# Compound 278E, structurally modified from tanshinone, induces monocytic differentiation and regulates proto-oncogene expression in human leukemic HL-60 cells

Hui-Fen Liao[a,c,d,g], Shin-Yi Shyu[e], Yueh-Hsiung Kuo[f], Yuh-Cheng Yang[a] and Yu-Jen Chen[a,b,d]

**Tanshinone derivative compounds, isolated from *Salvia miltiorrhiza* Bunge (Labiatae), have been reported as microtubule inhibitors with antimitotic activity. In this study, we examined the growth-inhibiting and differentiation-inducing effect of these compounds on human leukemic HL-60 cells. The expression of protein kinase C (PKC) and proto-oncogenes in 278E-treated cells was also assessed. All tanshinone derivative compounds exhibited growth-inhibitory effects on HL-60 cells, but only 278E induced cell differentiation. Morphological observation of 278E-treated HL-60 cells showed a greater percentage of monocytes and macrophages (Mo/Mϕ). Treatment with 5 μg/ml 278E resulted in a marked increase in the percentages of superoxide-producing (up to 95.5 ± 1.8%) and non-specific esterase-positive cells (up to 80.3 ± 9.1%). The differentiated cells also expressed cell surface antigens characteristic of Mo/Mϕ, including CD11b, CD14 and CD68. Neither cellular changes in isozymes of PKC nor translocation of these isozymes from cytosol to cell membrane were seen in 278E-treated HL-60 cells. 278E caused a downregulation of c-*myc* as well as an up-regulation of c-*fms*, c-*jun* and c-*fos*. *Anti-Cancer Drugs* 16:175–183 © 2005 Lippincott Williams & Wilkins.**

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Departments of [a]Medical Research and [b]Radiation Oncology, Mackay Memorial Hospital, Taipei, Taiwan, [c]Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan, [d]Graduate Institute of Sport Coaching Science, Chinese Culture University, Taipei, Taiwan, [e]Institute of Ecology and Evolutionary Biology and [f]Department of Chemistry, National Taiwan University, Taipei, Taiwan and [g]Department of Molecular Biology and Biochemistry, National Chiayi University, Chiayi, Taiwan.

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Correspondence to Y.-J. Chen, Department of Radiation Oncology, Mackay Memorial Hospital, 92 Chung San North Road, Section 2, Taipei 104, Taiwan.
Tel: +886 2 28094661; fax: +886 2 28096180;
e-mail: chenmdphd@yahoo.com

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## Introduction

Acute myeloid leukemia (AML) is a hematological malignancy characterized by a differentiation block that leads to an accumulation of immature cells. Induction of differentiation is therefore becoming the treatment of choice in a subset of AML. Substances such as 1,25-dihydroxyvitamin $D_3$ inhibit proliferation and induce differentiation in AML HL-60 cells to the monocyte/macrophage (Mo/Mϕ) lineage [1,2], whereas all-*trans*-retinoic acid causes differentiation to morphologically and functionally mature granulocytes [3,4].

Activation of protein kinase C (PKC) and c-*myc* protein expression are associated with the induction of monocytic differentiation in tumor necrosis factor-α-treated HL-60 cells [5]. PKC can be divided into three groups, including conventional (PKC-α, -βI, -βII and -γ), novel (PKC-δ, -ε, -η and -θ) and atypical (PKC-ξ and -λ/ι) isozymes [6]. RACK1 (receptor for activated C kinase-1), PKC-anchoring protein is involved in PKC-mediated signal transduction and subcellular PKC localization. It was reported to coordinate the binding of activated PKC and select pleckstrin homology domains *in vitro* [7,8]. Amplification of c-*myc* in HL-60 cells [9] is downregulated during monocytic differentiation [10,11]. Expression of proto-oncogenes such as c-*fms*, c-*fos* and c-*jun* is also involved in monocytic differentiation of HL-60 cells [10]. The correlation between the expression of proto-oncogenes and leukemic cell proliferation and differentiation is of crucial importance for clarifying the mechanisms by which differentiation occurs.

*Salvia miltiorrhiza* Bunge (SM), a commonly prescribed herb in the treatment of cardiovascular disorders (called blood stasis in traditional Chinese medicine), is widely used in China, Japan and Taiwan. Its extracts inhibit platelet aggregation [12], and may reduce atherosclerosis by lowering cholesterol as well as antioxidant effects that prevent endothelial damage and inhibit oxidative modification of low-density lipoprotein in hypercholesterolemic animals [13]. Several phenolic compounds isolated from SM are effective in protecting liver microsomes, hepatocytes and erythrocytes against peroxidative damage [14]. SM extract

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exhibits cytotoxic effects with induction of apoptosis in human hepatoma HepG2 cells [15]. Salvinal (also named tanshinone 278A), isolated from SM, is a novel microtubule inhibitor with antimitotic activity in human tumor cells [16]. However, the differentiation-inducing activity of components of SM has not been investigated.

In a preliminary study, we discovered that 5-(3-hydroxypropyl)-7-methoxy-2-(3′-methoxy-4′-benyloxyphenyl)-3-benzo[*b*]furan (278E, structure shown in Fig. 1), a synthetic derivative of 5-(3-hydroxypropyl)-7-methoxy-2-(3′-methoxy-4′-hydroxyphenyl)-3-benzo[*b*]furancarbaldehyde (278A) [17], inhibited the growth of HL-60 cells, accompanied by morphological changes characteristic of differentiation. In the present study, we conducted experiments with human leukemic HL-60 cells to analyze induction of differentiation by 278E and the possible molecular mechanisms involved.

## Materials and methods

### Cell culture and materials

The human acute myeloid leukemic cell line HL-60 (ATCC, Manassas, VA) was used in this study. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and maintained in an exponential growth state. The tanshinone derivative compounds 278A, B, C, D, E, 9A and 9B were kindly provided by Professor Yueh-Hsiung Kuo (Department of Chemistry, National Taiwan University, Taipei, Taiwan). For assay of cytotoxicity, cells were incubated in 35-mm Petri dishes at an initial concentration of $1 \times 10^5$/ml in the presence of various concentrations of tanshinone derivative compounds (0, 1, 2, 3, 4, 5 and 6 μg/ml).

### Cell growth inhibition

Tanshinone-treated HL-60 cells were collected by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, NJ). The numbers of viable cells were counted on days 1, 3 and 5 using the Trypan blue dye exclusion test.

### Maturation profile

After 5 days of treatments, cells were collected and cytocentrifuged onto a microscope slide using Cytospin (Shandon Southern Instrument, Sewickly, PA), stained with Wright's stain and observed under an inverted microscope (Olympus) with a magnification of × 1000. Based on morphology, the cells were classified as: (i) immature blasts, (ii) intermediates, or (iii) mature monocytes or macrophages (Mo/Mϕ) [18]. For assessing the differentiation-inducing effect of various combinations with PKC inhibitors (staurosporine or H7) and drugs (278E or TPA), cells were treated for 3 days and assayed by the same methods.

### Assay for superoxide production

The production of cytoplasmic superoxide by differentiated myeloid cells was detected by the nitroblue tetrazolium (NBT) reduction test [19]. Cells collected from day 5 cultures were suspended in RPMI 1640 medium at a concentration of $1 \times 10^6$ cells/ml and incubated for 30 min at 37°C with an equal volume of NBT test stock solution [containing 2 mg of NBT and 1 μM of phorbol myristate acetate/ml of phosphate-buffered saline (PBS)]. Cytospin preparations were counter-stained with 0.5% safranin O. The percentage of formazan-containing cells (based on counting 200 cells) was assessed microscopically.

### Assay for non-specific esterase (NSE) activity

The cells were seeded at an initial density of $1 \times 10^5$/ml in six-well plates and treated with different agents in a 5% $CO_2$ humidified atmosphere at 37°C for 5 days. Viable cells were determined by Trypan blue dye exclusion and cytocentrifuge smears of treated cells were assayed for NSE activity by using a Sigma 91-A kit.

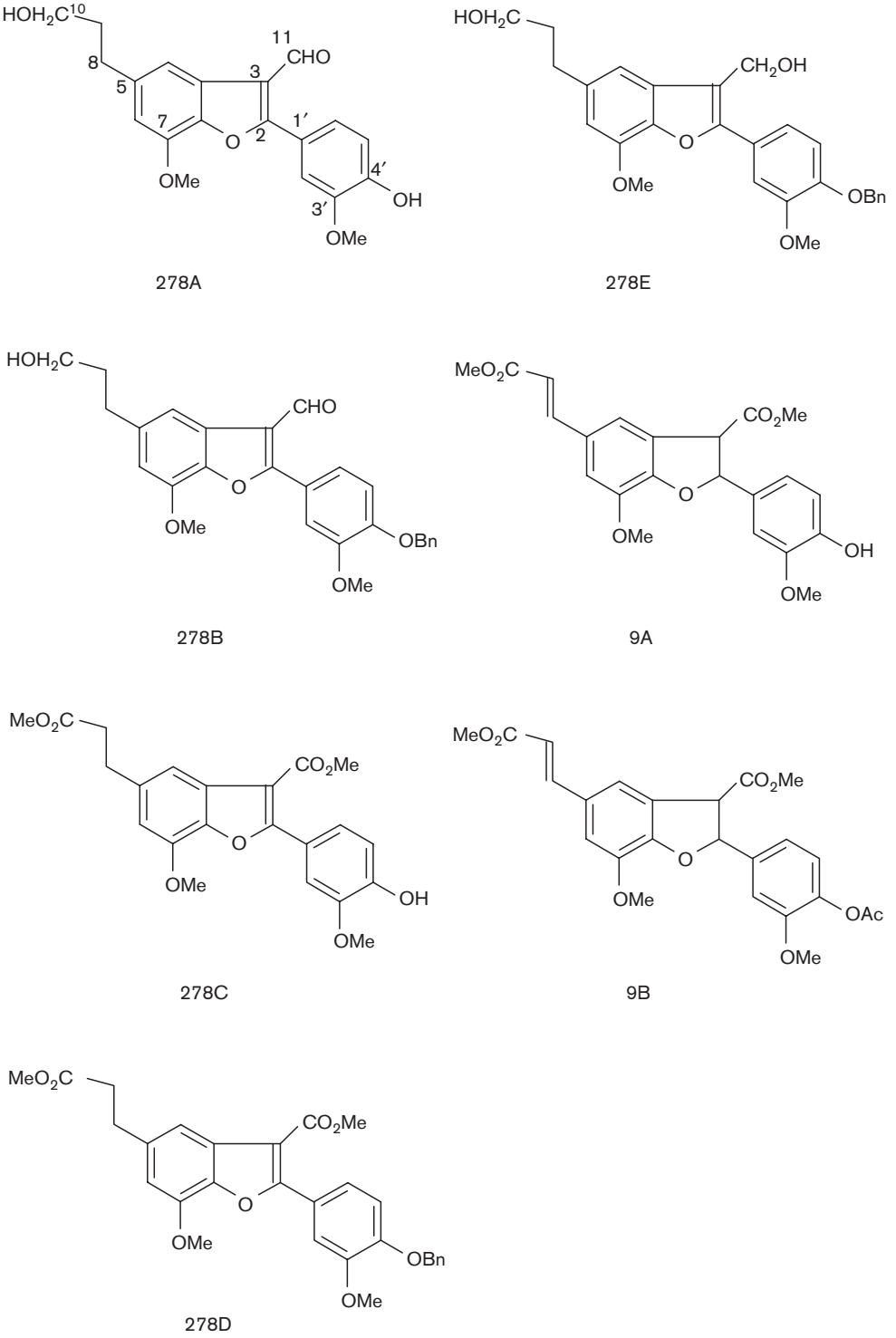
### Assay for differentiation antigens

An indirect immunofluorescence method was employed to detect the expression of monocyte-associated antigens on the surface of HL-60 cells after induction of differentiation. Cells collected from day 5 cultures were treated with primary monoclonal antibodies, washed with PBS and then exposed to fluorescein isocyanate (FITC) conjugated to a secondary antibody, goat $F(ab')_2$ anti-mouse IgG (Cappel, Cochranville, PA). Monoclonal antibodies against human monocytes included anti-CD11b and anti-CD14 (Serotec, Oxford, UK), and anti-CD68 (Dakopatts, Glostrup, Denmark). The percentage of positive cells was analyzed using a FACScan flow cytometer (Becton Dickenson, Franklin Lakes, NJ) and Lysis II software (Becton Dickinson). FITC conjugated to goat anti-mouse IgG was used to set background thresholds.

### Western blotting

Western blot analysis was performed as previously described [20]. Proteins were isolated from the cytoplasm and membrane of HL-60 cells after treatment with 6 μg/ml 278E for various lengths of time. The protein concentration was determined by a bicinchoninic acid assay kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg in each lane) were electrophoresed in 10% SDS–polyacrylamide gel at a constant current of 20 mA and 70–100 V. Then, the gel was transferred onto a nylon blotting membrane. The membrane was blocked with 5% defatted milk and immunoblotted with primary antibodies against various isozymes of PKC (Transduction, Lexington, KY) at room temperature for 3 h. This was followed by addition of horseradish peroxidase-labeled second antibodies (Transduction) and development using the enhanced chemiluminescence system (Amersham

**Fig. 1**




Structure of 278A and its derivatives. Me, methyl group; Bn, benzyl group; Ac, acetyl group.

Pharmacia, Piscataway, NJ). 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma, St Louis, MO), a PKC activator, was used as positive control.

### Northern blotting

Total RNA was isolated from HL-60 cells using an RNAzol B kit (Biotecx, Galveston, TX) and quantified

spectrophotometrically. Total cellular RNA (20 μg) from control or 278E-treated cells was electrophoresed on a 1.2% agarose gel and transferred to a nylon membrane. The blots were probed with radiolabeled cDNA of c-*myc*, c-*fms*, c-*fos* and c-*jun*. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control.

### Statistical analysis

Results are expressed as the mean ± SE of at least three experiments. Statistical comparisons were made by using Student's *t*-test or analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. All data analysis was carried out by using SPSS, version 8.0 (SPSS, Chicago, IL).

**Table 1 Effect of 278A and its derivatives on growth and differentiation of HL-60 cells**

| Treatment | $IC_{50}$ (μg/ml) | Differentiation |
|---|---|---|
| 9A | 0.49 | – |
| 9B | 0.49 | – |
| 278A | 0.53 | – |
| 278B | 1.39 | – |
| 278C | 1.49 | – |
| 278D | 1.48 | – |
| 278E | 3.48 | + |

HL-60 cells were seeded at a density of $10^5$ cells/ml, treated with 278A or its derivatives, and collected on day 5. Viable cells were counted and $IC_{50}$ was estimated. After cytospin and staining with Wright's dyes, cell morphology was observed. Positive differentiation was set when the percentage of mature monocytes/macrophages was >30% in 400 observed cells.

## Results

### Effect of 278A and its derivatives on the growth and differentiation in HL-60 cells

278A and all its derivatives (structure shown in Fig. 1) inhibited the growth of HL-60 cells, with the concentration yielding 50% inhibition ($IC_{50}$) ranging from 0.49 to 3.48 μg/ml for the various compounds (Table 1). However, only 278E induced cell morphologically observable differentiation.

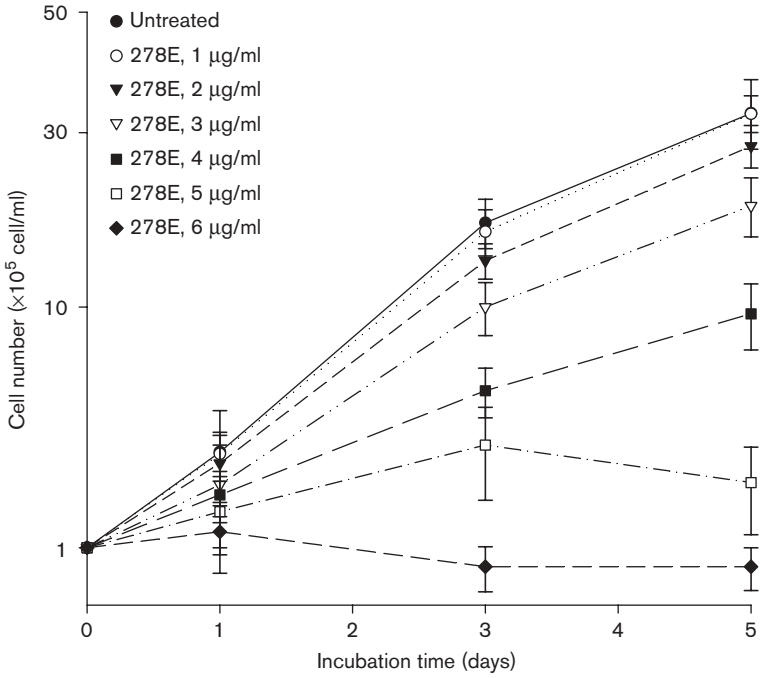
### Inhibition of cell growth by 278E

The growth of HL-60 cells was significantly inhibited by 278E in a dose- and time-dependent manner (Fig. 2). A concentration of 0.5 μg/ml 278E had a minimal effect on cell viability compared with untreated cells. However, concentrations up to 5 μg/ml caused marked growth inhibition (85.5 ± 2.8% on day 3 and 91.7 ± 1.7% on day 5).

### Morphological changes in HL-60 cells treated with 278E

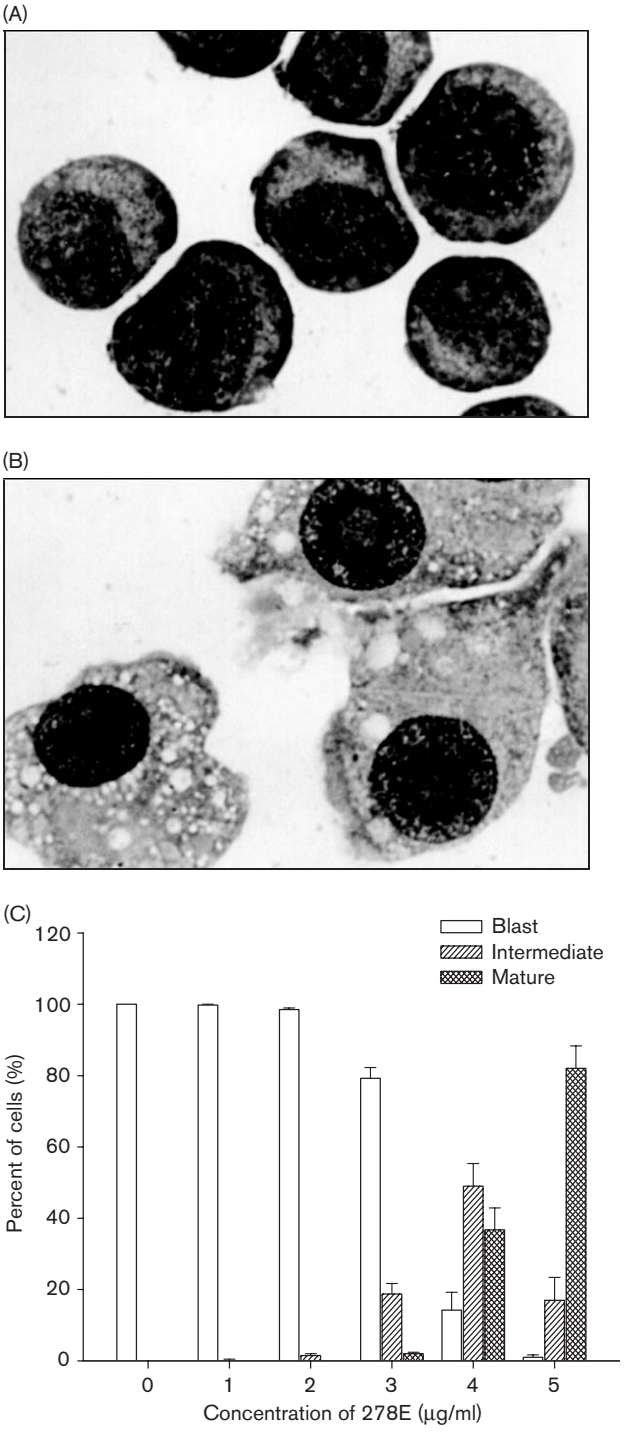
Untreated HL-60 cells are morphologically myeloblast-like cells, with a round cell contour, scanty cytoplasm containing some granules, and an ovoid-shaped nucleus with some nucleoli (Fig. 3A). After 5 days of incubation with 5 μg/ml 278E, many cells exhibited changes characteristic of Mo/Mϕ such as a lower nucleus/cytoplasm ratio, a less basophilic cytoplasm, scanty nucleoli, evident pseudopodia and many phagosomal vesicles

**Fig. 2**




Dose-dependent growth inhibition of human leukemic HL-60 cell induced by 278E. HL-60 cells were seeded initially at $10^5$ cells/ml and treated for 5 days with or without various concentrations of 278E. The number of viable cells was counted using the Trypan blue dye exclusion test. Data are the mean of four separate experiments.

**Fig. 3**




Induction of differentiation in HL-60 cells by 278E. HL-60 cells were cultured at a density of $10^5$ cells/ml and treated with or without various concentrations of 278E for 5 days. Smears were then prepared and the maturation profile analyzed by examining the morphology of 200 cells on Wright's stained under a microscope ( × 1000). (A) Morphology of untreated cells. (B) Morphology of 278E-treated cells. (C) Percentage of differentiated cells induced by 278E with various concentrations. Results from six separate experiments are expressed as the mean ± SEM.

(Fig. 3B). The percentage of mature Mo/Mϕ was 0% in untreated HL-60 cells and 82.0 ± 6.3% after 5 days of treatment (Fig. 3C).

**Changes in superoxide production and NSE activity**

Immature blast cells had little superoxide production. Treatment with 5 μg/ml 278E resulted in a marked

increase in the percentage of superoxide-producing cells (up to 95.5 ± 1.8%) and NSE-positive cells (up to 80.3 ± 9.1%) (Table 2). These changes occurred in a dose-dependent manner.

### Expression of monocyte-associated antigens

The percentage of CD11b-, CD14- and CD68-bearing cells was low ( < 3%) in untreated HL-60 cells. As the dose of 278E increased to 5 μg/ml, the percentage of CD14-positive cells increased to 63.6 ± 8.9%. Similar results were seen in HL-60 cells expressing CD11b and CD68 (Table 3 and Fig. 4).

### Induction of monocytic differentiation by various combination treatments

Table 4 demonstrates the results of PKC inhibitors. The pretreatment of PKC inhibitor staurosporine and H7 to HL-60 cells suppressed the differentiation-inducing activity of TPA, but not of 278E. PKC inhibitors *per se* had no differentiation-inducing effect on HL-60 cells.

### Expression of PKC isozymes

The activation of cellular PKC is characterized by downregulation of its expression. Treatment with 6 μg/ml 278E did not induce downregulation of cellular PKC isozymes (Fig. 5A) nor was there translocation of PKC isozymes from cytosol to the cell membrane (Fig. 5B).

**Table 2 NBT reduction and NSE activity of HL-60 cells treated with 278E**

| Concentration of 278E (μg/ml) | Positive cells (%) | |
|---|---|---|
| | NBT reduction | NSE |
| 0 | 0 | 0 |
| 1 | 0.3 ± 0.3 | 1.5 ± 1.5 |
| 2 | 1.8 ± 1.2 | 6.3 ± 2.7 |
| 3 | 9.3 ± 2.8 | 42.8 ± 9.3[a] |
| 4 | 59.0 ± 12.6[a] | 77.8 ± 6.9[a] |
| 5 | 95.5 ± 1.8[a] | 80.3 ± 9.1[a] |

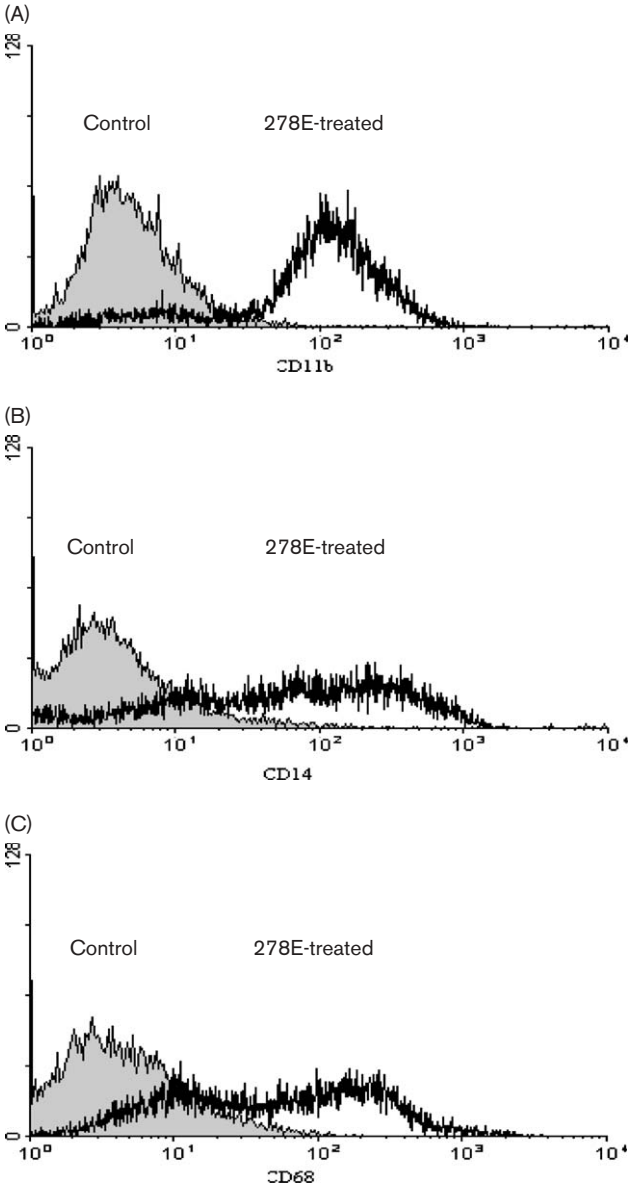
HL-60 cells were seeded at a density of a $10^5$ cells/ml, treated with various concentrations of 278E, and collected at day 5 for assessment. Data are expressed as mean ± SE at least three independent experiments.
[a]$p < 0.05$, ANOVA with Tukey's test was used for comparison.

**Table 3 Surface marker expression on HL-60 cells induced by 278E**

| Concentration of 278E (μg/ml) | Positive cells (%) | | |
|---|---|---|---|
| | CD11b | CD14 | CD68 |
| 0 | 1.9 ± 0.4 | 2.4 ± 0.7 | 1.9 ± 0.4 |
| 1 | 1.8 ± 0.5 | 1.5 ± 0.4 | 1.4 ± 0.4 |
| 2 | 2.6 ± 0.6 | 2.1 ± 0.6 | 1.8 ± 0.4 |
| 3 | 18.1 ± 6.5[a] | 5.8 ± 2.0 | 5.6 ± 2.1 |
| 4 | 62.1 ± 10.4[a] | 47.4 ± 10.6[a] | 34.5 ± 9.9[a] |
| 5 | 79.6 ± 9.8[a] | 63.6 ± 8.9[a] | 53.7 ± 10.0[a] |

HL-60 cells were seeded at a density of $10^5$ cells/ml, treated with various concentrations of 278E and collected at day 5 for assessment. Data are expressed as mean ± SE at least three independent experiments.
[a]$p < 0.05$, ANOVA with Tukey's test was used for comparison.

**Fig. 4**




Expression of surface marker in 278E-treated HL-60 cells by flow cytometric assay. Cells were seeded at a density of $10^5$ cells/ml, treated with various concentrations of 278E and collected at day 5. (A) CD11b. (B) CD14 and (C) CD68.

However, the positive control TPA did induce rapid translocation of PKC-α in HL-60 cells (Fig. 5B).

### Expression of proto-oncogenes

Expression of c-*myc* was markedly suppressed in HL-60 cells after 16 h treatment with 6 μg/ml 278E (Fig. 6A). In contrast, expression of c-*fms*, c-*jun* and c-*fos* was enhanced by 6 μg/ml 278E after 16, 48 and 48 h of treatment, respectively (Fig. 6B–D).

**Table 4 Induction of monocytic differentiation by various combination treatments**

| Treatment | Mo/Mϕ (%) |
|---|---|
| None | 0 |
| Staurosporine 10 nM | 0 |
| H7 15 μM | 0 |
| TPA 10 ng/ml | 76.8 ± 7.2 |
| Staurosporine 10 nM + TPA 10 ng/ml | 35.0 ± 2.3 |
| H7 15 μM + TPA 10 ng/ml | 30.2 ± 4.5 |
| 278E 6 μg/ml | 56.1 ± 6.2 |
| Staurosporine 10 nM + 278E 6 μg/ml | 55.6 ± 4.7 |
| H7 15 μM + 278E 6 μg/ml | 51.3 ± 5.2 |

HL-60 cells were seeded at a density of $10^5$ cell/ml, treated with various combinations and collected at day 3. After cytospin and Wright's staining, morphology was observed in 400 cells for each group under a light microscope.

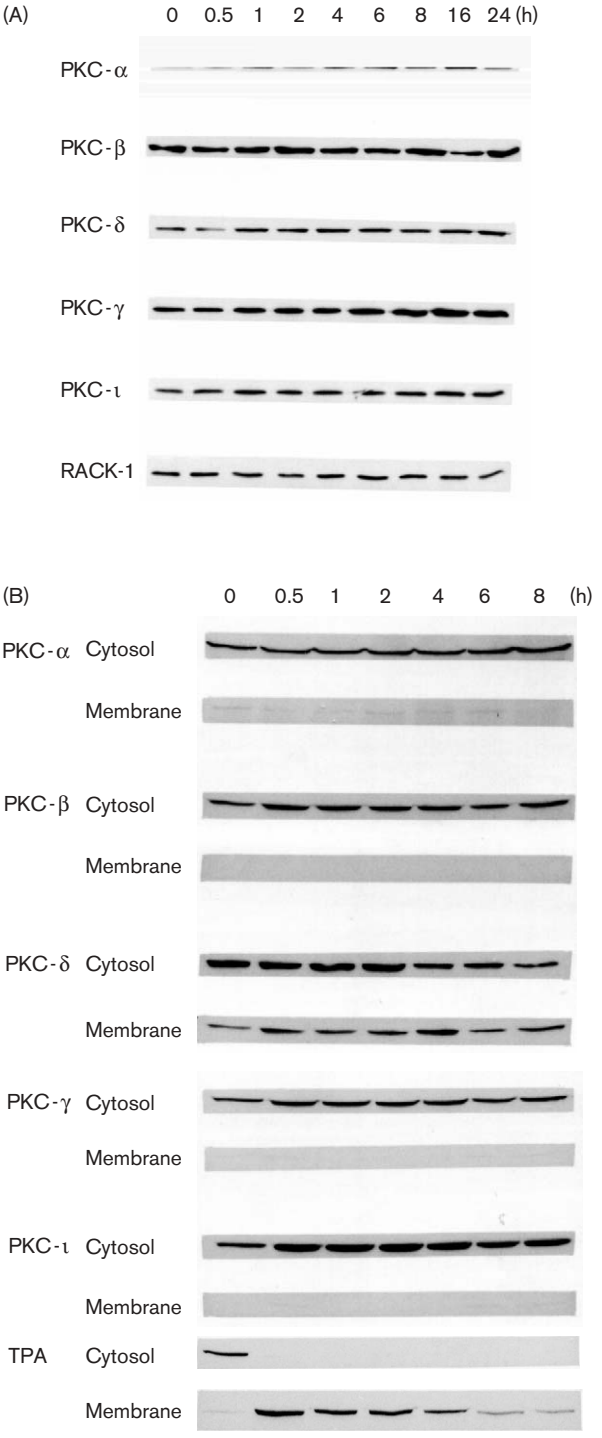
## Discussion

In this study, we demonstrated that 278E inhibits cell growth and induces monocytic differentiation of human myeloid leukemic HL-60 cells. This effect is PKC independent, and is accompanied by downregulation of c-*myc* as well as up-regulation of c-*fms*, c-*jun* and c-*fos* mRNA expression.

Activation of PKC involved in the induction of differentiation along the monocytic lineage of leukemic cells such as U937 [21] and HL-60 cells [22]. However, treatment with 278E caused neither a downregulation nor translocation of PKC in HL-60 cells. Pretreatment with the PKC inhibitors, H7 and staurosporine, did not block the growth-inhibiting and differentiation-inducing activity of 278E, indicating that 278E may not be a PKC activator and that it induces differentiation of HL-60 cells to Mo/Mϕ through a PKC-independent pathway. HL-60 cells have been reported to have endogenous amplification of a c-*myc*-related DNA sequence [9] and loss of this amplification may result in expression of the c-*fms* proto-oncogene that has been found during monocytic differentiation [23,24]. Up-regulation of c-*jun* and c-*fos* mRNA expression is involved in proliferation and differentiation during hematopoiesis [25]. Moreover, AP-1, a product of the co-expression of c-*jun* and c-*fos*, binds a negative element located in the c-*myc* DNA sequence to downregulate c-*myc* expression [26]. We demonstrated that 278E induced downregulated c-*myc* (at 16 h) and up-regulated c-*fms* (at 16 h), c-*jun* (at 48 h) and c-*fos* (at 48 h) mRNA expression in HL-60 cells in the course of monocytic differentiation. This implies that 278E is a monocytic differentiation-inducing compound capable of regulating differential expressions of these proto-oncogenes.

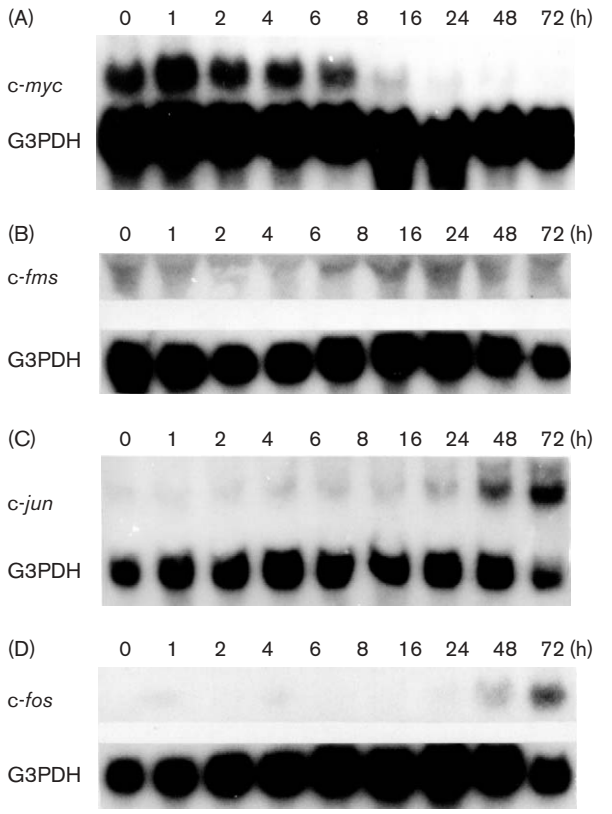
We found that all five derivatives of 278A (salvinal) possess growth inhibitory activity, but only 278E induced monocytic differentiation. It has the lowest growth inhibition. Comparison of the chemical structures revealed that the 278B and 278E have the same backbone except for a unique functional group at carbon atom 3, i.e.

**Fig. 5**




Expression of PKC isozymes in the 278E-treated HL-60 cells by Western blot analysis. Cells were treated with 6 μg/ml 278E for the indicated periods of time. Equal amounts of proteins (50 μg/lane) from whole-cell lysates were separated by electrophoresis on a 10% SDS–PAGE gel and immunoblotted with anti-PKC isozyme monoclonal antibodies. (A) PKC isozymes expression. (B) Intracellular distribution of PKC isozymes.

**Fig. 6**




Gene expression in the 278E-treated HL-60 cells. Cells treated with 6 μg/ml 278E for the indicated periods of time. Total RNA (20 μg/ml) was extracted and analyzed by Northern blotting. Expression of (A) c-*myc*, (B) c-*fm*, (C) c-*jun* and (D) c-*fos*.

278B has a 3-benzo[*b*]furancarbaldehyde group and 278E has a 3-benzo[*b*]furan group at C-3. It may be this structure that accounts for the effects of 278E on HL-60 cells.

With AML, there is increasing interest in using agents to induce differentiation rather than simply killing cells with cytotoxic agents. All-*trans*-retinoic acid and arsenic trioxide have been extensively investigated in clinical trials of treatment of acute promyelocytic leukemia [27,28]. Given the evidence that 278E induces monocyte differentiation, this may have potential in the treatment of acute myeloid leukemia.

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