ORIGINAL ARTICLE

Francesca Civoli · Susan B. Pauig · Larry W. Daniel

Differentiation of HL-60 cells distinguishes between cytostatic and cytotoxic effects of the alkylphospholipid ET-18-OCH_3

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Abstract The synthetic dialkylphospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) inhibits growth of the acute myelogenous leukemia cell line HL-60. Incubation of HL-60 cells with dimethylsulfoxide causes the cells to differentiate to a granulocyte-like phenotype and become quiescent. Incubation of the DMSO-treated cells with ET-18-OCH_3 results in a reduction in cell numbers due to cytotoxicity. In contrast, treatment of undifferentiated HL-60 cells with lower concentrations of ET-18-OCH_3 leads to growth inhibition. These data indicate that the model of differentiated HL-60 cells currently used for the study of resistance to growth inhibition is inappropriate. HL-60 cells can be used to measure growth inhibition and at higher doses cytotoxicity. However, the differentiated, nonproliferative, cells can only be used to measure direct cytotoxicity. Therefore, the results of studies directly comparing the effects of ET-18-OCH_3 in proliferative HL-60 cells and quiescent DMSO-treated HL-60 cells should be reevaluated. An evaluation of the effects of low concentrations of ET-18-OCH_3 (0.5–1.5 μM) in proliferating HL-60 cells indicated that ET-18-OCH_3 was an effective cytostatic agent at nontoxic concentrations. In summary, studies on the mechanism of action of ET-18-OCH_3, or related ether lipids, should carefully investigate differences in the effects at cytostatic versus cytotoxic concentrations.

Key words ET-18-OCH_3 · HL-60 cells · Differentiation · Cytotoxicity · Cytostasis

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F. Civoli[1] · S.B. Pauig · L.W. Daniel (✉)
Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157–1016, USA
Fax (910) 716-7671

Present address:
[1] Department of Developmental Biology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Introduction

The synthetic dialkylphospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and structurally related ether-linked lipids selectively inhibit neoplastic cell growth compared to normal cells [1, 3, 4, 16]. Leukemia cells also differ in sensitivity to ET-18-OCH_3. For example, the HL-60 cell line is more sensitive to growth inhibition than the K562 cell line. The ether lipids alter proliferative signal transduction pathways, and this has been suggested as an important part of their mechanism of action [4, 9, 10, 15]. ET-18-OCH_3 has also been shown to nonspecifically disrupt membrane integrity and function [13].

Protein kinase C (PKC) is inhibited by ET-18-OCH_3 both in vitro and in intact cells and has been suggested as a target for the ether lipids [3, 5, 6, 9, 11, 12, 14]. However, recent studies argue against the role of PKC in the cytotoxic action of ET-18-OCH_3 [8]. Some of the controversy may be due to the model systems used and the failure to distinguish between cytostatic and cytotoxic activities. One suggested model for studying the resistance to ET-18-OCH_3 is the HL-60 cell line differentiated to granulocytes by treatment with dimethylsulfoxide (DMSO). Vallari et al. [17] and others [7, 8] have shown that higher concentrations of ET-18-OCH_3 are required to reduce cell numbers in the DMSO-treated cells compared with undifferentiated cells. However, since the differentiated cells are nonproliferative this seems to be an inadequate model to study inhibition of cell proliferation. In the present study, we reevaluated this model taking into consideration effects upon proliferation versus cytotoxic effects.

Materials and methods

Materials

The HL-60 cell line was obtained from the American Type Culture Collection. ET-18-OCH_3 was a gift for Medmark Pharma, Munich,

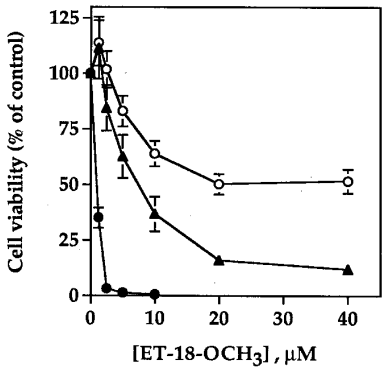

Fig. 1 Effect of ET-18-OCH_3 on growing, stationary and differentiated HL-60 cells. HL-60 cells in log-phase growth were transferred to 96-well plates (5×10^4 cells/well in 100 μl of medium) with the indicated concentrations of ET-18-OCH_3 and incubated for 18 h before the MTT assay was performed during the following 6 h. (● cells growing during the assay period). Alternatively, cells were transferred to 96-well plates and incubated for 5 days with (○) or without DMSO (1.3% v/v) (▲) before the addition of ET-18-OCH_3. The values presented are means ± SEM (n = 4)

Germany. The cell culture medium, serum and antibiotics were purchased from Gibco/Life Technologies, Gaithersburg, Md. Plasticware was from Costar, Cambridge, Mass. The Cell Titer 96 kit for the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was from Promega Corporation, Madison, Wis.

Cell culture

The HL-60 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, L-glutamine (290 μg/ml), penicillin (100 units/ml) and streptomycin (70 μg/ml). All incubations were carried out in an atmosphere containing 5% CO_2 humidified at 37 °C. For the MTT assay, exponentially growing HL-60 cells were transferred to 96-well plates (5×10^3 cells/well in 100 μl medium). In some assays, ET-18-OCH_3 was added immediately after transfer to the 96-well plates. Alternatively, HL-60 cells were transferred to the 96-well plates and incubated for 5 days with DMSO (1.3% v/v) or without DMSO before treatment with ET-18-OCH_3.

Assays for viable and nonviable cells

The MTT assay for viable cells, which is based upon the cellular conversion of a tetrazolium salt into a blue formazan product, was used. The assay was purchased as a kit (Cell Titer 96, Non-Radioactive Cell Proliferation/Cytotoxicity Assay; Catalog #G4000) and used according to the manufacturer's directions. Absorbance at 570 nM was measured using a Molecular Devices microtiter plate reader. We also used trypan blue dye and hemacytometer counting in some experiments to determine the numbers of viable and nonviable cells.

Assay for granulocytic differentiation

Differentiation in response to DMSO was measured by determining nitroblue tetrazolium reducing ability as described by Baehner and Nathan [2]. Briefly, the HL-60 cells were incubated for 30 min at 37 °C in an equal volume of phosphate-buffered saline, pH 7.3, containing 0.1% nitroblue tetrazolium (NBT) w/v, 1% DMSO v/v and 10 n*M* 12-*O*-tetradecanoylphorbol-13-acetate. After incubation slides were prepared using a Shandon Elliott cytospin centrifuge and at least 200 cells per slide were counted. The differentiation assays were done in triplicate.

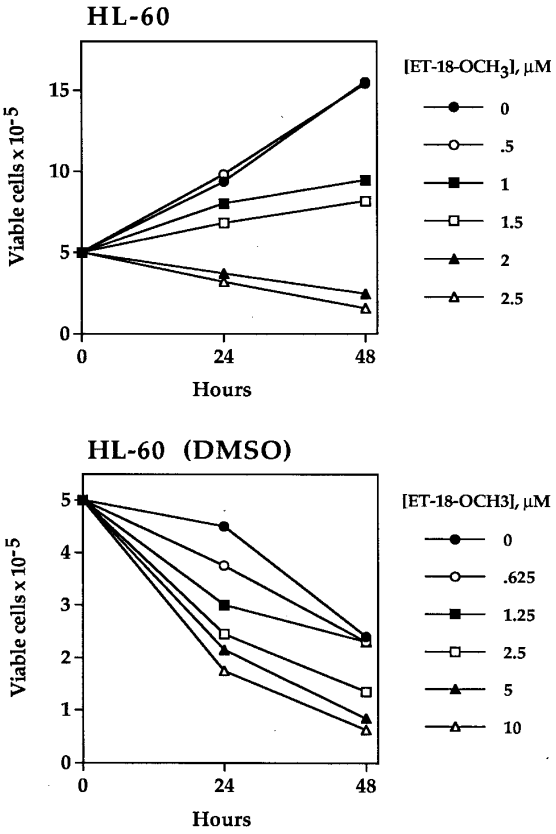

Fig. 2 A, B Comparison of ET-18-OCH_3 effects on HL-60 cells (**A**) and DMSO-differentiated HL-60 cells (**B**). HL-60 cells were treated with DMSO (1.3% v/v) for 5 days then resuspended (5×10^5 cells/ml) in fresh medium containing the indicated concentration of ET-18-OCH_3. The control cells were in the log-growth phase and resuspended at 5×10^5/ml with the indicated concentrations of ET-18-OCH_3. Cell viability was determined by hemacytometer counting using trypan blue. The data presented are the mean of duplicate cell counts from a representative one of two experiments

Results

When exponentially growing HL-60 cells were incubated with ET-18-OCH_3 for 18 h, there was a marked concentration-dependent decrease in viable cell numbers compared with control cells not incubated with ET-18-OCH_3 (Fig. 1). In contrast, HL-60 cells treated for 5 days with DMSO were relatively resistant to ET-18-OCH_3. Similar results have been reported previously [7, 8, 10, 17]. We also found that HL-60 cells incubated in the 96-well plates for 5 days without DMSO were significantly less sensitive to ET-18-OCH_3 (Fig. 1). The DMSO-treated cells were 17.5 ± 0.8% positive in the NBT assay for cell differentiation. The cells not treated with DMSO but incubated for 5 days in the 96-well plates became quite dense (3.4×10^6 cells/ml) but did not differentiate (no NBT-positive cells were noted). These results indicate that differentiation per se may not be responsible for the differences in sensitivity noted in Fig. 1 and imply that the growth state of the cells influences sensitivity.

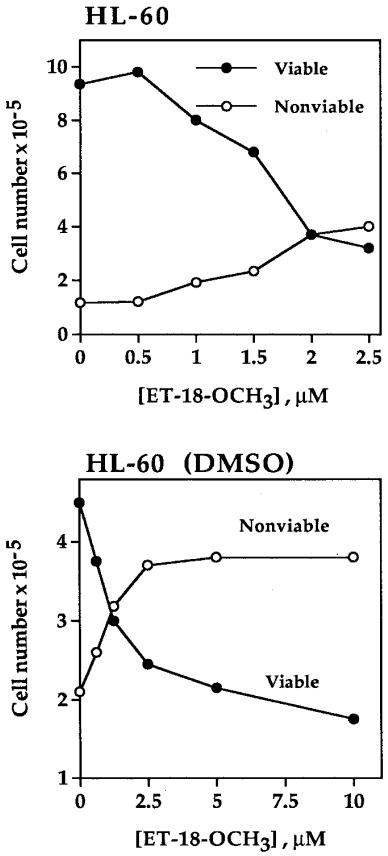

Fig. 3 A, B Effect of ET-18-OCH_3 on cell viability. HL-60 cells (**A**) and DMSO-differentiated HL-60 cells (**B**) were cultured as described in the legend to Fig. 2. Viable and nonviable cell numbers were determined 24 h after ET-18-OCH_3 treatment by hemacytometer counting with trypan blue dye. The data presented are the mean of duplicate counts from one of two similar experiments. Similar results were observed when the cells were incubated for 48 h before counting

We next compared the effects of ET-18-OCH_3 on the growth rate of HL-60 cells and DMSO-treated cells. In the undifferentiated HL-60 cells, low concentrations of ET-18-OCH_3 (~1 μ*M*) inhibited cell growth and higher concentrations were cytotoxic (Fig. 2A). In the DMSO-treated HL-60 cells (20.1 ± 2.3% NBT positive), ET-18-OCH_3 was cytotoxic (Fig. 2B). HL-60 cells treated with DMSO for 5 days were incapable of proliferation. Therefore, in the untreated HL-60 cells both cytostatic and cytotoxic effects could be observed. In the DMSO-treated cells only cytotoxicity was observed. To confirm this observation we measured the number of viable and nonviable cells as a function of ET-18-OCH_3 concentration. In the undifferentiated HL-60 cells, ET-18-OCH_3 caused a decrease in viable cells without a comparable increase in nonviable cells (Fig. 3). In DMSO-treated HL-60 cells, the ET-18-OCH_3-induced decrease in viable cells was matched by a corresponding increase in dead cells. This resulted in a marked decrease in the total number (viable plus nonviable) of HL-60 cells with little decrease in the total number of differentiated HL-60 cells (Fig. 4).

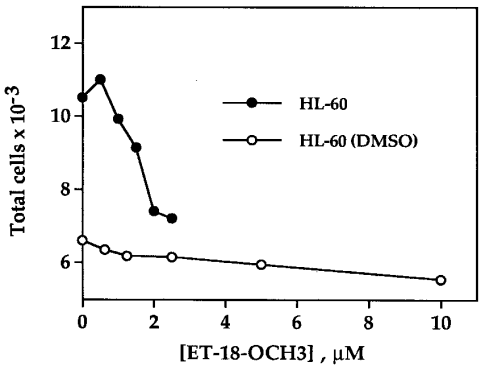

Fig. 4 Effect of ET-18-OCH_3 on total numbers of HL-60 and DMSO-differentiated HL-60 cells. ET-18-OCH_3 reduced the total number of HL-60 cells observed at 24 h of treatment due to inhibition of cell growth. ET-18-OCH_3 did not appreciably reduce the number of differentiated HL-60 cells because the decrease in viable cell numbers was matched by an increase in nonviable cells

Discussion

These results indicate that the model of DMSO-differentiated HL-60 cells used to study ET-18-OCH_3 resistance should be reevaluated. Our results separate cytostatic and cytotoxic effects of ET-18-OCH_3 in HL-60 cells and indicate that cytostatic effects occur at low noncytotoxic doses. However, only cytotoxicity due to nonspecific detergent-like effects can be measured in the DMSO-treated, nonproliferative cells. These results may explain previous controversial observations that the mechanism of action of ET-18-OCH_3 could be by interference with signal transduction or disruption of membrane integrity if the former results in cytostasis and the latter results in cytotoxicity.

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