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Interaction of Antileukemia Agents Adriamycin and Daunomycin with Sphinganine on the Differentiation of Human Leukemia Cell Line HL-60[1]

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ABSTRACT

A slight induction of granulocytic differentiation of HL-60 cells occurred after treatment with antileukemia chemotherapeutic agents Adriamycin (ADM) and daunomycin (DM). Addition of an inhibitor (sphinganine, SP) of protein kinase C (PKC) enhanced 2–4-fold the ADM or DM-induced differentiation. This phenomenon was accompanied by a slightly augmented antiproliferative effect. The enhancement of differentiation induction in these treatments seemed to be absolute, since the combination treatment (ADM-SP or DM-SP) showed about 2.5–3.6 times as many differentiated cells as the treatment with the anticancer drugs ADM or DM alone. Further characterization of the interaction of ADM and DM with SP on differentiation of HL-60 cells was carried out. Whereas the addition of SP in the fresh medium after the removal of ADM or DM (0.5 h treatment) enhanced the induction of differentiation, a pretreatment (24 h) of the cells with SP followed by continuous exposure to ADM or DM did not show such enhancement effect. The addition of SP at as late as 48 h after the administration of ADM or DM potentiated the induction of differentiation to the same extent as in the simultaneous combination of ADM-SP or DM-SP. Similar results were obtained in the experiments with another PKC inhibitor, staurosporine. These results indicated that inhibition of PKC activities may play an important role in the later events during the induction of differentiation elicited by ADM or DM. The use of the antileukemia drugs ADM and DM in combination with an inhibition of PKC activity results in enhancement of induction of differentiation and suggests a new strategy and a promising approach to the treatment of leukemia.

INTRODUCTION

It is widely appreciated that ADM[3] and DM are cytotoxic compounds. Essentially all growing cells are susceptible to growth inhibition by these agents. ADM and DM are clinically active against a number of human cancers, including both hematological and solid tumors (1, 2). Several mechanisms have been proposed to explain their antitumor properties (3–8). It has been suggested that ADM and DM enter cells passively and leave cells by a mechanism that appears to involve the gp150–180 product of the *mdr1* gene, the amplification of which is associated with the "multiple drug-resistant" phenotype (9–12). PKC is thought to play a role in multidrug resistance. Changes in PKC activity have been reported in multidrug-resistant MDR sublines (13–15). Recent results by Chambers *et al.* (16) have indicated that PKC-mediated phosphorylation stimulates the drug transport activity of P-glycoprotein. In the studies of the biological effects elicited by ADM, Posada *et al.* (17) have shown that PKC activity is altered by ADM and this modulation appears to be linked to the cytotoxic effect of the drug as well as the acquired ability of cell to resist the cytotoxic action.

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[3] The abbreviations used are: ADM, Adriamycin; DM, daunomycin; SP, sphinganine; PKC, protein kinase C; RA, retinoic acid.

It has been noted that a slight induction of morphological differentiation of HL-60 cells occurs after treatment with certain antileukemia chemotherapeutic agents, particularly ADM and DM (18). Although the cellular target site and the mechanism of action of these differentiation inducers have not been clearly established, it is quite possible that PKC is implicated in such leukemia cell differentiation. Since PKC is thought to play some roles along the pathway of the induction of differentiation (19–22) and the possibility of the involvement of PKC in the biological effect elicited by ADM, studies of the interaction of ADM and DM with SP, a potent inhibitor of PKC (21), were carried out. The elucidation of the influence of different ADM-SP and DM-SP administration schedules and sequences on the proliferation and induction of differentiation of HL-60 cells was also made. The addition of sphinganine simultaneously or at as late as 48 h after administration of ADM or DM was found to increase the percentage of ADM- or DM-treated HL-60 cells that exhibited mature morphology.

MATERIALS AND METHODS

Drugs and Chemicals. Adriamycin, daunomycin, staurosporine, and sphinganine were purchased from Sigma Chemical Co., St. Louis, MO. The dye, Wright, was from Sigma, and the Giemsa stain was purchased from Aldrich Chemical Co. The lipid sphinganine was dissolved in absolute ethanol at 10^{-2} M.

Cells. The HL-60 promyelocytic leukemic cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotics in a 5% CO_2 humidified incubator at 37°C.

Cell Growth Determination. HL-60 cells (approximately 2×10^5/ml) cultured in 75-cm^2 plastic tissue culture flasks were incubated with Adriamycin for 30 min. Cells were then washed 3 times with phosphate-buffered saline before fresh medium was added. Cultures were incubated at 37°C. At various times after the washing procedure, cultures were harvested. Cell numbers were obtained by counting cell suspensions with hemocytometer.

Wright-Giemsa Stain. Morphological assessment of the induced cells was performed using the Wright-Giemsa staining method. Cells (5×10^4) were prepared on slides by Cytospin (Shandon Southern) and stained with Wright-Giemsa stain. The morphology of cells was examined under a light microscope (×1000). The criteria for the morphology judging was according to those previously described (23). Triplicate 200-cell counts were performed. Because sphinganine is a normal intermediate of sphingolipid biosynthesis, exogenously supplied sphinganine is metabolized by cells to complex sphingolipids (24). Therefore to maintain cellular levels during the 4–5-day differentiation period, sphinganine was supplied daily.

RESULTS

Drug Effects on HL-60 Cell Proliferation. Initial experiments demonstrated that ADM and DM inhibited HL-60 cell growth in a dose- and time-dependent fashion. Following a 72-h exposure, increasing concentrations of ADM (0.02–0.04 μM) inhibited cell proliferation by 30–69%, compared to controls (Fig. 1). ADM (0.04 μM) reduced cell growth by 23, 40, and 69%

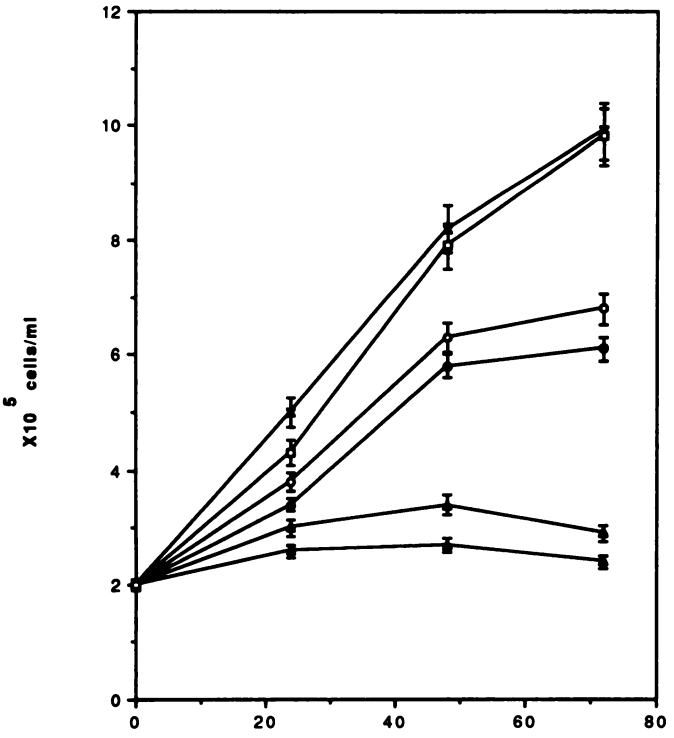

Fig. 1. Cell growth in HL-60 cells after treatment with ADM in the absence or presence of SP. HL-60 cells were incubated with 0.02–0.04 μM ADM in the absence or presence of SP for various times (indicated). Cultures were then harvested. Cell numbers were obtained by counting cell suspensions with hemocytometer. *Points*, mean of triplicate samples; *bars*, SD. The figure is representative of the results obtained in several (at least three) replications of the experiments. ×, control cells without drug treatment; □, cells treated with 2.5 μM SP alone; ○, 0.02 μM ADM treatment without SP; ●, 0.02 μM ADM with 2.5 μM SP treatment. △, 0.04 μM ADM treatment without SP; ▲, 0.04 μM ADM with 2.5 μM SP treatment.

after 8, 24, and 72 h of exposure (Fig. 1). Similarly, increasing concentration of DM (0.004–0.008 μM) inhibited cell growth by 21–46% (Fig. 2). DM (0.008 μM) reduced cell growth by 20, 35, and 46% after 8, 24, and 72 h of exposure (Fig. 2). The ability of 2.5 μM SP, a protein kinase C inhibitor, to slightly augment the antiproliferative effect of ADM (0.02–0.04 μM) or DM (0.004–0.008 μM) is shown in Figs. 1 and 2, respectively. In the presence of 0.02 μM ADM, cell number increased from $2.0 \pm 0.1 \times 10^5$ to $6.8 \pm 0.4 \times 10^5$ (69% of control cell number) between 0 and 72 h; whereas cell number increase to a lesser extent from $2.0 \pm 0.1 \times 10^5$ to $6.1 \pm 0.3 \times 10^5$ (62% of control cell number) if SP were also present. Similar slight enhancement of antiproliferative effect was also noted in the DM-SP combination treatment (Fig. 2). SP (2.5 μM) alone had litttle effect on inhibition of the cell growth of HL-60 cells after 72 h incubation (Fig. 2).

Drug Effects on HL-60 Cell Differentiation. A slight induction of morphological differentiation of HL-60 cells occurred after treatment with ADM or DM (Table 1). SP alone had little effect on the extent of differentiation of HL-60 cells during the 4-day incubation. Simultaneous treatment with either ADM (0.02–0.04 μM) or DM (0.004–0.008 μM) and 2.5 μM SP resulted in an about 2–4-fold increase in the percentage of differentiated cells. The enhancement of differentiation induction in these treatments seemed to be absolute, since the combination treatment (ADM-SP or DM-SP) showed about 2.5–3.6 times as many differentiated cells as the treatment with the anticancer drugs ADM or DM alone (Table 2).

Drugs Administered Sequentially. In an attempt to study the mechanism of the interaction of ADM or DM with SP in the induction of differentiation further, the drugs were administered to HL-60 cells in different schedules and sequences. Pretreatment (24 h) of the cells with SP followed by continuous exposure to ADM or DM, failed to potentiate the induction of differentiation (Table 1). SP supplied daily to the SP-pretreated (24 h) cells in the continuous exposure to ADM or DM potentiated the induction of differentiation to the same extent as in the simultaneous combination of ADM-SP or DM-SP (Table 1). Therapeutically, it is generally agreed that following drug administration the circulating and tissue concentrations decline as excretion processes take place. The tumor will probably be exposed to the drug for a short period of time. The effect of short exposure of ADM or DM with the addition of SP on induction of differentiation was therefore carried out. A slight induction of differentiation was observed on ADM (0.5–1.0 μM) or DM (0.1–0.2 μM) short exposure (30 min) cells. About 20–30% of the cells resembled mature granulocytes at day 4 after the removal of ADM or DM (Table 3). The addition of SP (2.5 μM) to the fresh culture medium after the removal of ADM or DM also resulted in about 2–4-fold enhancement of differentiation induction. At day 4 after the removal of ADM (0.5–1.0 μM) or DM (0.1–0.2 μM), about 50–70% of the cells

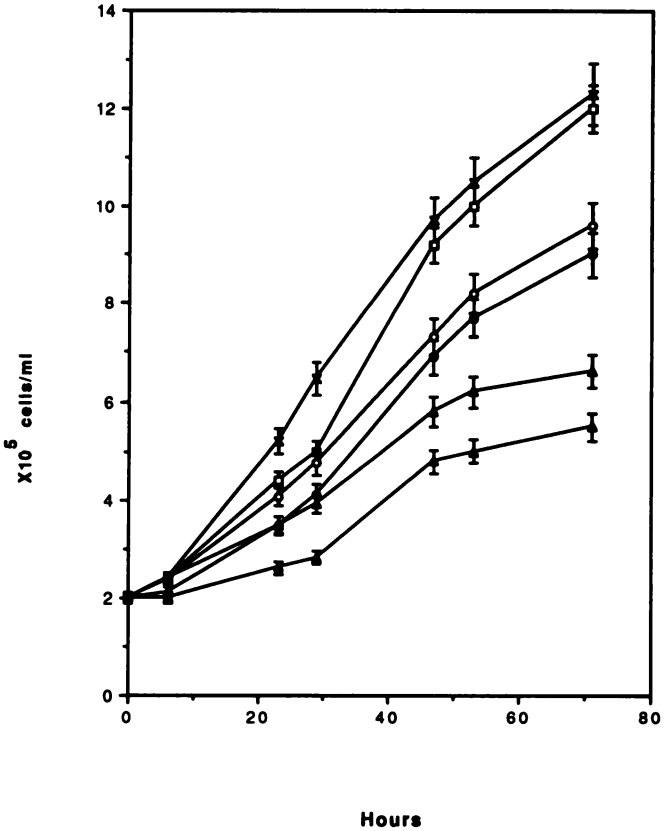

Fig. 2. Cell growth in HL-60 cells after treatment with DM in the absence or presence of SP. HL-60 were incubated with 0.004–0.008 μM DM in the absence or presence of SP for various times (indicated). Cultures were then harvested. Cell numbers were obtained by counting cell suspensions with hemocytometer. *Points*, mean of triplicate samples; *bars*, SD. The figure is representative of the results obtained in several (at least three) replications of the experiments. ×, control cells without drug treatment; □, cells treated with 2.5 μM SP alone; ○, 0.004 μM DM treatment without SP; ●, 0.004 μM DM with 2.5 μM SP treatment. △, 0.008 μM DM treatment without SP; ▲, 0.008 μM DM with 2.5 μM SP treatment.

Table 1 *Effects of sphinganine in the ADM and DM continuous treatment on the induction of differentiation*

HL-60 cells were cultured in 75-cm² plastic tissue culture flasks. ADM (0.02–0.04 μM) and DM (0.004–0.008 μM) with or without 2.5 μM SP (pretreated for 24 h and/or continued to be supplied daily) was added to the culture medium for 4 days. Differentiation was assessed by morphology change as described in "Materials and Methods."

Drug	Dose (μM)	SP pretreatment (24 h)	SP daily	Blast[a]	Pro	Mature cells			
						Myelo	Granulocytes		
							Meta	Band	Seg
	0	−	−	3	92	5	0	0	0
	0	−	+	4	94	1	1	0	0
	0	+	−	2	93	4	1	0	0
	0	+	+	2	92	5	1	0	0
ADM	0.02	−	−	5	82	11	2	0	0
	0.02	−	+	5	56	37	2	0	0
	0.02	+	−	5	82	6	7	0	0
	0.02	+	+	4	54	39	3	0	0
	0.04	−	−	5	76	14	5	0	0
	0.04	−	+	1	46	40	12	1	0
	0.04	+	−	5	75	18	2	0	0
	0.04	+	+	4	44	44	8	0	0
DM	0.004	−	−	2	84	10	3	1	0
	0.004	−	+	1	44	50	4	1	0
	0.004	+	−	2	82	12	3	1	0
	0.004	+	+	1	46	49	3	1	0
	0.008	−	−	1	79	16	3	1	0
	0.008	−	+	1	25	42	30	2	0
	0.008	+	−	3	78	15	3	1	0
	0.008	+	+	1	28	39	32	0	0

[a] Blast, myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded (stab) neutrophil; Seg, segmented neutrophil. Triplicate 200-cell counts were performed. The table is representative of the results obtained in several (at least three) replications of the experiment. Cell viability was over 95% under these conditions.

with SP resembled mature granulocytes. These results suggested that the biological action of ADM and DM may have to precede the inhibition of PKC activities during the induction of differentiation. The influence of the changes of PKC activities on the ADM- or DM-induced differentiation may be critical in the later events during the granulocytic differentiation of HL-60 cells. To test this hypothesis further, the combination effects of ADM-SP or DM-SP on differentiation were examined. SP was added at 0, 12, 24, or 48 h after the administration of ADM or DM. Our results showed that the addition of SP at as late as 48h after ADM or DM administration enhanced the induction of differentiation to the same extent as in the simultaneous combination of ADM-SP or DM-SP (Table 4).

Moreover, the idea that SP is working through PKC is further supported by experiments with another PKC inhibitor, staurosporine (25). Staurosporine (2 nM) alone had little effect on the cell growth and the extent of differentiation of HL-60 cells during the 4-day incubation. Similar to the results in the experiments above with SP, simultaneous treatment with either ADM (0.02–0.04 μM) or DM (0.004–0.008 μM) and 2.0 nM staurosporine resulted in an about 2–4-fold increase in the percentage of differentiated cells (Table 5) while causing a slight enhancement of anti-proliferative effects of ADM or DM (data not shown). Addition of staurosporine at as late as 48 h after ADM or DM administration also enhanced the induction of differentiation to the same extent as in the simultaneous combination of ADM or DM and staurosporine (data not shown).

DISCUSSION

In this study, we have demonstrated that the treatment with antileukemia chemotherapeutic agents ADM and DM in combination of SP, a PKC inhibitor, results in the absolute enhancement of differentiation of HL-60 cells. The biological effect elicited by ADM or DM seems to have to precede the action of SP in such absolute enhancement of differentiation. The addition of SP in the fresh medium after the removal of ADM or DM results in the enhancement of differentiation. Pretreatment with SP followed by continuous exposure to ADM or DM does not show such an enhancement effect. The addition of SP at as late as 48 h after ADM or DM administration could potentiate the induction of differentiation to the same extent as in the simultaneous combination of ADM-SP or DM-SP. Our results indicated that inhibition of PKC activities may play an important role in the later events during the granulocytic differentiation of HL-60 cells elicited by ADM or DM. Recent studies by Ishikura *et al.* (26) have shown that the treatment with hydroxyurea, which block the cells at the G_1-S boundary results in the absolute enhancement of differentiation of HL-60 cells induced by RA or $1\alpha,25(OH_2)$-vitamin D_3. It is possible that following the blocking of cell cycle at some points by cell cycle-specific antitumor agents, ADM and DM, the inhibition of PKC activities at the later stages may lead to the turning on of some critical signals and results in the absolute enhancement of the induction of differentiation. The studies in this report indicating the involvement of PKC have shed some light on the understanding of the mechanism of action of ADM and DM on the

Table 2 *Number of the differentiated cells in sphinganine treatment with ADM or DM*

2.5 μM SP treatment with	Dose (μM)	No. of differentiated cells[a] (ratio to the ADM or DM treatment alone)
ADM	0.02	2.7 ± 0.16[b]
	0.04	2.5 ± 0.19
DM	0.004	3.6 ± 0.16
	0.008	3.0 ± 0.17

[a] The numbers of differentiated cells was calculated as

$$\frac{\%\ \text{morphologically mature cells}}{100 \times \text{viable cell number}}$$

The values represent the ratio of the number of differentiated cells in the combination (ADM-SP or DM-SP) treatment to the number of those in the ADM or DM treatment alone.

[b] Mean ± SD of independent sets of triplicate experiments.

Table 3 *Potentiation of differentiation by the posttreatment with sphinganine upon removal of ADM or DM after 0.5-h treatment*

HL-60 cells were cultured in 75-cm² plastic tissue culture flasks. Various doses of ADM (0.5–1.0 μM) and DM (0.1–0.2 μM) were added to the culture medium. After 30 min of incubation , the drugs were removed (washed 3 times with 1 × phosphate-buffered saline). Fresh medium with or without 2.5 μM sphinganine (supplied daily) was added. Differentiation was assessed by morphology change as described in "Materials and Methods" at day 4.

					Mature cells			
						Granulocytes		
Drug	Dose (μM)	SP daily	Blast[a]	Pro	Myelo	Meta	Band	Seg
	0	–	1	95	4	0	0	0
	0	+	1	94	5	0	0	0
ADM	0.5	–	1	77	20	2	0	0
	0.5	+	1	43	40	12	4	0
	1.0	–	1	71	27	1	0	0
	1.0	+	1	32	52	12	3	0
DM	0.1	–	1	71	17	10	1	0
	0.1	+	1	36	45	14	4	0
	0.2	–	1	64	22	10	3	0
	0.2	+	1	29	53	13	4	0

[a] Blast, myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded (stab) neutrophil; Seg, segmented neutrophil. Triplicate 200-cell counts were performed. The table is representative of the results obtained in several (at least three) replications of the experiment. Cell viability was over 95% under these conditions.

Table 4 *Effects of the addition of sphinganine at various times in the ADM and DM continuous treatment on the induction of differentiation*

HL-60 cells were cultured in 75-cm² plastic tissue culture flasks. ADM (0.02 μM) and DM (0.008 μM) were added to the culture medium. Sphinganine (2.5 μM) was added to the culture medium at various times after administration of ADM or DM. Differentiation was assessed by morphology change as described in "Materials and Methods" at day 4.

					Mature cells			
						Granulocytes		
Drug	Dose (μM)	SP daily (h)	Blast[a]	Pro	Myelo	Meta	Band	Seg
	0	–	2	93	5	0	0	0
	0	+/0[b]	3	94	3	0	0	0
ADM	0.02	–	5	83	10	2	0	0
	0.02	+/0	3	56	39	2	0	0
	0.02	+/12	3	52	41	4	0	0
	0.02	+/24	3	53	39	5	0	0
	0.02	+/48	2	55	38	5	0	0
DM	0.008	–	2	82	12	3	1	0
	0.008	+/0	1	43	39	13	4	0
	0.008	+/12	1	42	38	12	7	0
	0.008	+/24	1	45	32	18	4	0
	0.008	+/48	2	59	29	7	3	0

[a] Blast, myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded (stab) neutrophil; Seg, segmented neutrophil. Triplicate 200-cell counts were performed. The table is representative of the results obtained in several (at least three) replications of the experiment. Viability of the cells was over 95% under these conditions.

[b] The first addition of sphinganine was added at 0, 12, 24, or 48 h after the administration of ADM or DM. The sphinganine was further supplied daily afterwards.

Table 5 *Effects of staurosporine in the ADM and DM continuous treatment on the induction of differentiation*

HL-60 cells were cultured in 75-cm² plastic tissue culture flasks. ADM (0.02–0.04 μM) and DM (0.004–0.008 μM) with or without 2 nM staurosporine (ST) was added to the culture medium for 4 days. Differentiation was assessed by morphology change as described in "Materials and Methods."

					Mature cells			
						Granulocytes		
Drug	Dose (μM)	ST (2 nM)	Blast[a]	Pro	Myelo	Meta	Band	Seg
	0	–	3	93	4	0	0	0
	0	+	4	92	3	1	0	0
ADM	0.02	–	4	81	12	3	0	0
	0.02	+	5	66	28	1	0	0
	0.04	–	3	78	13	6	0	0
	0.04	+	1	46	42	10	1	0
DM	0.004	–	2	82	11	4	1	0
	0.004	+	1	59	36	3	1	0
	0.008	–	1	80	15	3	1	0
	0.008	+	1	39	40	15	5	0

[a] Blast, myeloblast; Pro, promyelocyte; Myelo, myelocyte; Meta, metamyelocyte; Band, banded (stab) neutrophil; Seg, segmented neutrophil. Triplicate 200-cell counts were performed. The table is representative of the results obtained in several (at least three) replications of the experiment. Viability of the cells was over 95% under these conditions.

induction of differentiation. Previously, PKC has been implicated to play a role in RA- and phorbol ester-induced differentiation. Earlier studies by Stevens *et al.* (21, 27) showed that SP enhances the RA-induced but on the other hand inhibits the phorbol ester-induced differentiation of HL-60 cells. Hoffman and Newlands (28) recently reported that inhibition of PKC blocks ADM-induced erythroid differentiation. Our results of enhancement of ADM-induced differentiation by SP suggested that the regulation of this differentiation program may be in some way similar to RA-induced differentiation, but different than that involved in phorbol ester-induced differentiation and the ADM-induced erythroid differentiation. The mechanistic basis of understanding the apparent synergism observed between the growth inhibitors and blockers of PKC is under current investigation.

The modulation of PKC activities has been linked to the cytotoxic effect of the ADM as well as the acquired ability of cells to resist the cytotoxic action (17). These results raise the possibility that the cell signaling mechanisms, particularly those involving PKC, may play an important role in mediating the biological action of the anticancer drug ADM. Our present results further indicated that morphological differentiation may be one of the important biological action of ADM and DM that is regulated by PKC.

ADM and DM are potent antineoplastic agents, possessing significant potential on the treatment of most hematological malignancies (1, 2). The use of ADM and DM in patients is, however, limited due to their toxicity such as cardiac toxicity (29). Much attention has been paid on the averting their toxicity and increasing their therapeutic efficacy (30). From a clinical point of view, the use of the standard antileukemia drugs such as ADM and DM at their low subtoxic doses, in combination with a modulation of PKC activity, results in potentiating the induction of differentiation and thereby suggests a new strategy and a promising approach to the treatment of leukemia.

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