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Induction of Differentiation and Down-Regulation of c-*myb* Gene Expression in ML-1 Human Myeloblastic Leukemia Cells by the Clinically Effective Anti-Leukemia Agent Meisoindigo

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ABSTRACT. Meisoindigo, a second generation derivative of indirubin, is an effective chemotherapeutic agent with very low toxicity used in the treatment of chronic myeloid leukemia. To determine the nature of this activity, the effect of a nontoxic concentration (0.72 μg/mL) of this compound on ML-1 human myeloblastic leukemic cells was examined. At such a concentration, differentiation induction was found to be the most pronounced drug effect. During the 3-day drug incubation period, the viable cell number remained essentially constant, with approximately 48% of the cells demonstrating a mature phenotype with increased acid phosphatase activity and nitroblue tetrazolium dye reduction. As observed with other DNA-specific agents, induction of ML-1 differentiation by meisoindigo was accompanied by the down-regulation of c-*myb* gene expression. These data suggest that induction of leukemic cell differentiation associated with decreased c-*myb* expression may be one of the mechanisms of the antitumor action of meisoindigo. BIOCHEM PHARMACOL 51;11:1545–1551 , 1996.

KEY WORDS. meisoindigo; new chemotherapeutic agent; ML-1 cells; chronic myeloid leukemia; cell differentiation; c-*myb* expression

Cancer can be considered a disorder of cell differentiation [1]. Numerous studies have shown that human leukemic cell lines retain their ability to differentiate *in vitro* when treated with natural differentiation factors or when exposed to a variety of chemical compounds including clinically active anticancer agents [2–4]. It has been shown that DNA-specific inhibitors, such as cytarabine or daunorubicin, are capable of effectively inducing the differentiation of various myeloid leukemic cell lines, whereas inhibitors of RNA or protein synthesis are unable to achieve this result [5, 6]. It has been demonstrated that c-*myb* proto-oncogene plays a very important role in regulating the proliferation and differentiation of hemopoietic cells [7–9]. Initiation of differentiation by natural differentiation factors or chemical inducers is associated with down-regulation of c-*myb* expression [10–12], suggesting that c-*myb* is involved in the differentiation process.

Traditional Chinese medicine, based to a large extent on the use of plant extracts, constitutes a valuable approach for new antineoplastic drug discovery. This is well illustrated by the successful therapeutic response obtained with indirubin for the treatment of chronic myeloid leukemia [13]. Because of poor solubility, poor absorption, and irritation of the gastrointestinal tract by indirubin, a search for new agents of this type with high efficacy and low toxicity was carried out. A series of indirubin derivatives was synthesized, and their structure-activity relationships were studied. It was found that methylisoindigotin, abbreviated as meisoindigo, exhibited higher activity than indirubin against rodent tumors. As a second generation of indirubin (see Fig. 1 for their structures), meisoindigo has been used in the clinic and shown to be active against chronic myeloid leukemia with lower toxicity than indirubin [14]. We have reported that this agent exerts strong inhibitory activity on the biosynthesis of DNA and RNA in W256 cells [15]. Inhibition of microtubule assembly was also observed [16]. However, the mechanism of the antitumor action of meisoindigo is still unclear, particularly since its therapeutic effects are achieved in the absence of toxicity. Because it has been demonstrated previously that the therapeutic action of DNA-specific antitumor agents is exerted through the initiation of tumor cell maturation [17], we investigated whether meisoindigo also acts similarly on human myeloblastic leukemia cells.

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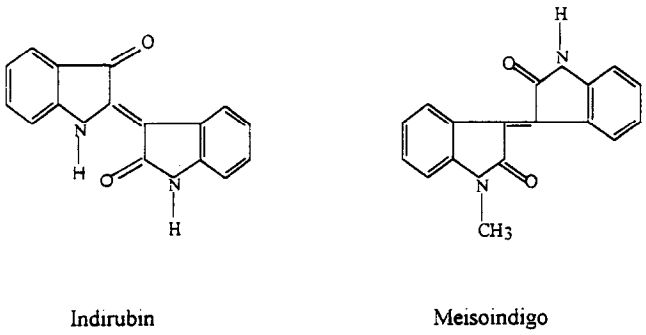


Indirubin Meisoindigo

FIG. 1. Chemical structures of indirubin and meisoindigo.

MATERIALS AND METHODS

Reagents

Meisoindigo was synthesized in the Institute of Materia Medica, the Chinese Academy of Medical Sciences, Beijing, China. The oncogene probe was purchased from Oncor (Gaithersburg, MD, U.S.A.). [^{32}P]dCTP and labeling kits RPN.1601Z and N.5500 were purchased from Amersham (Arlington Heights, IL, U.S.A.).

Cell Culture

ML-1, a line of human myeloblastic leukemia cells, was maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated FBS*, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37°.

Assay of Growth- and Differentiation-Associated Characteristics

The inhibitory effect of meisoindigo on ML-1 cell growth was estimated by trypan blue dye exclusion. Cell numbers were counted with a hemocytometer, and the cumulative cell number was calculated by multiplying the cell counts by the dilution factor derived from refeeding the cultures. Cell differentiation was assessed by the increase of AP activity, the NBT test, and morphological change. Cell cycle distribution was determined cytofluorometrically as described previously [6, 17].

AP activity was determined by measuring the enzymatic hydrolysis of *p*-nitrophenyl phosphate [18]. After treatment of ML-1 cells with various concentrations of meisoindigo, cells (1 mL) were collected by centrifugation and washed once with PBS. The cell pellet was resuspended and incubated with 40 μL of 0.05% Triton X-100 for 30 min at 0° followed by the addition of substrate buffer (sodium citrate, 38 mM, pH 4.8; Triton X-100, 0.1%; *p*-nitrophenyl phosphate, 4.2 mM) and incubation at 37° for an additional 15 min. The reaction was stopped immediately by chilling on ice and adding 0.8 mL of 0.1 N NaOH. The absorbance at 410 nm was then measured using a spectrophotometer. NBT reduction was assayed by a modification of the procedure described by Takuma *et al.* [19]. Briefly, ML-1 cells (3×10^5 cells/mL were incubated in 96-well flat-bottom plates for 60 min at 37° in the presence of 1 mg/mL NBT and 100 ng/mL TPA. After incubation, the reaction was stopped by adding 2 N hydrochloric acid, the supernatant solution was removed by pipette, and the cells were dried overnight at 37°. Formazan was dissolved in DMSO, and its absorbance at 540 nm was measured by an autoreader.

For assessing changes in morphology, slides of viable cells were prepared by Cytospin centrifugation of aliquots of the appropriate cell suspensions, and after staining with Wright-Giemsa differential counts were made with a light microscope; at least 250 cells were counted for each experimental condition. All assay data were derived from three separate experiments.

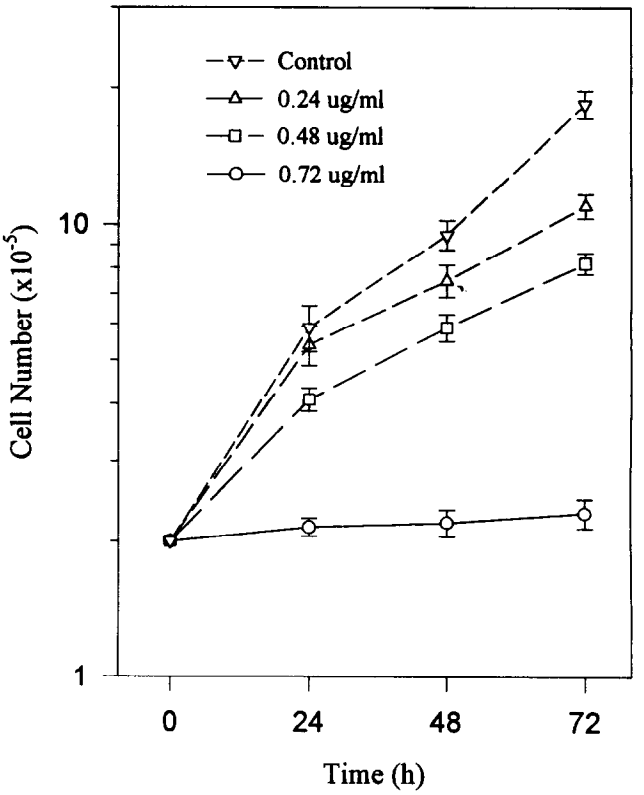
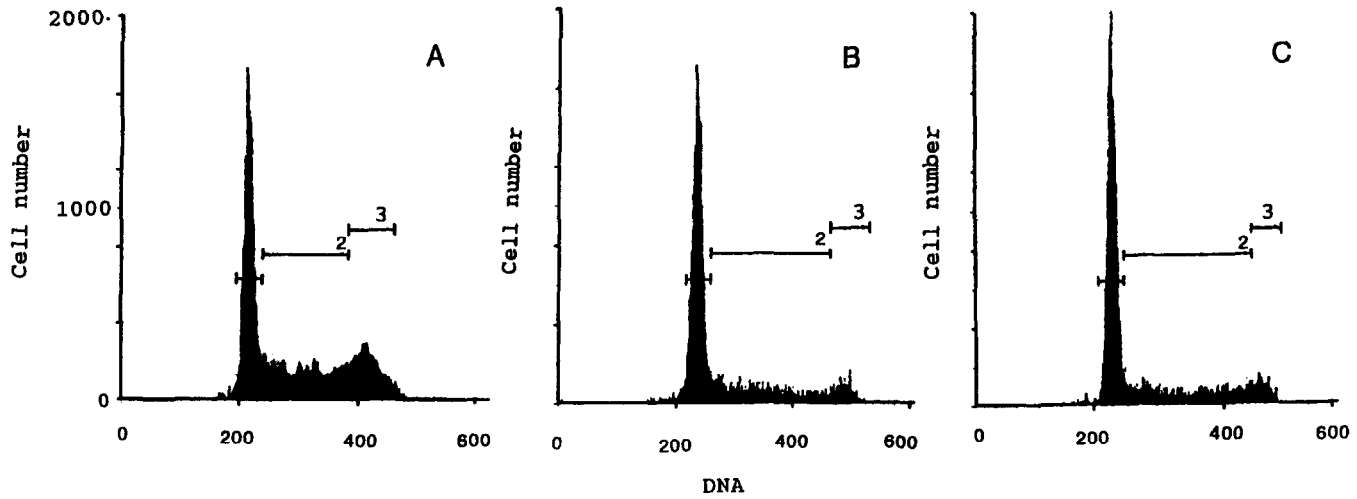


FIG. 2. Growth inhibitory effect of meisoindigo on ML-1 cells. ML-1 cells were incubated in 24-well flat-bottom plates in the absence (control) or presence of the indicated concentrations of meisoindigo. Cell growth was estimated by trypan blue dye exclusion as described in Materials and Methods. The data (means ± SEM) were obtained from four separate experiments with triplicate samples.

RNA Extraction and Northern Blotting

ML-1 cells were harvested from confluent cultures, washed extensively with serum-free medium, and synchronized by

* *Abbreviations:* FBS, fetal bovine serum; AP, acid phosphatase; NBT, nitroblue tetrazolium dye; and TPA, tetradecanoyl phorbol acetate.



	Control	Meisoindigo (0.72 ug/ml)	Daunomycin (5x10-9 M)
G1	36.10	65.82	68.76
S	41.95	24.87	22.96
G2+M	18.60	6.36	6.60

FIG. 3. Effect of meisoindigo on the ML-1 cell cycle. The cells were exposed to 0 (A: control), meisoindigo (B, 0.72 μg/mL) or daunomycin (C, 5 × 10^{-9}M) for 24 hr and the experiment was performed as described in Materials and Methods. This illustration is representative of three separate experiments with similar results.

incubating for another 2 days in RPMI 1640 medium containing 0.05% serum. This treatment arrested the majority (80%) of cells in G_1. The cells were washed twice with pre-warmed, serum-free RPMI 1640 medium and exposed to meisoindigo (0.72 μg/mL) for the time periods indicated in Fig. 7. Cells were harvested and washed once with cold PBS, and total RNA was extracted by the guanidinium/phenol method as described previously [20]. Quantitative analysis of RNA was conducted spectrophotometrically. Twenty micrograms of total RNA was fractionated on 1.2% agarose-formaldehyde gels and transferred to GeneScreen (NEN Research Products, Boston, MA, U.S.A.). The c-*myb* probe was radiolabeled randomly according to the company protocol. Prehybridization and hybridization were performed by procedures described by Sariban *et al.* [21]. The autoradiograms were analyzed by densitometer (Molecular Dynamics, Sunnyvale, CA) and normalized by 28S RNA.

RESULTS

Differentiation Induction of Meisoindigo

Treatment of ML-1 cells with different concentrations of meisoindigo in the presence of 10% FBS resulted in inhibition of cell growth. As shown in Fig. 2, during 3 days of exposure of the cells to 0.72 μg/mL of meisoindigo, the cell number remained almost constant without a significant decrease of viability. This concentration was therefore used as the optimal dose level.*

As shown in Fig. 3, exposure of ML-1 cells for 24 hr to 0.72 μg/mL of meisoindigo resulted in a significant accumulation in G_1 (65.82% in the treatment group compared with 36.10% in the control, $P < 0.05$). This effect was similar to that obtained with another DNA-specific agent, daunomycin. As demonstrated previously, the accumulation of cells in G_1 is generally followed by cell differentiation.*

Figure 4 shows the time- and concentration-dependent increase in AP activity in ML-1 cells; 72 hr after exposure of the cells to 0.72 μg/mL meisoindigo. AP activity, a marker of ML-1 cell differentiation, increased almost 2-fold. Higher (0.96, 1.2 μg/mL) or lower (0.24, 0.48 μg/mL) concentrations of the agent resulted in less AP activity (Fig. 4A). At the higher concentrations (0.96 μg/mL and above), dead cells were observed. Induction of AP activity by meisoindigo was found to be time dependent (Fig. 4B). The longer the exposure, the higher the AP activity.

The induction of ML-1 cell differentiation by meisoindigo was further supported by the finding that this agent

* Bloch A, DNA-specific antineoplastic agents as biological response modifiers. *Proceedings of the Seventh Symposium on Host Defense Mechanisms Against Cancer, Hakone, 8–10, November 1985*, pp. 296–302.

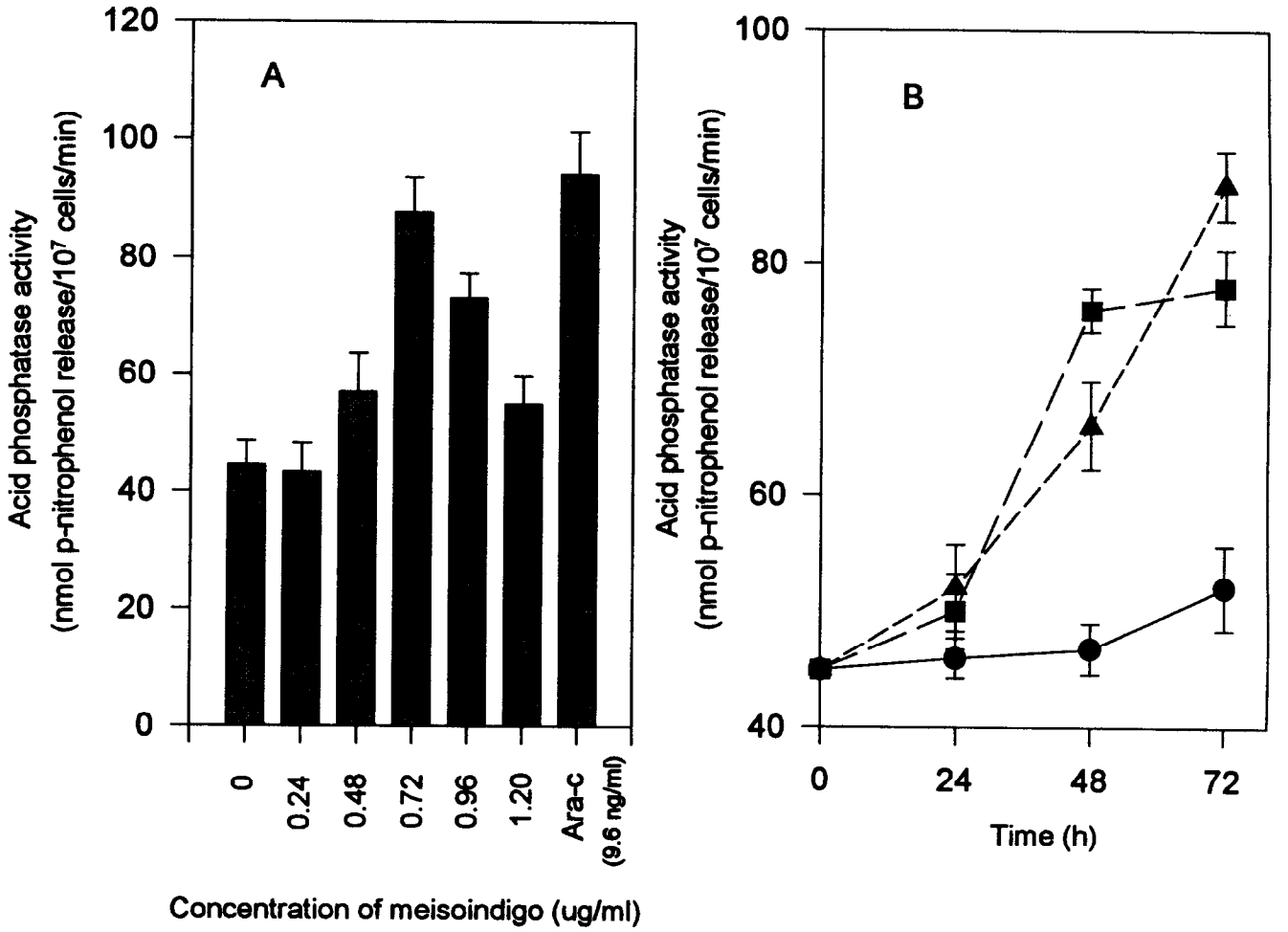


FIG. 4. Concentration- and time-dependent changes in AP activity induced by meisoindigo. ML-1 cells were treated with the indicated concentrations of meisoindigo for 72 hr or with the same concentrations of the agent for the indicated periods of time, and AP activity was measured as described in Materials and Methods. (A) Concentration–response at 72 hr. (B) Time–response at (–●–) control, (–▲–) 0.72 μg/mL, and (–■–) 0.96 μg/mL meisoindigo. Data are means ± SEM of three independent experiments.

enhanced the ability of ML-1 cells to reduce NBT. As shown in Fig. 5, after incubation of ML-1 cells for 3 days in the presence of meisoindigo, the formazan formation increased 12-fold in a concentration-dependent manner. Moreover, the increased AP activity and NBT-reducing ability were paralleled by an increase in the maturation of the ML-1 cells. On day 5, approximately 48% of the viable cell population (Table 1 and Fig. 6) matured with morphologic characteristics of monocytes or macrophages. Only 3% maturation was present in the untreated group.

Down-Regulation of c-myb Expression

The graph at the bottom of Fig. 7 shows the extent of *c-myb* expression as a function of time following treatment of ML-1 cells with meisoindigo, which is based on information contained in panels A and C of Fig. 7. *c-myb* was expressed at a high level in ML-1 cells during normal growth. The 0-time point shows the level of *c-myb* expressed in cells maintained in G_1 by the presence of only 0.05% serum. Upon removal of serum by extensive washing with serum-free medium, *c-myb* expression declined to a lower level maintained during the incubation period (Fig. 7A). These data indicate that growth arrest of the cells is not sufficient to extinguish *c-myb* expression. However, 3 hr treatment with meisoindigo, *c-myb* expression was decreased remarkably, and this low mRNA level was maintained for up to 7 hr (Fig. 7C). This result suggests that the decrease in *c-myb* mRNA associated with the commitment of ML-1 cells to differentiation was induced by meisoindigo.

DISCUSSION

Induction of the differentiation of neoplastic cells with antitumor drugs may contribute an alternative strategy for the treatment of malignant diseases [22–24, *]. The present study demonstrated that meisoindigo, like other DNA-specific agents, inhibits ML-1 cell growth through its ability to induce cell differentiation.

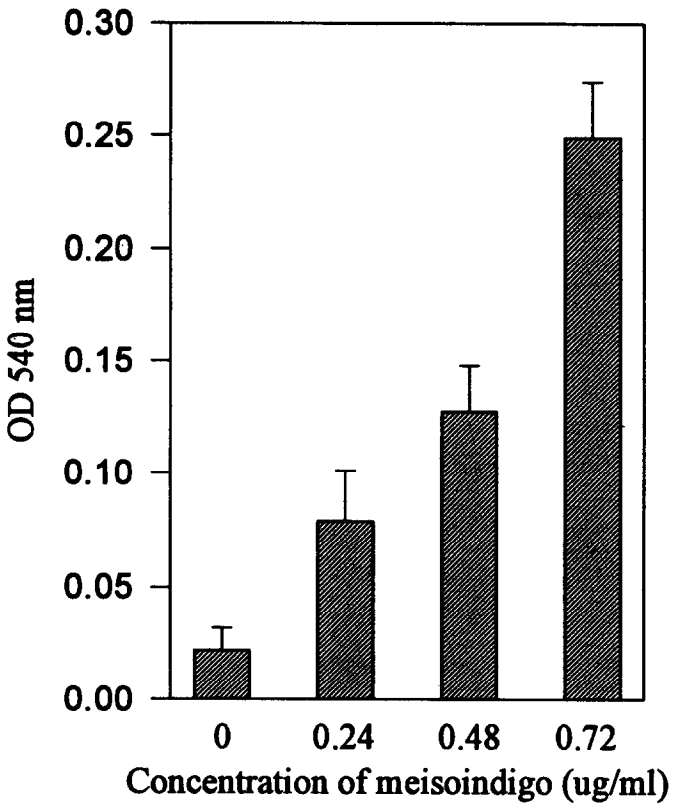


FIG. 5. Increase of NBT reduction by ML-1 cells following treatment with meisoindigo. ML-1 cells were exposed for 3 days to different concentrations of meisoindigo. The cells were harvested and washed with prewarmed serum-free RPMI 1640 medium, and then NBT reduction was measured as described in Materials and Method. The data (means ± SEM) were obtained from three separate experiments.

DNA-specific agents have been considered as biological response modifiers.* Since the proliferation program involves DNA synthesis and transcription while the differentiation program is silent, DNA-specific agents interrupt the expression of the proliferation program through their modification of biological response, such as inhibition of DNA synthesis, replication, or transcription, causing the cells to accumulate in G_1. As a result of this interruption, the transformed cell regains its potential to mature. This process is initiated in the presence of an appropriate differentiation signal. Our findings indicate that meisoindigo, like other DNA-specific agents, causes ML-1 cells to differentiate terminally. Recent studies have provided evidence that c-*myb* plays an important role in the control of cell proliferation and differentiation as well as in the development of a variety of tumors [25–28]. This gene is expressed predominantly at immature stages of development in the hematopoietic system. Levels of c-*myb* mRNA decrease remarkably

* Bloch A, DNA-specific antineoplastic agents as biological response modifiers. *Proceedings of the Seventh Symposium on Host Defense Mechanisms Against Cancer, Hakone, 8–10, November 1985*, pp. 296–302.

TABLE 1. Morphological maturation of ML-1 cells induced by meisoindigo

Concentration of the agent (μg/mL)	Percent of the maturation	
	3 days	5 days
Control	1.2 ± 1.0	3.2 ± 1.6
0.24	7.5 ± 1.9	10.4 ± 2.9
0.48	14.7 ± 2.3	35.7 ± 4.1
0.72	32.4 ± 4.7	48.3 ± 5.7

ML-1 cells were exposed to indicated concentrations of meisoindigo for 3 or 5 days in normal culture conditions. Slides of viable cells were prepared by Cytospin centrifugation of 50-μL aliquots of the cell suspensions, and after staining with Wright-Giemsa differential counts were made with a light microscope; at least 250 cells were counted for each experimental condition. All assay data (means ± SEM) were derived from three independent experiments.

during chemically induced differentiation of several hematopoietic tumor cell lines. Recent experiments have demonstrated that c-*myb* is an important oncogene that is up-regulated by growth factors and down-regulated by differentiation factors at an early stage [12, 25]. The findings presented here indicate that c-*myb* expression in ML-1 cells decreased following treatment with meisoindigo. This early down-regulation of c-*myb* in ML-1 cells was also obtained when the cells were treated with TPA [11]. Therefore, the early modulation of c-*myb* expression by meisoindigo may be a biochemical effect related to the induction of differentiation. These data indicate that the regulatory effect of meisoindigo on cell differentiation and c-*myb* oncogene ex-

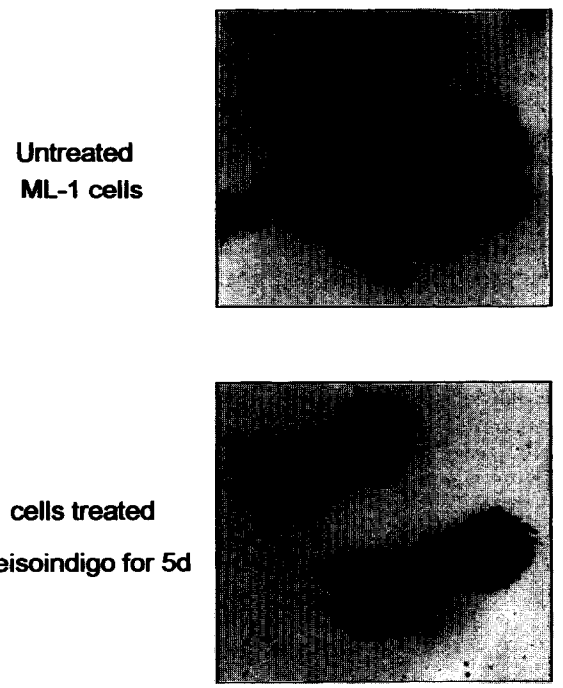


FIG. 6. Examples of morphological change of ML-1 cells after treatment with meisoindigo. ML-1 cells were exposed to 0.72 μg/mL for 5 days, and slides of viable cells were prepared by Cytospin centrifugation of 50-μL aliquots of the cell suspensions and stained with Wright-Giemsa.

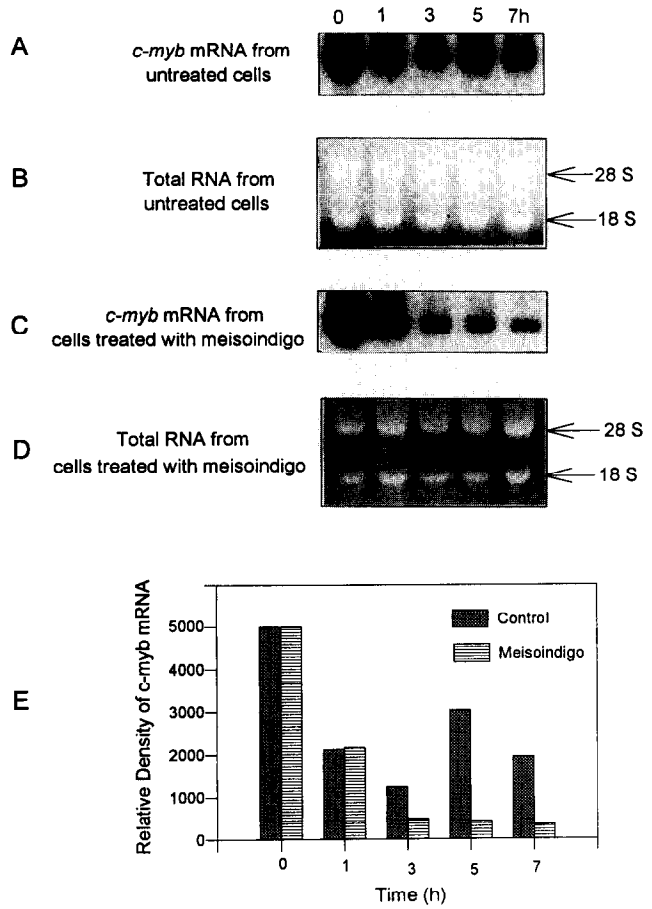


FIG. 7. c-*myb* expression after treatment of ML-1 cells with meisoindigo. c-*myb* mRNA in untreated cells (A) or in the cells treated with 0.72 μg/mL meisoindigo for the indicated periods of time (C) was measured by northern blotting. (B) and (D) show the total RNA from untreated control or from cells treated with the agent. The autoradiograms from the northern blotting were quantitated by densitometry and normalized by 28S RNA (E). The figures represent examples of three separate experiments with similar results.

pression plays an important role in the antitumor action of the agent, which may explain its strong antitumor action and low toxicity.

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