Betulinic acid enhances 1α,25-dihydroxyvitamin D_3-induced differentiation in human HL-60 promyelocytic leukemia cells

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Betulinic acid (BA) is a pentacyclic triterpene found in a number of medicinal plants and has been shown to cause apoptosis in a number of cell lines. We report here that BA may also have an effect on HL-60 cell differentiation. BA was cytotoxic to HL-60 cells with an IC_{50} of 5.7 μM after a 72-h treatment. Flow cytometry analysis showed that after exposure to 1–12 μM of BA for 72 h, approximately 10% of viable cells were in the sub-G_1, presumably apoptotic, phase. At the same time differentiation was induced in approximately 10% (at 1 μM BA) to a maximum of 20% (at 6 μM BA) of cells as judged by the NBT-reduction test, and the expression of membrane markers CD11b and CD14. On the other hand, at 1 and 5 nM, 1α,25-dihydroxyvitamin D_3 (DHD_3) induced differentiation in approximately 10 and 70% of cells, respectively. At 1 nM DHD_3, the addition of 1 μM BA increased differentiated cells from 10 to 43% and with 3 μM BA the increase was to 80%. BA also enhanced the effects of DHD_3 in the expansion of the G_1 cell population with a concomitant decrease of S phase cells. The effects of DHD_3 and BA on CD11b and CD14 expression were inhibited by PD98059, a MEK inhibitor. Our results suggest that BA may enhance the effect of DHD_3 in inducing mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase-mediated HL-60 cell differentiation. *Anti-Cancer Drugs* **15:619–624**

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Introduction

1α,25-dihydroxyvitamin D_3 (DHD_3) is capable of inducing differentiation in acute promyelocytic leukemia (APL). However, the clinical use of DHD_3 has been limited by its serious side-effects such as hypercalcemia. One plausible solution to this problem is the simultaneous use of an agent that enhances the differentiation-inducing action of DHD_3, therefore allowing the use of a lower dose of DHD_3.

Betulinic acid (BA) is a pentacyclic triterpene (Fig. 1) of natural origin from various plants such as *Tryphyllum peltatum*, *Ancistrocladus heyneaus*, *Diospyros leucomelas*, *Tetracera boliviana*, *Zizyphus joazeiro* and *Syzygium formosanum* [1]. It induces mitochondria-dependent apoptosis in neuroblastoma cells by causing loss of transmembrane potential of mitochondria, releasing soluble apoptogenic factors such as cytochrome *c* that can activate caspases and endonucleases [2]. BA can also induce apoptosis in melanoma through the activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase [3]. BA has been reported to inhibit human immunodeficiency virus type 1 (HIV-1) via interfering with HIV-1 entry into cells [4]. In addition, BA was also found to inhibit topoisomerase I and II [5,6].

In the present study, we demonstrated that BA enhanced DHD_3-induced monocytic cell differentiation. The action was abolished by inhibition of extracellular signal-regulated protein kinase (ERK) activity, suggesting the involvement of ERK signaling.

Materials and methods

Chemicals and antibodies

BA was purchased from Aldrich (St Louis, MO), DHD_3 was from Alexis Biochemicals (Lausen, Switzerland), nitroblue tetrazolium (NBT), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), dimethylsulfoxide (DMSO) and propidium iodide (PI) were from Sigma (St Louis, MO). MAP kinase inhibitor, PD98059, was purchased from Calbiochem-Novabiochem (La Jolla, CA). RNase was obtained from ICN (Aurora, OH) and R-phycoerythrin (PE)-conjugated anti-CD11b and anti-CD14 were from Caltag (Burlingame, CA).

Cell culture and proliferation assay

The human promyelocytic leukemic cell line, HL-60, was cultured in RPMI-1640 medium (Gibco/BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (Gibco/BRL). Cells were subcultured 2 or 3 times weekly to maintain a log-phase growth. Cells were seeded at 5000

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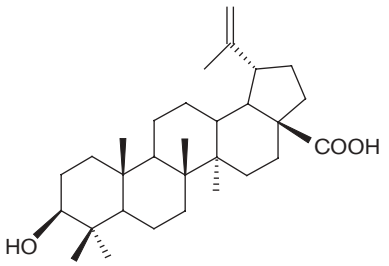
Fig. 1



Structure of BA.

cells/well in 96-well plates and incubated for 72 h with BA, DHD_3 or their combination. Cell proliferation was determined by the MTT assay. Briefly 20 µl of MTT (5 mg/ml) were added to each well and samples were incubated at 37°C for 4 h. Dye crystals accumulated in viable cells were dissolved with 100 µl of stop solution (15% SDS w/v in 0.04 N HCl/50% isobutanol v/v) and absorbance was read at 570 nm with a microtiter plate reader.

Assessment of differentiation

Cell differentiation was initially assessed by nitroblue tetrazolium (NBT) reduction assay. Cells were incubated with drugs for 72 h. The MAP kinase inhibitor, PD98059, which inhibits MEK1, was added at 20 µM 2 h before the addition of drugs. After treatment, 1×10^6 cells were harvested, washed with phosphate-buffered saline (PBS), incubated with freshly prepared NBT/TPA solution (1 mg/ml NBT with 1 µg/ml TPA in serum-free medium) for 20 min at 37°C and finally washed with 70% methanol to remove non-reduced NBT. Cells were lysed by the addition of 500 µl of 2 M KOH overnight. Nitroblue diformazan deposit was dissolved in 600 µl/sample of DMSO and absorbance at 570 nm was read.

The expression of differentiation cell-surface markers CD11b and CD14 was determined by flow cytometry. Aliquots of 1×10^6 cells were harvested, washed twice with PBS, resuspended in 100 µl of PBS and then incubated for 45 min at room temperature with 5 µl of a commercial preparation of PE-conjugated anti-human CD11b or CD14 monoclonal antibody. Cells were washed 3 times with ice-cold PBS, resuspended in 0.5 ml PBS and analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest software (Verity Software House, Topsham, ME).

Cell cycle distribution

Aliquots of 1×10^6 cells from the differentiation-induction experiments were washed twice with ice-cold PBS, fixed in 70% ethanol at −20°C overnight, washed with PBS and incubated in 1 ml of PBS containing 100 µg of RNase at 37°C for 30 min. Propidium iodide (PI) staining solution was added to a final concentration of 40 µg/ml. Analysis was carried out on a FACSCalibur flow cytometer (Becton Dickinson).

Statistical methods

All experiments were repeated at least 2 times. For drug enhancement studies, two compounds (A and B) were considered showing enhancement if the effect of their mixture (AB) was larger then the sum of their individual effects, i.e. $AB > (A + B)$. Data was compared after subtraction of the respective background control values. $p \leq 0.05$ from one-way analysis of variance (ANOVA) was considered to be statistically significant.

Results

Effects of BA on HL-60 cell proliferation

After exposure to 1–12 µM of BA for 72 h, cell number, as estimated by the MTT assay, reduced in a dose-dependent manner (Fig. 2A). At the highest concentration of BA used in this study, i.e. 12 µM, cell number was reduced to 40% of the untreated control. Flow cytometry analysis showed that about 10% of treated cells were in the sub-G_1, presumably apoptotic, phase (Fig. 2B), suggesting that the decrease of cell number was at least partly caused by apoptosis.

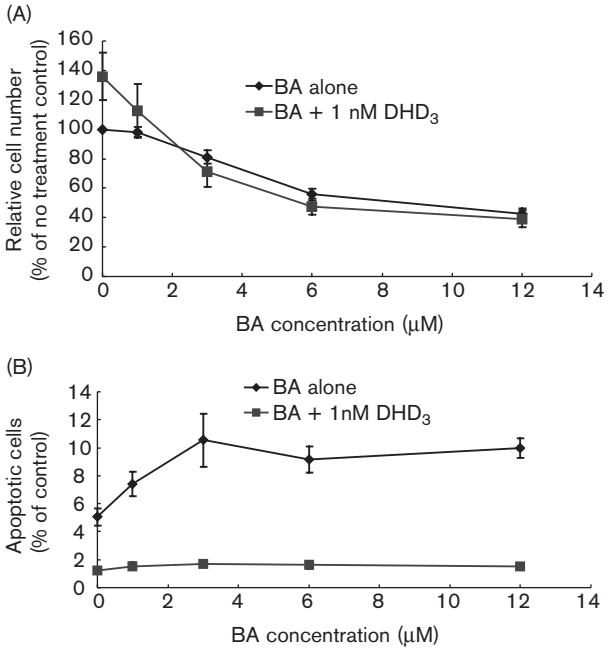
The addition of 1 nM DHD_3 to BA (3–12 µM) treatment did not alter the extent of total cell number decrease (Fig. 2A), but less than 2% (compared to 10% in BA treatment alone) of cells were in the sub-G_1 (presumably apoptotic) phase (Fig. 2B). It has been widely reported that DHD_3 initially increases cell proliferation, which is followed by cell differentiation and maturation [7].

Enhancement of HL-60 cell differentiation

In differentiated HL-60 cells, the presence of TPA stimulates superoxide anions (O_2^-) production that reduces water-soluble NBT to dark-blue nitroblue diformazan (NBD) deposits. BA (3–12 µM) by itself did not significantly increase the reduction of NBT over a period of 72 h (Fig. 3). An increase of NBT reduction was observed starting at 1 nM DHD_3. The addition of BA (3–12 µM) to 1 nM DHD_3 noticeably augmented the production of NBD to a level comparable to the level achieved by 5 nM of DHD_3 alone.

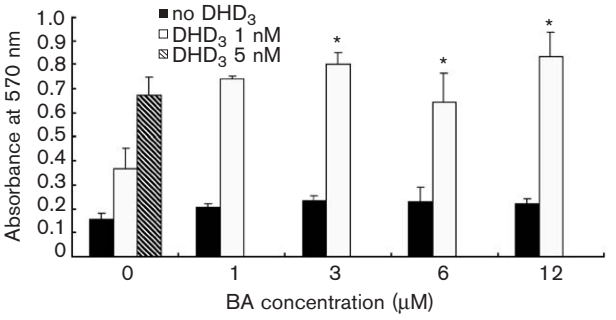
To further ascertain the occurrence of HL-60 cell differentiation, membrane differentiation markers were immunolabeled and measured by flow cytometry. A typical experiment is shown in Figure 4(A). The addition of BA to 1 nM of DHD_3 caused a significant increase in the expression of both CD11b and CD14 surface markers expression (c and g, respectively) as compared to individual drug treatment controls (a, b, e and f). This experiment was repeated at least 3 times and numerical

Fig. 2



Effects of BA and DHD_3 on HL-60 cells proliferation. HL-60 cells were treated with various concentrations of BA (0, 1, 3, 6 or 12 μM) in the absence or presence of 1 nM DHD_3 for 72 h. (A) Cell proliferation was determined by the MTT assay and DHD_3 alone stimulated cell proliferation. Data are presented as percent of no treatment control. (B) Apoptotic HL-60 cells were estimated by flow cytometry and data are presented as percent of no treatment control. Data represents mean ± SE of three independent experiments.

Fig. 3



Effects of BA on DHD_3-stimulated HL-60 cells differentiation as estimated by the NBT assay. HL-60 cells were treated with DHD_3 and/or BA for 72 h, and cell differentiation was estimated by the NBT assay. DHD_3 at 5 nM was included as a positive control. Data represents mean ± SE of three independent experiments. Each data point from combined drug treatments was compared to the sum of respective individual drug treatments. *Significant difference at $p < 0.05$.

data are present in Fig. 4(B). DHD_3 alone at 1 nM induced the expression of CD11b and CD14 in 8.32 ± 0.56% (no treatment control of 0.49 ± 0.24%) and 11.22 ± 1.94% of cells (no treatment control 0.79 ± 0.34%), respectively (Fig. 4B). BA alone caused the expression of CD11b and CD14 in 25.58 ± 9.63% and 18.85 ± 3.44% of cells, respectively. Simultaneous treatments by 1 nM DHD_3 and 3–12 μM BA could achieve a synergy, and the enhanced results (79.64 ± 5.44% for CD11b, and 58.78 ± 4.49% for CD14) were higher than the summation of their individual effects.

Enhancement of DHD_3-induced G_1 arrest by BA

DHD_3 at 1 nM altered cell cycle distribution with an increased G_1 population after 72 h of incubation ($p = 0.018$) (Fig. 5). No change in cell cycle distribution was observed after BA (3–6 μM) treatment (Fig. 5). The addition of various concentrations of BA (3–6 μM) to 1 nM DHD_3 resulted in a does-dependent and statistically significant increase in the G_1 population with a concomitant reduction in the S phase population. Less than 2% of cells were apoptotic under these conditions.

The differentiation effect was diminished by PD98059 pretreatment

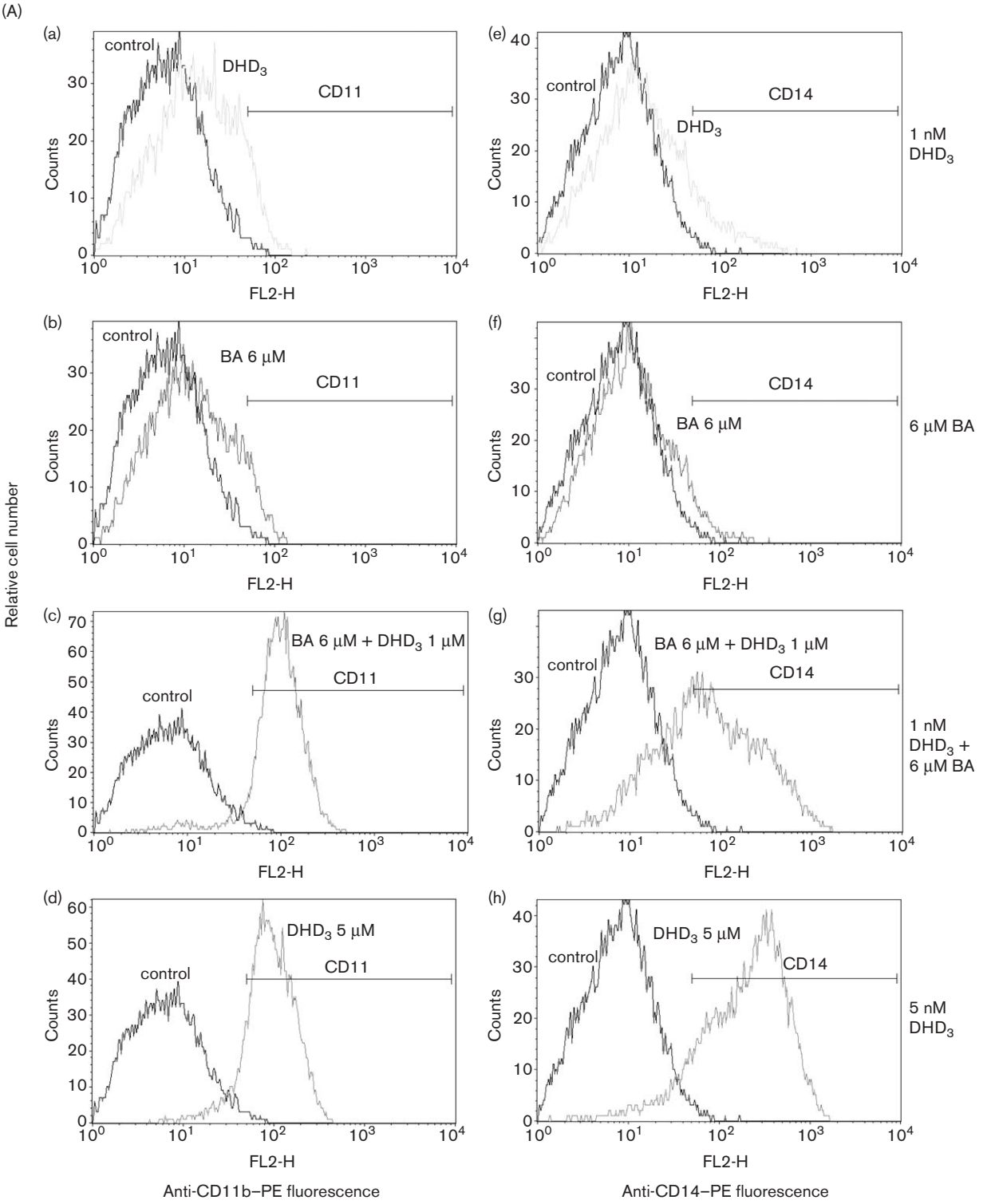
It has been reported that MEK/ERK signaling is involved in DHD_3-induced HL-60 cell differentiation [8]. We investigated if MEK/ERK signaling was involved in the action of BA on enhancing DHD_3-induced HL-60 cell differentiation. Pre-incubation of the cells with 20 μM PD98059, a MEK1 inhibitor that inhibits ERK activation, 2 h before the combined DHD_3 and BA treatment completely eliminated the expression of CD11b and CD14 (Fig. 6). This result suggested that the activation of ERK, the presumed signal pathway involved in DHD_3-stimulated HL-60 cell differentiation, is also involved in the action of BA on enhancing the DHD_3 action. This would imply that BA does not change the basic mechanism for HL-60 differentiation.

Discussion

BA at the concentration range of 1–12 μM was toxic to HL-60 cells (IC_{50} 5.7 μM) and induced apoptosis in about 10% of surviving cells. Part of the toxic effect may be due to its inhibitory effects on topoisomerase I and II in some cell types, and therefore may affected DNA replication [5,6].

HL-60 cell has been widely used as a model for studying pre-myolocytic cell differentiation and the identification of differentiation-inducing agents. The effective concentration range for DHD_3 to induce HL-60 cell differentiation was 1–100 nM [8]. Under our experimental conditions, about 70% of HL-60 cells expressed differentiation markers CD11b and CD14 after a 72-h treatment with 5 nM DHD_3. At 1 nM DHD_3 only about 10% of cells were positive in the NBT reduction test or for differentiation marker expression. The number of differentiated cells was significantly increased by the addition of BA, at concentrations as low as 1 μM.

Fig. 4



BA enhanced CD marker expression in HL-60 cells stimulated by DHD_3. HL-60 cells were treated with BA and/or DHD_3 for 72 h. The expression of monocytic membrane differentiation markers CD11b and CD14 was estimated by fluorescence monoclonal antibody labeling and flow cytometry. (A) Results from a typical flow cytometry experiment (out of three) showing monocytic differentiation marker expression detected by fluorescence monoclonal antibody labeling. (B) Data in histogram show values as means ± SE of three independent experiments. Each data point from combined drug treatments was compared to the sum of respective individual drug treatments. *Significant differences at $p < 0.05$.

Fig. 4 (*Continued*)

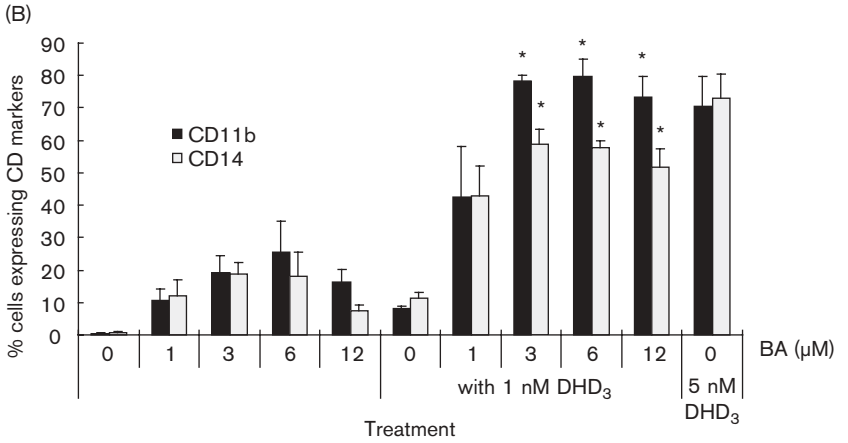
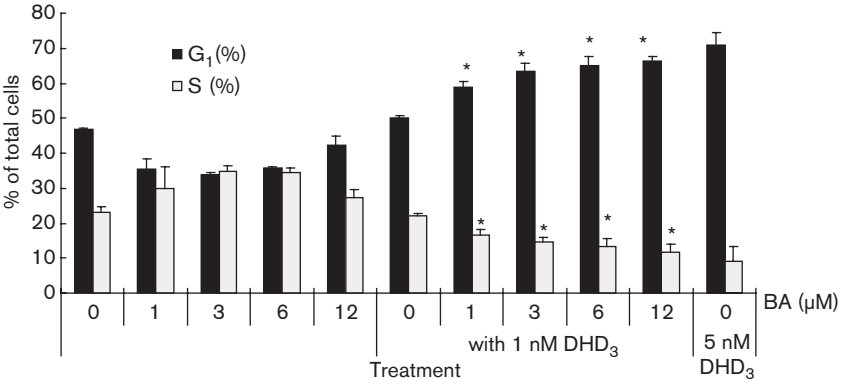


Fig. 5



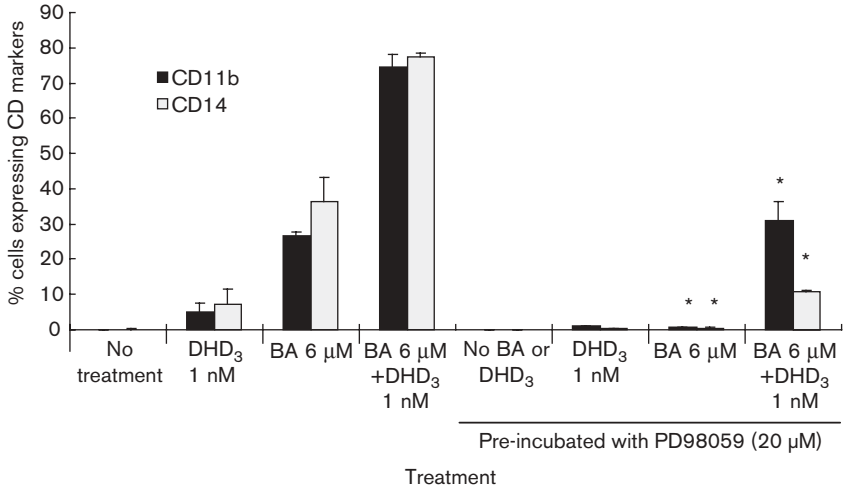
Effect of BA and/or DHD_3 on HL-60 cell cycle. Cells were treated for 72 h and cell cycle distribution was determined by flow cytometry. Data represent means ± SE of three independent experiments. $*p < 0.01$ compared to 1 nM DHD_3 treatment.

Differentiated cells are normally inactive in cell division and arrested in G_1 phase of the cell cycle. Significant G_1 arrest was observed when HL-60 cells were treated with DHD_3. This G_1 arresting action of DHD_3 was also enhanced with the addition of BA, but cell number was not affected under the same conditions. Brown et al. has suggested that DHD_3 initially accelerates cell proliferation, which is followed by cell differentiation and maturation [7]. A single DHD_3-treated HL-60 cell would give rise to 10 or more matured monocytes. Our observations on the action of BA in enhancing DHD_3 induced NBT reduction, membrane marker expression and G_1 cell cycle arrest provide corroborative evidence that BA and DHD_3 act synergistically in inducing differentiation in HL-60 cells.

MEK/ERK signaling has been shown to regulate the events of cell proliferation, survival and differentiation. Although some have suggested that MEK/ERK signal is more related to cell proliferation rather than differentiation in leukemia cells [9], many studies have suggested that MEK/ERK signaling plays an important role in monocytic and granulocytic differentiation of different leukemic cells induced by various differentiation agents, including DHD_3 [10–12]. In our study, the individual and combined effects of BA and DHD_3 in inducing monocytic differentiation marker CD11b and CD14 expression was completely abolished when the cells were pretreated with PD98059, a MEK inhibitor. This suggests that BA did not change the basic signaling mechanism for the induction of differentiation in HL-60 cells.

Another MAP kinase, JNK, is reported to be involved in the DHD_3-induced HL-60 monocytic differentiation [13]. Our results (data not shown) showed that the application of the JNK inhibitor, SP600125, did not

Fig. 6



Effect of the MEK1/2 inhibitor, PD98059, on the action of BA and DHD_3 on the stimulation of HL-60 cell differentiation. HL-60 cells were pre-treated with 20 μM PD98059 2 h before the addition of 6 μM BA and 1 nM of DHD_3. After 72 h, cells were analyzed by flow cytometry. Data represent means ± SD of three independent experiments. *Significant difference at $p < 0.05$ compared with respective samples without PD98059 pre-treatment.

inhibit HL-60 cell differentiation induced by DHD_3 or BA.

In a recent study, it was reported that chemicals such as parthenolide could enhance DHD_3-induced HL-60 cell differentiation through inhibition of NF-κB [14]. BA was also reported to suppress tumor necrosis factor-induced NF-κB activation in non-leukemic cell lines [15]. One may hypothesize that inhibiting or lowering NF-κB is the molecular mechanism underlying the action of BA on enhancing DHD_3-induced HL-60 cell differentiation. Studies along this line are now underway in our laboratory.

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