However, after treatment with 30 μM indirubin, as shown in Fig. 1A, the population was significantly spread along the axis of side scatter, but not forward scatter. The spread of data is usually expressed as the standard deviation (SD). In flow cytometry, the coefficient of variation (CV = SD/mean channel number) is used to express the spread of data. The CV of the side scatter histogram obtained from the cells treated with 30 μM indirubin was much larger than that of the control cells (Fig. 2).

(Figure 2)

3.3. Process of cell death induced by indirubin

The process of cell death induced by indirubin was examined using annexin V-FITC and PI staining. Treatment with 30 μM indirubin for 3 h increased the population of cells exhibiting PI fluorescence (dead cells) with and without exhibiting FITC fluorescence (exposed PS), and

cells exhibiting no PI fluorescence (living cells) with exposed PS (Fig. 3A). Indirubin at 10 μM induced cell death in the population to a lesser extent than that at higher concentrations (Fig. 3B).

(Figure 3)

3.4. Changes in levels of $[Zn^{2+}]i$ and $[Ca^{2+}]i$ by indirubin

A significant increase in $[Ca^{2+}]i$ and/or $[Zn^{2+}]i$ induces cell death (Zhivotovsky and Orrenius, 2011; Oyama et al., 2010, 2012, 2014). Therefore, the effect of indirubin (3–30 μM) on cells incubated with FluoZin-3-AM for $[Zn^{2+}]i$ and Fluo-3-AM for $[Ca^{2+}]i$ was examined to determine if intracellular Zn^{2+} and/or Ca^{2+} is involved in the mechanism of indirubin-induced cell death. Indirubin at 3 μM did not affect both Fluo-3 and FluoZin-3 fluorescence, indicating no change in $[Ca^{2+}]i$ and $[Zn^{2+}]i$ levels at this concentration. Although indirubin (10–30 μM) significantly increased the fluorescence intensity of Fluo-3, it did not affect FluoZin-3 fluorescence (Fig. 4). Removal of external Ca^{2+} completely inhibited the indirubin-induced augmentation of Fluo-3 fluorescence, suggesting that indirubin increased the membrane Ca^{2+} permeability. However, the involvement of intracellular Ca^{2+} and Zn^{2+} is doubtful because indirubin at lethal concentrations (10–30 μM) only mildly affected the $[Ca^{2+}]i$ level and had no effect on $[Zn^{2+}]i$ level.

(Figure 4)

3.5. Changes in $[O_2^-]i$ and [GSH]i by indirubin

A significant increase in oxidative stress also induces cell death (Ryter et al., 2007). Therefore, the effect of indirubin on the $[O_2^-]i$, a highly reactive oxygen species, was examined using BES-So-AM. The fluorescence intensity of BES-So in the cells incubated with indirubin (10–30 μM) for 1 h was lower than that in the control cells (Fig. 5). A decrease in the cellular antioxidant, [GSH]i increases cell vulnerability to oxidative stress (Fernández-Checa et al.,

1998). Therefore, the effect of indirubin on the [GSH]i was examined using 5-CMF-DA. As shown in Fig. 5, no change in the fluorescence intensity of 5-CMF in the cells incubated with indirubin was observed compared to that in the control cells.

(Figure 5)

3.6. Effects of indirubin on the plasma membrane potential (PMP) and mitochondrial membrane potential (MitoMP)

Attenuation of DiOC5(3) fluorescence corresponds to the depolarization of PMP (Labeed et al., 2003). Treatment of cells with indirubin (10–30 μM) for 1 h significantly attenuated the fluorescence intensity of DiOC5(3) (Fig. 6A), indicating that it induced the depolarization of PMP. The results are summarized in Fig. 6B.

(Figure 6)

Increase in the ratio of green/red JC-1 fluorescence intensities corresponds to depolarization of MitoMP (Smiley et al., 1991). Treatment of cells with indirubin (10–30 μM) increased this ratio, indicating that it induced the depolarization of MitoMP (Fig. 7).

(Figure 7)

3.7. Effects of indirubin on cells treated with H_2O_2 or A23187

Significantly increased $[Ca^{2+}]$i level or oxidative stress is a common cause of cell death induced by chemicals. Therefore, synergistic effects of indirubin (10–30 μM) with A23187 (a calcium ionophore) or H_2O_2 on the cells under conditions of increased $[Ca^{2+}]$i or oxidative stress, respectively were evaluated. As shown in Fig. 8, indirubin acted synergistically with A23187 and H_2O_2 to increase cell lethality.

(Figure 8)

4. Discussion

4.1. Characteristics of indirubin-induced in vitro cytotoxicity

Among all the dyes studied, indirubin exerted diverse cellular effects on rat thymic lymphocytes. Incubation with indirubin (10–30 μM) for 3 h significantly increased the number of shrunken dead cells (Fig. 1A). Cell shrinkage being one of the earlier phenomena during apoptosis (Bortner and Cidlowski, 1998; Maeno et al., 2000), cell death may have been due to apoptosis. However, two types of dead cells were observed: those bound to annexin V, one of the early markers for apoptosis (Koopman et al., 1994), and those not bound to annexin V (Fig. 3). Although indirubin (30 μM) significantly increased the population of annexin V-bound living cells (Fig. 3), the side scatter (cell granularity) and its coefficient of variation significantly increased, whereas no significant change in the forward scatter (cell size) was observed (Fig. 2). Therefore, it is likely that indirubin induces apoptosis and necrosis.

Indirubin decreased the cellular content of $[O_2^-]$i that was generated in the mitochondria (Fig. 5). Further, it reduced mitochondrial activity as indicated by depolarization of the mitochondrial membrane (Fig. 7). Moreover, it depolarized the plasma membrane (Fig. 6) and increased the $[Ca^{2+}]$i level by increasing plasma membrane Ca^{2+} permeability. Depolarization of the plasma membrane, changes in its membrane potential, and an increase in $[Ca^{2+}]$i level is associated with altered physiological functions of non-excitable cells, such as lymphocytes (Han and Kang, 2009; Lam and Wulff, 2011; Feske et al., 2012). Opening and closing of K^+ channels regulate membrane K^+ permeability, one of the important determinants of membrane potential. Charybdotoxin, a specific blocker of the Ca^{2+}-dependent K^+ channel, inhibits the proliferation of T lymphocytes (Price et al., 1989). Non-specific K^+ channel blockers inhibit the activation of B lymphocytes, resulting in an attenuation of lymphocytic progression through the cell cycle (Amigorena et al., 1990). Based on the evidence, indirubin may modify some functions of lymphocytes. Intracellular Ca^{2+} is involved in many physiological functions, and an imbalance in its level disturbs many intracellular signaling pathways (Berridge et al., 1998). Thus, a

sustained increase in $[Ca^{2+}]i$ by indirubin may be linked to cell injury and death.

4.2. Further risk

It is considered that the indigo plants are used not only as colorants but also as bioactive substances for functional foods. According to the abstracts (Tsuji et al., 2016, 2017) presented in the Annual Meeting of Japan Society of Nutrition and Food Science, the animal studies showed that the foods containing 1–2 % leaf powder of indigo plant (*Polygonum tinctrium*) could be used as a diet food.

Aryl hydrocarbon (Ah) receptors, also called dioxin receptors, are implicated in dioxin-induced toxicity (Birnbaum, 1994). Indirubin and indigo are known to be potent ligands for Ah receptors (Adachi et al., 2001). The binding affinity of indirubin is greater than that of the most potent dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); the concentration of indirubin required to activate Ah receptors is lower than that of TCDD. In the present study, 10–30 μM of indirubin was necessary to induce cell death (Fig. 1B). These concentrations are higher than those required for ligand binding to the yeast Ah receptor (Adachi et al., 2001). Mean concentrations of indirubin and indigo in human urine were reported to be 0.19 ± 0.11 nM and 0.22 ± 0.26 nM, respectively (Adachi et al., 2001). The actual concentrations of indirubin to fully activate Ah receptors under in vitro conditions were 10–100 nM (Adachi et al., 2001). Because indirubin is known to be most potent activator of Ah receptors, the indirubin concentrations for the activation of Ah receptors were much lower than those to elicit adverse actions on rat lymphocytes. The extract of *P tinctoria* contains 0.043 % indigo and 0.009 % indirubin (Lee et al., 2016). Therefore, 1 kg of the extract contains 343 μM of indirubin (molecular weight 262.27). If functional or health foods containing the constituents of dye plants are proposed, the dose of these plant extracts should be carefully adjusted to prevent the risk of adverse effects on the health of consumers.

4.3. Other problems

There may be many processing of plants to produce functional foods. Although we do not have enough information on the processing, the post-harvest treatments affect the content of indigo precursors (Oberthür et al., 2004). It may be also required to study the fat content of functional foods containing dye compounds. Because these compounds are lipophilic, their bioavailability can be influenced by the fat content. Thus, the processing may affect the contents of dye compounds. The plants contain several types of bioactive compounds such as tryptanthrin and isantin (Chiang et al., 2013; Chang et al., 2015). Therefore, it is necessary to examine adverse actions of compounds present in the plants before producing functional foods.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgements

This study was supported by the Grant-in-Aid for Scientific Research (C26340039) from the Japan Society for the Promotion of Science (Tokyo, Japan).

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Figure legends

Figure 1. Changes in cell lethality induced by indican, indoxyl, indigo, and indirubin. (A) Indirubin-induced change in the cytogram (forward scatter intensity versus PI fluorescence intensity). The dotted line indicates the population of cells exhibiting PI fluorescence. The effect was examined after 3 h of incubation with indirubin. The cytogram was constructed from 2500 cells. (B) A concentration-dependent increase in indirubin-induced cell lethality. All values are represented as the mean ± SD of 8 samples. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN).

Figure 2. Change in the forward and side scatter of intact cells by indirubin. (A) Indirubin-induced change in the cytogram (forward scatter versus side scatter) constructed from 2000 intact cells. The effect was examined after 3 h of incubation with indirubin. (B) Indirubin-induced change in the mean scatter intensity (upper 2 columns) and its coefficient of variation (lower 2 columns) of intact cells. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN).

Figure 3. Indirubin-induced change in the cell population labeled with annexin V-FITC and PI. (A) Indirubin-induced change in the cytogram (PI fluorescence versus FITC fluorescence) constructed from 2500 cells. N, Intact living cells; A, Annexin V-positive living cells; P, Annexin V-negative dead cells; AP, Annexin V-positive dead cells. The effect was examined after 3 h of incubation with indirubin (B) Indirubin-induced change in the respective populations. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN).

Figure 4. Effect of indirubin on the cells pretreated with Fluo-3-AM and FluoZin-3-AM. A1 and

A2 (upper panel) show indirubin-induced changes in the fluorescence intensity of Fluo-3 (mean ± SD of 4 samples) with and without external Ca^{2+}, respectively. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN). B1 and B2 (lower panel) indicate no indirubin-induced changes in the fluorescence intensity of FluoZin-3 (mean ± SD of 4 samples) with and without external Zn^{2+}, respectively.

Figure 5. Effect of indirubin on the redox state of cells. The upper panel indicates the effect on cells pretreated with BES-So-AM. Asterisks (**) indicate a significant difference between BES-So fluorescence ($P < 0.01$) in the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN). The lower panel shows no indirubin-induced change in 5-CMF fluorescence.

Figure 6. Effect of indirubin on the plasma membrane potential. (A) Indirubin-induced change in DiOC5(3) fluorescence in a population of 2500 cells treated without (CONTROL) or with indirubin (INDIRUBIN). (B) Indirubin-induced change in the fluorescence intensity of DiOC5(3). All values are presented as mean ± SD of 4 samples. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN). The dotted line indicates the fluorescence intensity of DiOC5(3) in dead cells.

Figure 7. Effect of indirubin on the mitochondrial membrane potential as estimated by the ratio of green/red JC-1 fluorescence intensities. All values are presented as mean ± SD of 4 samples. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN).

Figure 8. Effect of indirubin on cells treated with A23187 or H_2O_2. All values are presented as

mean ± SD of 4 samples. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control cells (CONTROL) and cells treated with indirubin (INDIRUBIN).

Table 1. Fluorescent probes used in this study

Fluorescent probes [Manufacture]	Purpose
Propidium iodide (PI) [Molecular Probes, Inc., Eugene, OR, USA]	Staining dead cells
Annexin V-FITC [Molecular Probes]	Detecting phosphatidylserine exposed on membranes
Fluo-3-AM [Dojin Chemicals, Kumamoto, Japan]	Estimating change in intracellular Ca2+ level
FluoZin-3-AM [Molecular Probes]	Estimating change in intracellular Zn2+ level
BES-So-AM [Wako Pure Chemicals, Osaka, Japan]	Detecting superoxide aninons
5-Chloromethylfluorescein diacetate (5-CMF-DA) [Molecular Probes]	Estimating change in cellular GSH content
3,3'-Dipentyloxacarbocyanine iodide (DiOC5(3)) [Molecular Probes]	Estimating plasma membrane potential
JC-1 [Molecular Probes]	Estimating mitochondrial membrame potentials

Figure 1

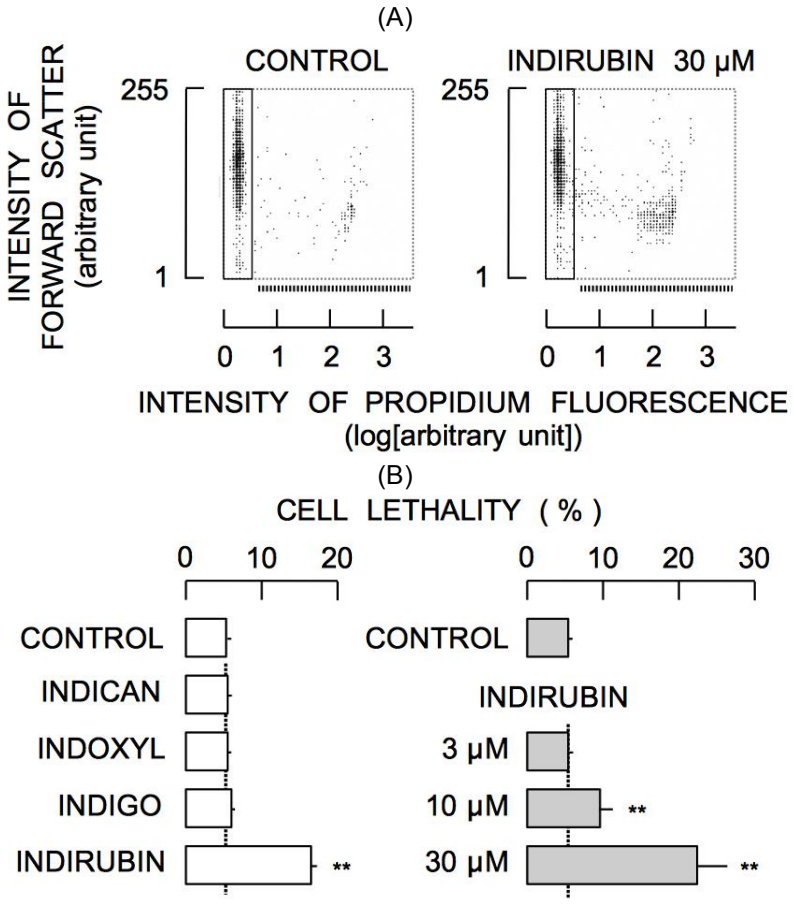

(A)
CONTROL
INDIRUBIN 30 μM
INTENSITY OF FORWARD SCATTER (arbitrary unit)
255
1
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
(B)
CELL LETHALITY (%)
0 10 20
0 10 20 30
CONTROL
INDICAN
INDOXYL
INDIGO
INDIRUBIN
**
CONTROL
INDIRUBIN
3 μM
10 μM
30 μM
**
**

Figure 2

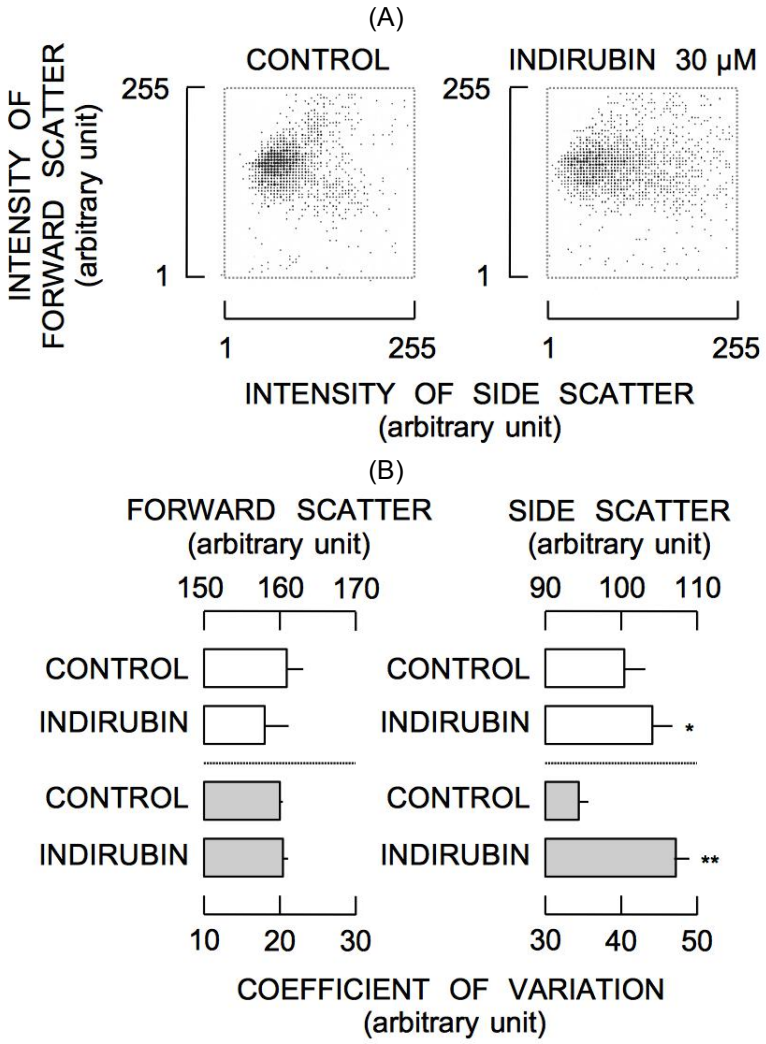

(A)
CONTROL
INDIRUBIN 30 μM
INTENSITY OF FORWARD SCATTER (arbitrary unit)
255
1
1
255
INTENSITY OF SIDE SCATTER (arbitrary unit)
(B)
FORWARD SCATTER (arbitrary unit)
SIDE SCATTER (arbitrary unit)
150 160 170
90 100 110
CONTROL
INDIRUBIN
*
**
10 20 30
30 40 50
COEFFICIENT OF VARIATION (arbitrary unit)

Figure 3

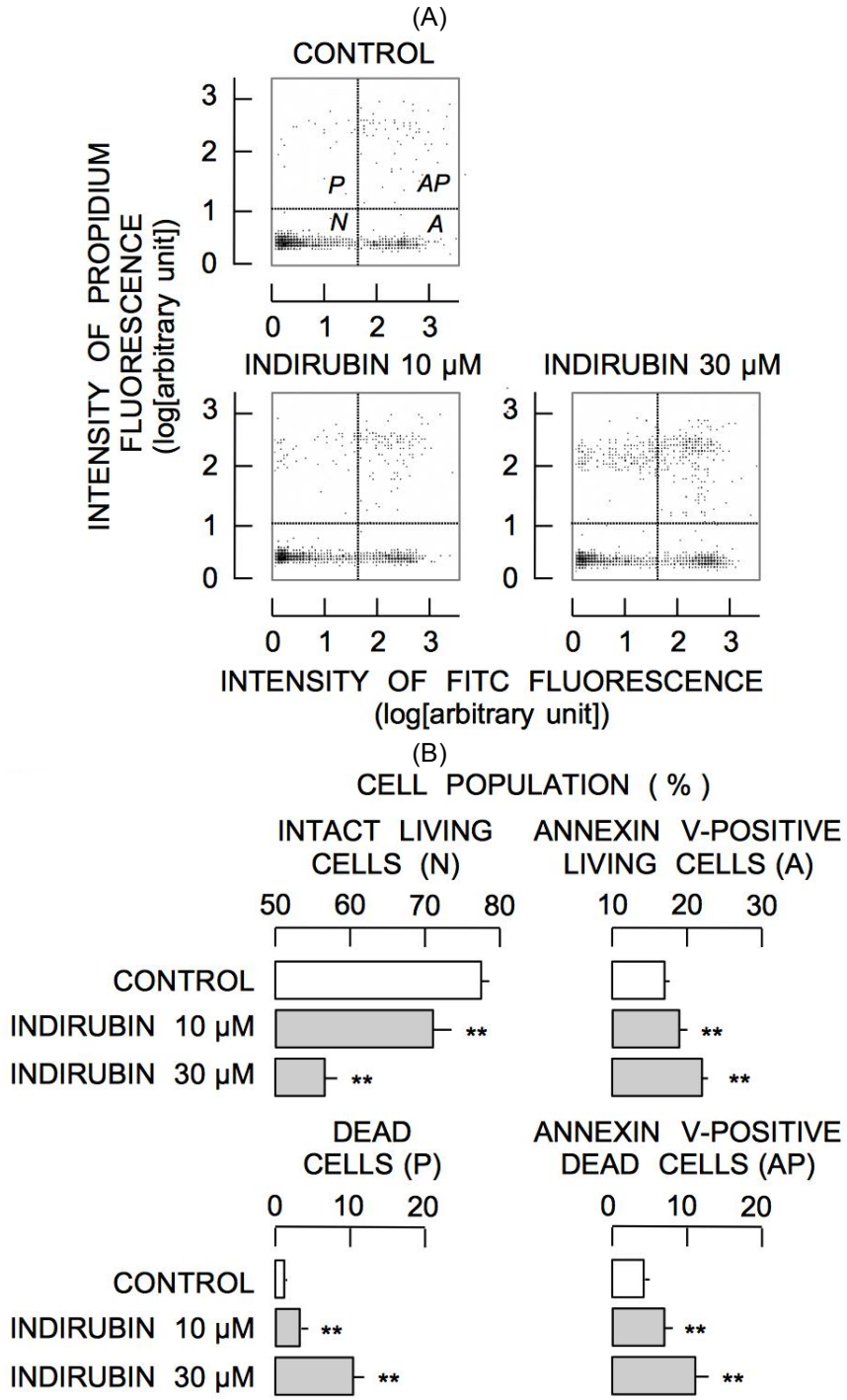

(A)
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM
P
AP
N
A
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
INTENSITY OF FITC FLUORESCENCE (log[arbitrary unit])
(B)
CELL POPULATION (%)
INTACT LIVING CELLS (N)
ANNEXIN V-POSITIVE LIVING CELLS (A)
DEAD CELLS (P)
ANNEXIN V-POSITIVE DEAD CELLS (AP)
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM
**

Figure 4

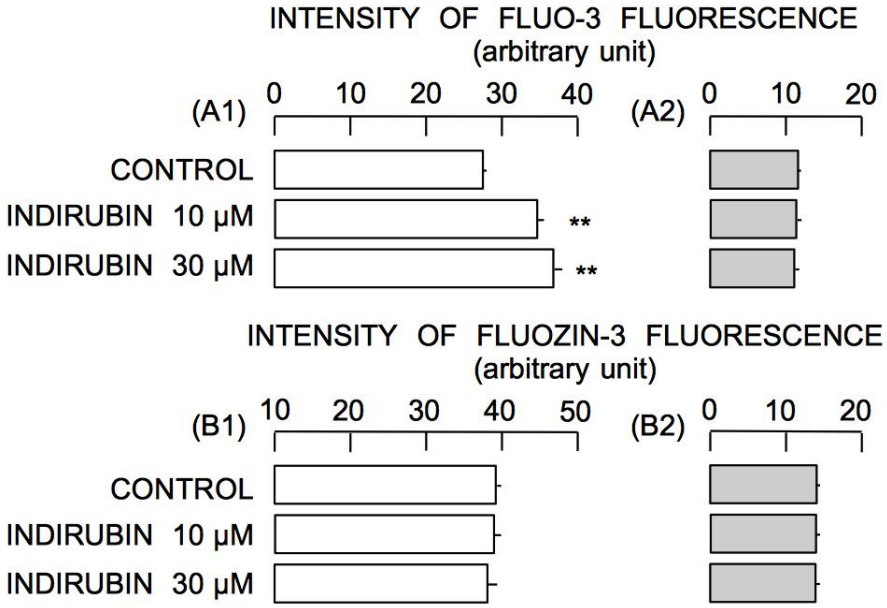

INTENSITY OF FLUO-3 FLUORESCENCE
(arbitrary unit)
(A1)
0 10 20 30 40
(A2)
0 10 20
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM
**
**
INTENSITY OF FLUOZIN-3 FLUORESCENCE
(arbitrary unit)
(B1)
10 20 30 40 50
(B2)
0 10 20
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM

Figure 5

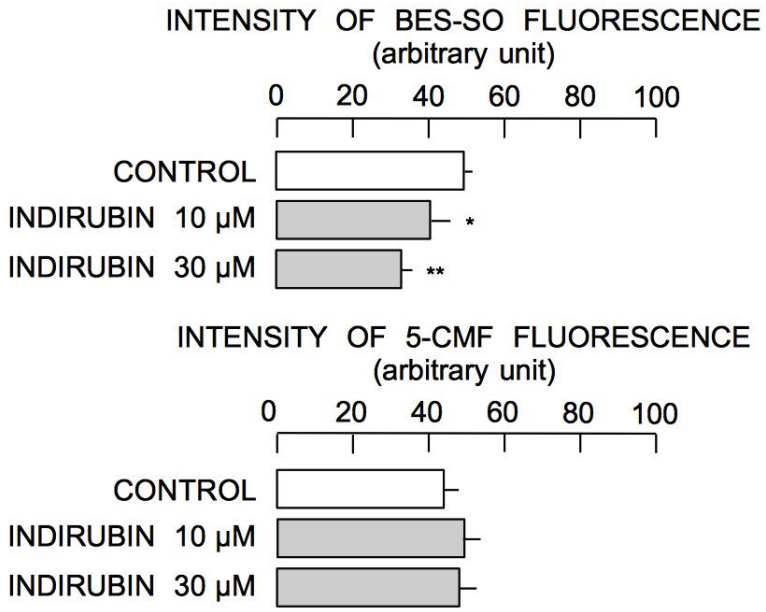

INTENSITY OF BES-SO FLUORESCENCE
(arbitrary unit)
0
20
40
60
80
100
CONTROL
INDIRUBIN 10 μM
*
INDIRUBIN 30 μM
**
INTENSITY OF 5-CMF FLUORESCENCE
(arbitrary unit)
0
20
40
60
80
100
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM

Figure 6

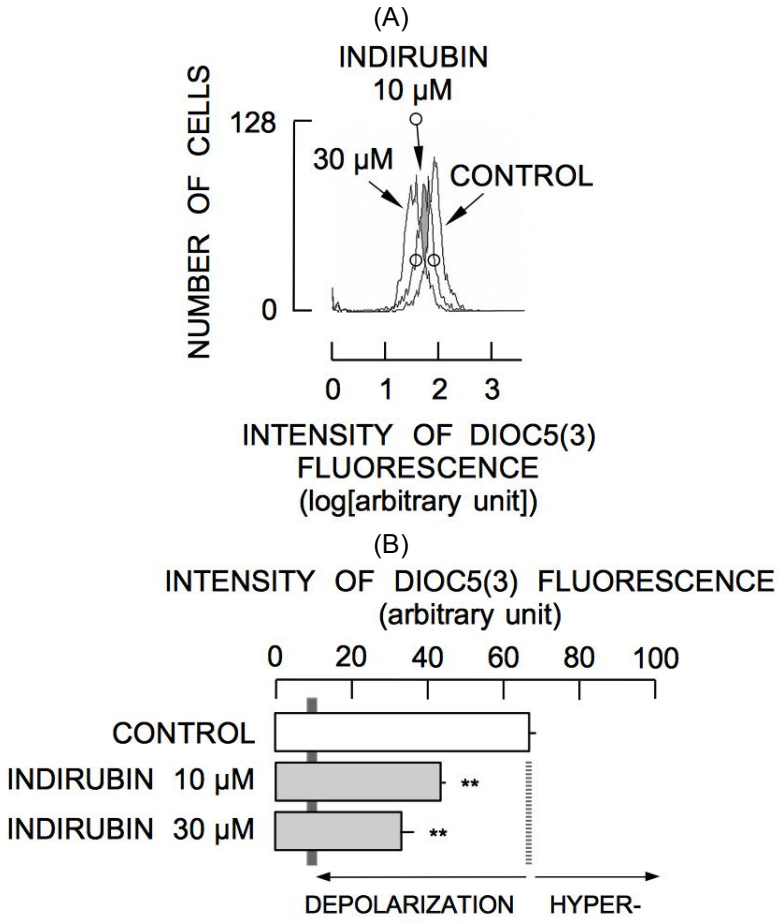

(A)
INDIRUBIN
10 μM
128
30 μM
CONTROL
NUMBER OF CELLS
0
0 1 2 3
INTENSITY OF DIOC5(3)
FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF DIOC5(3) FLUORESCENCE
(arbitrary unit)
0 20 40 60 80 100
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM
**
**
DEPOLARIZATION
HYPER-

Figure 7

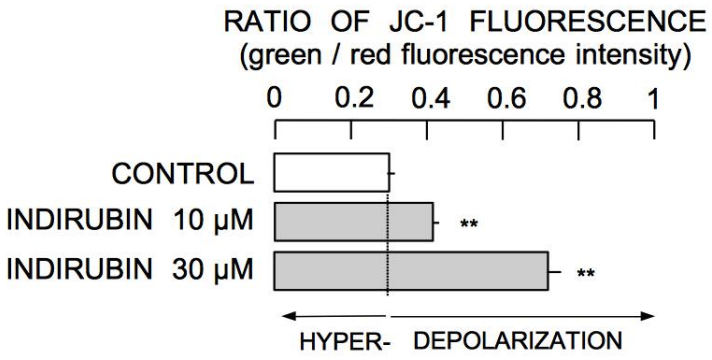
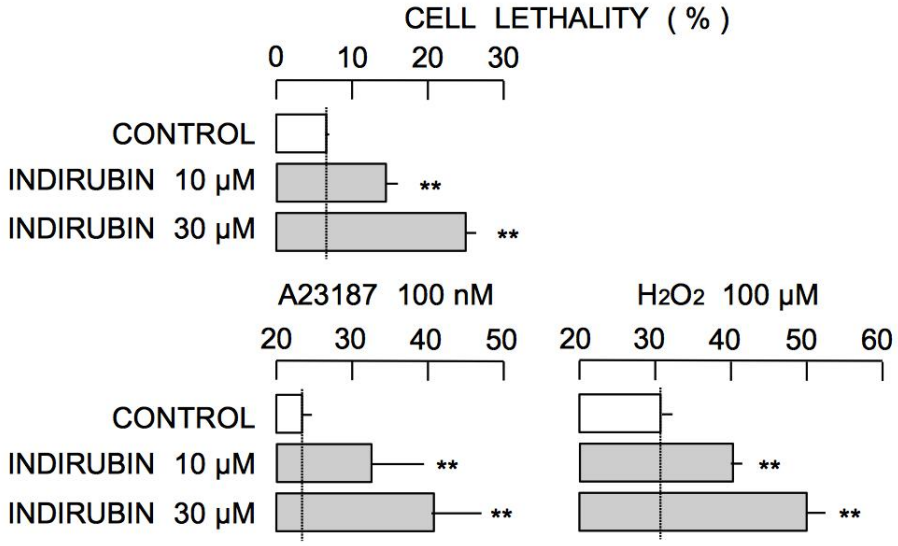

RATIO OF JC-1 FLUORESCENCE
(green / red fluorescence intensity)
0
0.2
0.4
0.6
0.8
1
CONTROL
INDIRUBIN 10 µM
**
INDIRUBIN 30 µM
**
HYPER-
DEPOLARIZATION

Figure 8


CELL LETHALITY (%)
0
10
20
30
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM
**
**
A23187 100 nM
20
30
40
50
CONTROL
INDIRUBIN 10 μM
INDIRUBIN 30 μM
**
**
H2O2 100 μM
20
30
40
50
60
**
**