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Dimethylamiloride-induced differentiation of HL-60 cells[1]

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KEY WORDS cell division; cell differentiation; HL-60 cells; amiloride; inhibitory concentration 50

ABSTRACT

AIM: To study the effect of 5-(*N*, *N*-dimethyl) amiloride (DMA) on the proliferation and differentiation of HL-60 cells *in vitro*. **METHODS**: MTT assay to test cytotoxicity; cell staining and NBT reduction to test cell differentiation. **RESULTS**: DMA inhibited HL-60 cells growth in a concentration-dependent manner, and IC_{50} value for 96 h was 31.7 (95 % confidence limits: 6.3 – 57.1) $\mu mol \cdot L^{-1}$. DMA also induced granulocytic differentiation in HL-60 cells. The percentage of differentiating cells increased from 6.5 % to 70 % after DMA 100 $\mu mol \cdot L^{-1}$ treatment for 3 d. The differentiating effect of DMA was distinguishable from amiloride, 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), and (5-(*N*-methyl-*N*-isobutyl) amiloride (MIA). None among the amiloride, EIPA, and MIA were capable of triggering the differentiation of HL-60 cells. **CONCLUSION**: DMA inhibited the proliferation of HL-60 cells and induced differentiation of HL-60 cells.

INTRODUCTION

Normal hemopoietic maturation involves the progressive differentiation of precursor cells into terminal differentiated form, ie, neutrophils, monocytes, and macrophages. HL-60 cells are human leukemia myeloid precursor cells that can be induced to undergo differentiation in response to a variety of biological and chemical agents[1].

Currently, chemotherapy of cancer is based principally on agents that are toxic to the cells. Several reports suggest that induction of cellular differentiation may supplement the use of cytotoxic drugs in several forms of cancer, such as the successful use of retinoic acid in the treatment of acute promyelocytic leukemia (APL)[2] or oral leukoplakia[3]. Interest is also developing for the use of other differentiation-inducing agents for evaluating their use in antitumor[4–6] therapy.

It has been reported that amiloride, an inhibitor of Na^+ passive flux across membranes, blocks dimethyl sulfoxide (Me_2SO)-induced differentiation of erythrocytic leukemia cell[7] and potentiates the Me_2SO-induced differentiation of HL-60 cells[8]. Several analogues of amiloride including of DMA, EIPA, MIA, *etc*, had a similar effect in potentiating the Me_2SO-induced differentiation in HL-60 cells[8]. However, it is not known whether amiloride or its analogue can trigger the differentiation directly without Me_2SO. We have therefore screened amiloride and several compounds of amiloride with mimic variant in *N*-5 group in HL-60 cells for evaluating their effects on differentiation. We have found that DMA directly induced HL-60 cells into terminal differentiation without Me_2SO, and the effect was distinguishable from amiloride and several other analogues of amiloride.

MATERIAL AND METHODS

Chemicals Amiloride, 5-(*N*, *N*-dimethyl) amiloride (DMA), 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), 5-(*N*, *N*-methyl-*N*-isobutyl) amiloride (MIA), 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT), and tetradecanoyl phorbol acetate (TPA) were obtained from Sigma Chemical Co, USA. Fetal calf serum and RPMI 1640 medium were purchased from Gibco, USA. Amiloride, EIPA, and MIA were dissolved in H_2O or Me_2SO in a stock of 30 $mmol \cdot L^{-1}$. DMA was dissolved in H_2O in a stock of 30 $mmol \cdot L^{-1}$.

Cell culture Human promyelocytic leukemia cells

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HL-60 were cultured in suspension of RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum, benzylpenicillin 100 kU/L, and streptomycin 100 mg/L (complete RPMI) in a humidified atmosphere containing 5 % CO_2 at 37 ℃. All experiments were performed using complete RPMI.

Cytotoxicity assay Exponentially growing HL-60 cells were planted in flat-bottomed 96-well plates at 1×10^4 cells/well. DMA was added in serial dilution to give a final volume of 200 μL/well. The plates were incubated for 24 h, 48 h, 72 h, and 96 h to test cell viability. The cells in each well were transferred to 1.5 mL Eppendorf tubes at indicated time, and MTT 20 μL (5 g·L^{-1}) was added to each tube, and the cells were incubated for additional 4 h at 37 ℃. The supernatant were aspirated after being centrifuged and formazan crystals were dissolved in Me_2SO 1 mL. The absorbance at 570 nm was read on spectrophotometer. Inhibitory concentration 50 (IC_{50}) for a particular drug was defined as the concentration causing 50 % decrease in cell growth.

Evaluation of cellular morphology For morphological assessment of the cells, 1×10^5 cells were taken onto slides, which were fixed in 100 % methanol and stained with Giemsa. Cells were scored as differentiated if they showed features of metamyelocytes or more differentiated forms. Differential counts were performed under light microscopy on a mininum of 200 cells for each experimental point.

NBT reduction assay HL-60 cells grown in complete RPMI 1640 medium for 72 h were counted, 3×10^5 cells were suspended in RPMI 1640 medium 0.2 mL, NBT 0.8 mL (0.125 %) and TPA 20 μL (10^{-5} g·L^{-1}) were added. The cells were incubated for 30 min at 37 ℃. The cells were resuspended in PBS 0.5 mL after being centrifuged to remove NBT and PMA, and then were transferred to a 24-well plate. The percentage of NBT-positive cells in each preparation was determined under reverse phase microscopy.

Statistical analysis Statistical analyses were made using t test. Data are expressed as $\bar{x} \pm s$, n was number of independent experiments.

RESULTS

Effect of DMA on HL-60 cells growth DMA depressed the growth of HL-60 cells in a concentration-dependent manner. The concentration-response curve is shown in Fig 1. After treatment of HL-60 cells with DMA for 96 h, the IC_{50} (95 % confidence limits) was 31.7 (6.3 – 57.1) μmol·L^{-1}.

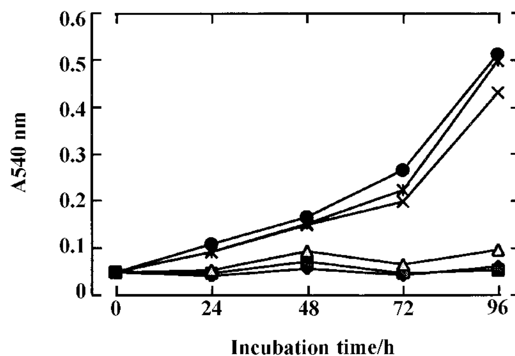

Fig 1. Growth inhibition by DMA on HL-60 cells. 1×10^4 cells were incubated with 300 μmol·L^{-1} DMA (◆), 200 μmol·L^{-1} DMA (■), 100 μmol·L^{-1} DMA (△), 10 μmol·L^{-1} DMA (×), 1 μmol·L^{-1} DMA (*), and without drugs (●) for 24 h, 48 h, 72 h, and 96 h. The cell proliferation was expressed as absorbance at 540 nm in MTT assay. The experiment was repeated twice.

Effect of DMA on HL-60 cell differentiation Untreated HL-60 cells, growing exponentially, had morphologic characteristic of a blastic leukemia cell line (Fig 2A). There was little evidence of spontaneous differentiation, as measured by either a low abundance of morphologically differentiated cells (<6.5 %, Tab 1) or a low frequency of cells competent to reduce the NBT dye (NBT+ cells, <5.5 %, Tab 1). After 72 h of treatment with DMA, HL-60 cells showed a morphologic differentiation (detected by the appearance of cells with band and segmented nuclei, and a decrease in the nuclear/cytoplasmic volume ratio).

The percentage of differentiated cells increased from 6.5 % to 70 % at 72 h of treatment with DMA 100 μmol·L^{-1}. To test whether DMA induced HL-60 cells

Tab 1. Differential counts and NBT reduction of HL-60 cells after incubation with various concentrations of DMA for 72 h. $\bar{x} \pm s$. $^cP < 0.01$ vs control.

DMA/ μmol·L^{-1}	Morphologically mature cells/% ($n=3$)	NBT positive cells/% ($n=3$)
0	6.5±0.6	5.5±0.4
25	21.1±3.0^c	27.7±2.0^c
50	40.3±3.1^c	36.0±4.0^c
100	70.5±4.5^c	60.3±4.9^c

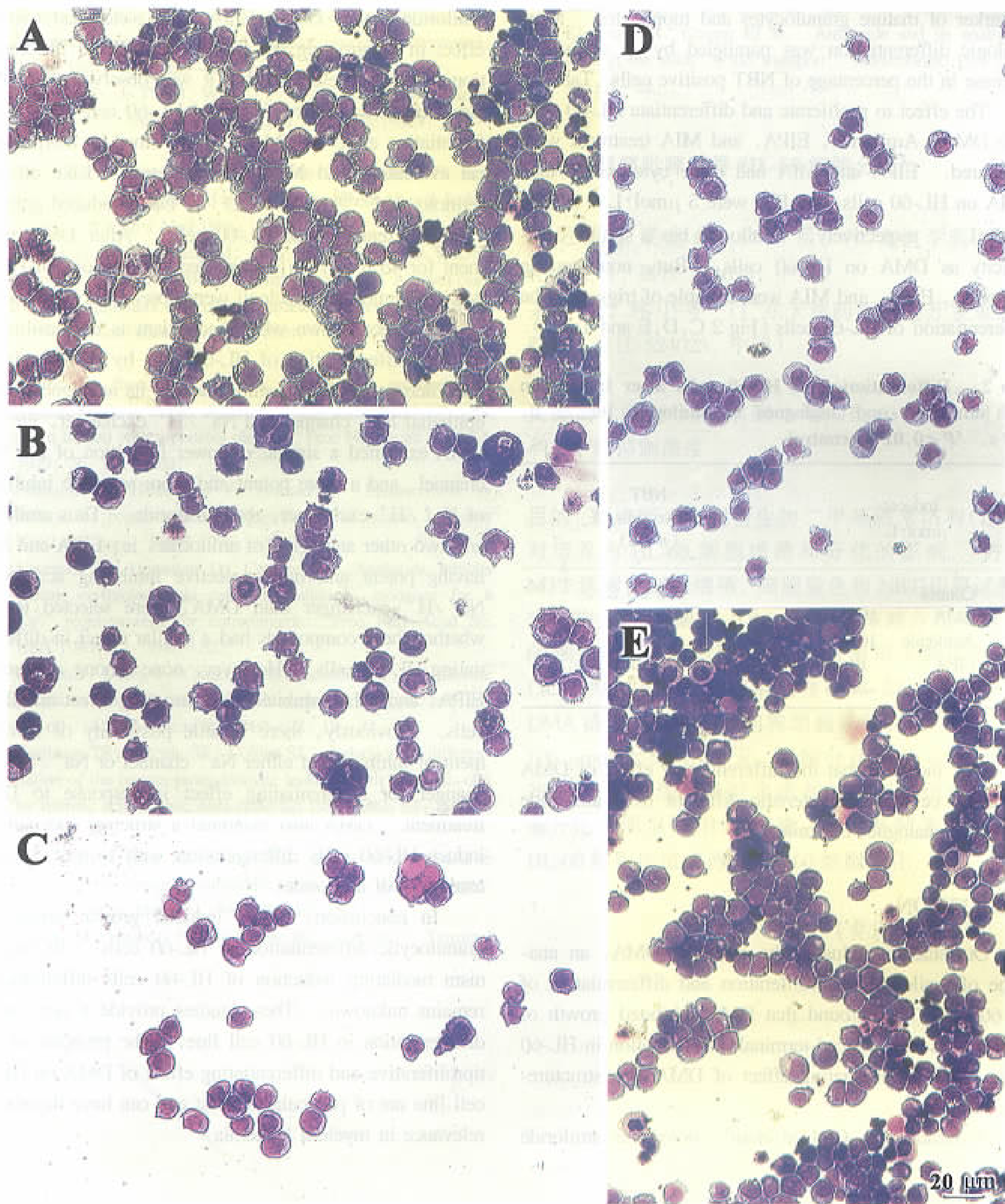

Fig 2. Morphorogical changes in HL-60 cells by treatment with amiloride, DMA, EIPA, and MIA: comparison of the morphological changes in HL-60 cells after 72 h of treatment with vehicle alone (A), 100 $\mu mol \cdot L^{-1}$ DMA (B), 100 $\mu mol \cdot L^{-1}$ amiloride (C), 10 $\mu mol \cdot L^{-1}$ EIPA (D), and 20 $\mu mol \cdot L^{-1}$ MIA (E). Arrows indentify typically differentiated cells with segmented nuclei. (×400).

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to functional differentiation, cellular reduction of NBT was used to measure the levels of superoxide production, a marker of mature granulocytes and monocytes. Morphologic differentiation was paralleled by an equivalent increase in the percentage of NBT positive cells (Tab 1).

The effect to proliferate and differentiate HL-60 cells with DMA, Amiloride, EIPA, and MIA treatment were compared. EIPA and MIA had more cytotoxicity than DMA on HL-60 cells, the IC_{50} were 5 $\mu mol \cdot L^{-1}$ and 15 $\mu mol \cdot L^{-1}$, respectively. Amiloride has a similar cytotoxicity as DMA on HL-60 cells. But, none among amiloride, EIPA, and MIA were capable of triggering the differentiation of HL-60 cells (Fig 2 C, D, E and Tab 2).

Tab 2. Differentiation of HL-60 cells after incubation with amiloride and analogues of amiloride for 72 h. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Inducer/ $\mu mol \cdot L^{-1}$		NBT positive cells/% ($n = 3$)
Control		6.2 ± 0.28
DMA	100	65.5 ± 10^c
Amiloride	100	6.0 ± 1.0
EIPA	10	6.3 ± 2.0
MIA	20	5.0 ± 2.6

This indicates that the differentiating effect of DMA in HL-60 cells is characteristic different from amiloride and other analogues of amiloride.

DISCUSSION

Our studies evaluated the effect of DMA, an analogue of amiloride on proliferation and differentiation of HL-60 cells. We found that DMA inhibited growth of HL-60 cells and induced terminal differentiation in HL-60 cells. The differentiating effect of DMA was structure-specific.

A decade ago, Carlson *et al*[8] noted that amiloride potentiated the Me_2SO-induced differentiation of HL-60 cells. They also found that several analogues of amiloride namely DMA, EIPA, MIA, etc, had a similar effect in potentiating Me_2SO-induced HL-60 differentiation. In the present study, it was observed that DMA alone triggered differentiation of HL-60 cells. The differentiating effect of DMA was confirmed by morphological evaluation and NBT reduction test. Like all-trans retinoic acid[9,10] and DMSO[11], DMA induced granulocytic differentiation of HL-60 cells. After DMA treatment for 48 h to 72 h, the nuclear morphology of HL-60 was segmented and nucleoli were observed to be absent.

It is not known what mechanism is responsible for induced differentiation of HL-60 cells by DMA. It has been demonstrated that amiloride and its analogues inhibit epithelial Na^+ channel and Na^+/H^+ exchanger, *etc*[12]. DMA exhibited a similar or lower inhibition of the Na^+ channel, and a more potent and more selective inhibition of Na^+/H^+ exchanger, than amiloride. Thus amiloride and two other analogues of amiloride, ie, EIPA and MIA having potent and more selective inhibiting activity of Na^+/H^+ exchanger than DMA, were selected to test whether these compounds had a similar effect in differentiating HL-60 cells. However, none among amiloride, EIPA, and MIA exhibited differentiating effect on HL-60 cells. Obviously, there is little possibility of involvement of inhibition of either Na^+ channel or Na^+/H^+ exchanger for differentiating effect in response to DMA treatment. DMA also exhibited a structure-speciality to induce HL-60 cells differentiation with respect to other analogues of amiloride.

In conclusion, DMA induced growth arrest and granulocytic differentiation in HL-60 cells. Its mechanism mediating induction of HL-60 cells differentiation remains unknown. These studies provide a new model differentiation in HL-60 cell line. The pronounced antiproliferative and differentiating effect of DMA on HL-60 cell line are of particular interest and can have therapeutic relevance in myeloid leukemia.

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二甲基氨氯吡咪诱导 HL-60 细胞分化[1]

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关键词　细胞分裂；细胞分化；HL-60 细胞；阿米洛利；半数抑制浓度

目的：研究阿米洛利衍生物二甲基阿米洛利（DMA）对培养的 HL-60 细胞增殖和分化的影响. 方法：MTT 法测定细胞增殖，细胞染色和 NBT 还原试验测定细胞分化. 结果：DMA 以浓度依赖方式抑制 HL-60 细胞增殖，药物作用 96 小时测得半数抑制浓度（IC_{50}）为 31.7（95 %可信限为 6.3－57.1）$\mu mol \cdot L^{-1}$. DMA 诱导 HL-60 细胞向粒细胞系分化. 加入 DMA 100 $\mu mol \cdot L^{-1}$ 作用 3 天，细胞分化率从 6.5 %升至 70 %. 阿米洛利及阿米洛利的另二个衍生物对细胞分化无明显作用. 结论：二甲基阿米洛利抑制 HL-60 细胞增殖和诱导 HL-60 细胞分化.

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