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Inhibition of Proliferation and Induction of Differentiation of Human and Mouse Myeloid Leukemia Cells by New Ethyleneglycol-type Nonphosphorus Alkyl Ether Lipids

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A variety of ethyleneglycol-type nonphosphorus alkyl ether lipids, ether derivatives of diethyleneglycol in which the two hydroxyl groups were substituted with long chain alkyl and quaternary ammonioalkyl groups, were synthesized and their effects on proliferation and differentiation of cultured human (HL-60) and mouse (M1) myeloid leukemia cells were studied. Incubation with these compounds inhibited the cellular proliferation, and the cells differentiated into morphologically and functionally mature granulocytes. Of the compounds tested, 1-[2-[2-(octadecyloxy)ethoxy]ethoxy]-butylpyridinium mesylate (EG-6) was the most effective in inducing differentiation of HL-60 cells. Almost maximal induction of differentiation and inhibition of growth of HL-60 cells on day 6 were observed when the cells were treated with EG-6 for 1 day and then cultured without EG-6 for a further 5 days. The inhibitory effect of EG-6 on the leukemic cells was over 100 times more than that of 2-[2-(dodecyloxy)ethoxy]ethyl 2-pyridinioethyl phosphate, a potent antileukemic ether phospholipid.

Key words: Differentiation — Growth inhibition — Myeloid leukemia — HL-60 cells — Alkyl ether lipids

Alkyl-lysophospholipids (ALP, glycerol-type alkyl ether lipids) are ether analogues of naturally occurring lysophospholipids. ALPs have cytocidal effects on neoplastic cells including leukemia cells, while sparing normal cells.[1,2] The direct antineoplastic effects of these compounds have been reported to be due to selective interference with phospholipid metabolism of neoplastic cells.[3–5]

A typical compound of this class is 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine (ET18-OCH_3),[6] which has been tested clinically in West Germany. However, unfortunately, it exhibited undesirable side effects.[7,8] Recently, alkyl thioether phospholipid (ilmofosine, BM 41.440)[9] and hexadecylphosphocholine (HPC)[10] have been reported and both compounds are now being tested clinically.

We have reported a newly synthesized ALP[(3-tetradecyloxy-2-methoxy)propyl-2-trimethylammonioethyl phosphate] which potently inhibited proliferation and induced differentiation of human (HL-60) and mouse (M1) myeloid leukemia cells into macrophages and granulocytes, but did not show any effect on the proliferation or differentiation of normal mouse bone marrow cells even at high concentrations.[11] However, it was found to have a marked side-effect in SL mice syngeneic to M1 cells.[12] To obtain differentiation inducers causing less severe side-effects, we synthesized various alkyl ether lipids and found that ethyleneglycol-type alkyl ether phospholipids had potent differentiation-inducing activities in HL-60 and M1 cells.[12] Among these synthetic lipids, 2-[2-(dodecyloxy)ethoxy]ethyl 2-pyridinioethyl phosphate (EGPL) (Fig. 1) showed antileukemic activity in mice inoculated with M1 cells.[13]

Based on these findings, we tried to develop more effective analogues of ethyleneglycol-type alkyl ether lipids for clinical use in differentiation therapy of myeloid leukemia. In this work, we synthesized several ethyleneglycol-type nonphosphorus alkyl ether lipids (NP-AELs) and examined their effects on the growth and differentiation of HL-60 and M1 cells *in vitro*. The structural features are: (a) a long-chain alkyl ether bonded to the diethyleneglycol backbone instead of the glycerol backbone, (b) an ether-linked polar head side chain with a cationic structure, and (c) the absence of the phosphate group. We found that 1-[2-[2-(octadecyloxy)ethoxy]-ethoxy]butylpyridinium mesylate (EG-6, Fig. 1) induced differentiation and inhibited proliferation of HL-60 and M1 cells at a much lower concentration than the effective concentration of EGPL.

MATERIALS AND METHODS

Chemicals The alkyl ether lipids tested were synthesized in our laboratories. We used 2-(2-octadecyloxy)ethoxyethanol as a starting material instead of 3-octadecyloxy-1,2-propanediol. Other syntheses were performed as de-

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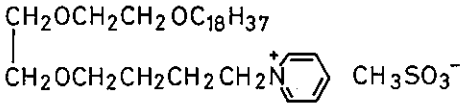
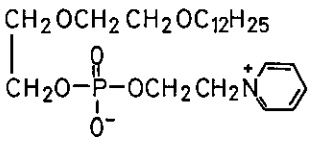


EG-6



EGPL

Fig. 1. Structures of EG-6 and EGPL.

scribed in previous reports.[14, 15] The purity and chemical structure of each compound were confirmed by TLC, NMR, and elemental analyses. These compounds were dissolved in ethanol.

Cell lines and cell culture Human myeloid leukemia HL-60 and K562 cells were cultured in suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air.[16, 17] Mouse myeloid leukemia M1 cells (clone T22) were maintained in Eagle's minimum essential medium with twice the normal concentrations of amino acids and vitamins, and supplemented with 10% calf serum.[18, 19]

Assays of cell growth and the properties of differentiated cells Cells (10^5/ml) were suspended in 5 ml of culture medium and cultured with or without alkyl ether lipids in Falcon 6-cm culture dishes. Cell numbers were counted in a Model ZM Coulter Courter (Coulter Electronics, Luton, England) 6 days (HL-60 and K562 cells) or 3 days (M1 cells) after various treatments. Nitroblue tertrazolium (NBT) reduction was assayed by microscopic or colorimetric assay as reported previously.[20, 21] Microscopic assay: the percentage of cells containing intracellular blue-black formazan deposits was determined by examination of a minimum of 200 cells. Colorimetric assay: The reaction was stopped by adding 5 *N* HCl (final concentration: 1 *N*). The suspension was stood for 1 h at room temperature and then centrifuged and the medium was discarded. The formazan deposits were solubilized by adding dimethylsulfoxide, and the absorption of the formazan solution at 560 nm was measured in a spectrophotometer. The percentage of cells that were morphologically similar to mature granulocytes was determined by examination of cell smears treated with May-Gruenwald-Giemsa stain.

RESULTS

Effects of ethyleneglycol-type NP-AELs on growth and differentiation of myeloid leukemia cells We synthesized a variety of ethyleneglycol-type NP-AELs possessing the octadecyl group and examined their effects in inducing differentiation of myeloid leukemia cells into mature granulocytes and macrophages. We found that ethyleneglycol-type NP-AELs in hibited the growth of HL-60 cells and induced their differentiation (Table I). The growth-inhibitory effects of the compounds were examined by determining their concentrations required to reduce the cell number to half that of untreated cultures (IC_{50}). The cells were cultured with the IC_{50} concentration of each compound for assay of morphological differentiation. These compounds (EG-1 to -6) had stronger effects on the growth and differentiation of HL-60 cells than EGPL, which had been proved to be therapeutically effective in leukemic mice in our laboratories.[13] Among these compounds, the 4-pyridinio-*n*-butyl ether (EG-6) had the greatest inhibitory effect on growth, with an IC_{50} value of 31.3 ng/ml, and was also the most effective in

Table I. Effects of Various Ethyleneglycol-type Nonphosphorus Alkyl Ether Lipids on Growth and Differentiation of HL-60 Cells

$CH_2OCH_2CH_2OC_{18}H_{37}$
|
$CH_2OCH_2CH_2R\ CH_3SO_3^-$

Compound	R	Growth inhibition IC_{50} (ng/ml)[a]	Morphological differentiation (% of mature myeloid cells)[b]
EG-1	$\overset{+}{N}(CH_3)_3$	325±12	10.7±2.1
EG-2		81.7±7.6	8.3±1.2
EG-3		99.0±1.0	7.3±1.2
EG-4		92.8±5.0	19.7±3.1
EG-5	$CH_2CH_2\overset{+}{N}(CH_3)_3$	148±19	16.3±2.5
EG-6		31.3±4.2	28.7±2.6
EGPL[c]		3270±350	17.3±2.1

a) IC_{50}, concentration required to reduce the cell number to half that of untreated cells±SD.
b) Mature myeloid cells, myelocytes, metamyelocytes, and banded and segmented neutrophils. HL-60 cells were cultured with the IC_{50} concentration of each compound for 6 days.
c) The structure is shown in Fig. 1.

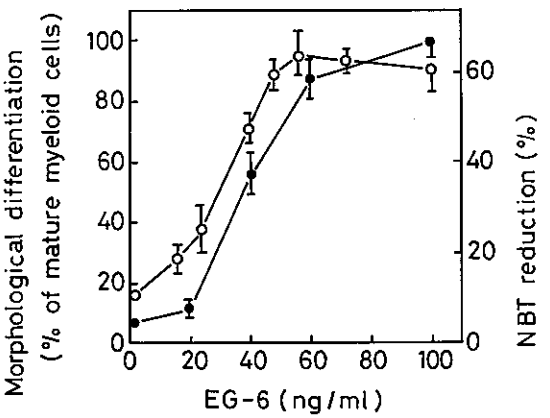


Fig. 2. Induction by EG-6 of morphological differentiation of HL-60 cells and of their ability to reduce NBT. HL-60 cells were cultured for 6 days in medium containing EG-6. ●, morphologically matured cells; ○, cells reducing NBT. Bars, SD (n=3).

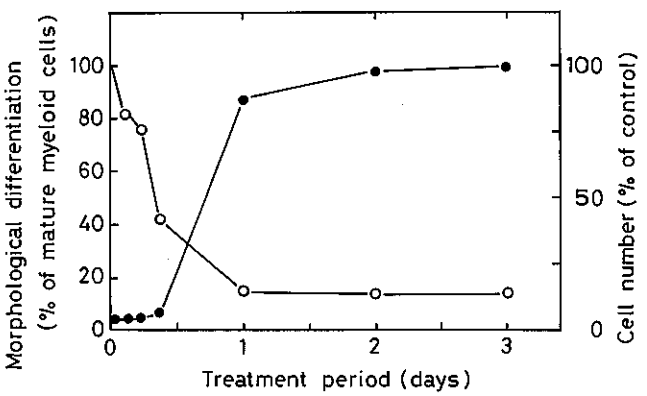


Fig. 4. Time course of induction of differentiation of HL-60 cells by EG-6. HL-60 cells were cultured with 80 ng/ml EG-6 for various periods, and then washed twice with phosphate-buffered saline and cultured in freash medium in the absence of EG-6 until day 6. Morphological differentiation and the cell number were examined on day 6. ●, morphologically matured cells; ○, cell number. Data are representative of 3 separate experiments. SDs were consistently below 10% of means.

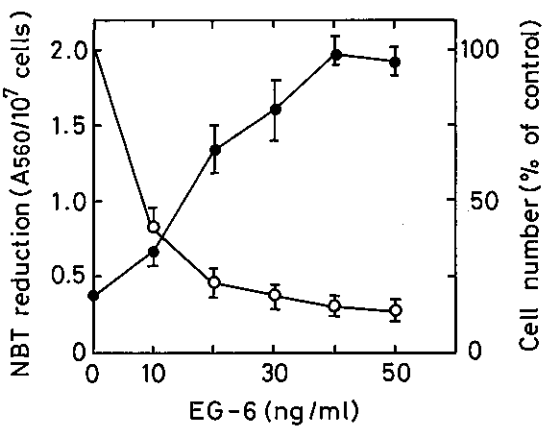


Fig. 3. Effect of EG-6 on growth and differentiation of M1 cells. M1 cells were cultured with various concentrations of EG-6 for 3 days, and then cell numbers and NBT reduction were determined. ●, NBT reduction; ○, cell number. Bars, SD (n=3).

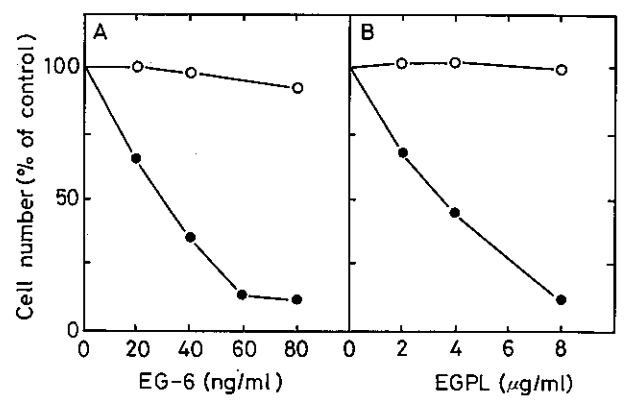


Fig. 5. Effects of EG-6 on growth of human myeloid leukemia HL-60 and K562 cells. Cells were treated with various concentrations of EG-6 (A) or EGPL (B) for 6 days and then cell numbers were determined. ●, HL-60 cells; ○, K562 cells. Data are representative of 3 separate experiments. SDs were consistently below 10% of means.

inducing differentiation. The cells which were treated with the IC_{50} concentration of EG-6 for 6 days remained as viable as those growing in the absence of EG-6 (>90%) when the viabilities were determined by trypan blue dye exclusion using a hemocytometer. The inhibitory effect of EG-6 was observed at a concentration of the order of 10^{-8} *M*, so that EG-6 was 100 times more potent than EGPL (IC_{50}=3270 ng/ml). The 2-(1-methylpyrrolidinio)ethyl ether (EG-4) was also effective in inhibiting growth and inducing differentiation of HL-60 cells, but its IC_{50} value was found to be 93 ng/ml, a value 3 fold higher than that of EG-6. The 2-pyridinioethyl ether (EG-2) showed almost the same IC_{50} value as EG-4 but was less effective than the latter in inducing differentiation (Table I).

HL-60 cells cultured without inducers were mostly promyelocytic, but on culture with increasing concentrations of EG-6, the percentage of mature granulocytes

increased progressively (Fig. 2). More than 90% of the cells had differentiated into myelocytic and more mature cells after treatment for 6 days. EG-6 also caused dose-dependent induction of NBT reduction, which is a typical functional marker of differentiated HL-60 cells (Fig. 2).

EG-6 also induced differentiation and inhibited growth of mouse myeloid leukemia M1 cells (Fig. 3). EG-6 dose-dependently induced NBT reduction (Fig. 3) and phagocytic activity of the cells (data not shown). The IC_{50} value (8.0 ng/ml) of EG-6 for M1 cells was 4-fold lower than that for HL-60 cells (IC_{50}=31.3 ng/ml).

Next, we examined whether continuous treatment of HL-60 cells with EG-6 was necessary for maximal effects on their growth and differentiation (Fig. 4). For this, HL-60 cells were cultured with 80 ng/ml EG-6 for various periods, and then washed twice with phosphate-buffered saline and recultured in fresh medium in the absence of EG-6 until day 6. Almost maximal induction of differentiation and inhibition of growth of the cells on day 6 were observed when the cells were treated with EG-6 for 1 day and then cultured without EG-6 for a further 5 days. These results indicate that continuous treatment of the cells with EG-6 is not necessary for induction of its maximal effects.

Effect of EG-6 on growth of other human myeloid leukemia cells Tidwell *et al.*[22] reported that a glycerol-type alkyl ether phospholipid (ET18-OCH_3) inhibited proliferation of HL-60 cells but not human myeloid leukemia K562 cells. The chemical structures of EG-6 and ET18-OCH_3 are different, so EG-6 may show a different target cell specificity. Therefore, we examined the effects of EGPL and EG-6 on the growth of HL-60 and K562 cells (Fig. 5). Both EGPL and EG-6 inhibited proliferation of HL-60 cells but not that of the K562 cells. These results indicate that the target cell specificity of EG-6 is similar to that of EGPL and ET18-OCH_3. We also examined the effects of EG-6 on the growth of other human myeloid leukemia cells. ML-1, U937 and THP-1 cells were inhibited similarly by EG-6, but the IC_{50} values for growth inhibition of these cells were found to be 50–70 ng/ml, slightly higher than that for HL-60 cells (data not shown).

DISCUSSION

In this work, we found that some ethyleneglycol-type NP-AELs inhibited proliferation of human (HL-60) and mouse (M1) myeloid leukemia cells and induced their differentiation. The structural feature of these new synthetic lipids are (a) a long-chain alkyl ether bonded to the diethyleneglycol backbone instead of the glycerol backbone, (b) an ether-linked polar-head side chain with a cationic structure, and (c) the absence of a phosphate group. Among these lipids, the 4-pyridinio-*n*-butyl ether EG-6 was the most effective in both inhibiting growth and inducing differentiation of these leukemia cells.

The absence of a phosphate group in this series of ethyleneglycol-type NP-AELs may be important for their activities on the cells. The phosphodiester bonds of ALP are probably susceptible to attack by phospholipase C or a related enzyme.[4] Ukawa *et al.*[15] recently reported that one of the apolar alcohols formed metabolically from ET18-OCH_3 was far less active toward HL-60 cells than the parent ALP. Fleer *et al.*[4] and Hilgard *et al.*[10] suggested that ALPs were activated during their metabolism, but the report of Ukawa *et al.* suggests that the active principles are not metabolites (apolar alcohols) but the ALPs themselves. We found that ethyleneglycol-type NP-AELs were much more effective against myeloid leukemia cells than the alkyl ether ethyleneglycophospholipid, EGPL. NP-AELs might be resistant to the action of phospholipase or related enzymes because they have no phosphate group and are metabolically more stable. Therefore, the active principles of ethyleneglycol-type NP-AELs may be the alkyl ether lipids themselves, not their metabolites.

The alkyl ether lipids we tested here are cationic compounds, whereas EGPL is a zwitterionic compound. This cationic feature may result in much higher affinity to leukemia cells as compared with the zwitterionic ether lipids, because the cell surface of malignant cells where alkyl ether lipids first make contact and exert antiproliferative activity has been shown to be much more electronegatively charged than that of normal cells.[23] Thus, the higher affinity to leukemia cells of these cationic compounds relative to the zwitterionic compound EGPL might be related to their higher activities than the latter in inducing differentiation and inhibiting proliferation of leukemia cells.

We have reported that an EGPL analog with the octadecyl group showed three times stronger growth-inhibitory activity than EGPL on HL-60 cells, although its differentiation-inducing activity was significantly lower.[12] The results indicate that the length of the alkyl group is important for the growth-inhibitory activity against leukemia cells.

In contrast to EGPL, a zwitterionic phospholipid, EG-6 is a cationic lipid characterized by the absence of the phosphate group. Despite this difference in structure, both alkyl ether lipids preferentially inhibited proliferation of HL-60 cells, but not that of K562 cells. Their mechanisms of actions are unknown, but possibly the structure of the alkyl ether bond at position 1 of the ethyleneglycol backbone in both lipids contributes to their target cell specificity.

Hoffman *et al.*[24] and Daniel *et al.*[25] reported that K562 cells incorporated markedly less ET18-OCH_3 com-

pared to HL-60 cells when incubated at the same drug concentration and they suggested that the cellular uptake of ALPs could be a factor in explaining the cytotoxic response of certain tumor cells. On the other hand, Fleer *et al.*[26] recently reported that the amount of HPC (another representative of ALPs) uptake was similar in HL-60 cells and K562 cells although HL-60 cells and K562 cells showed a twenty-fold difference in sensitivity. Thus, it will be important to examine whether the resistance to EG-6 in K562 cells can be explained in terms of the cellular uptake.

Maximal induction of differentiation of HL-60 cells was observed when the cells were treated with EG-6 for 1 or 2 days and then cultured in the absence of EG-6 until day 6. Similar results were obtained with M1 cells (data not shown). The effectiveness of short-term treatment of the cells with EG-6 for induction of differentiation may be attributed to the greater metabolic stability of EG-6 in the target cells as compared with other phospholipids, as described above. Furthermore, the differentiated HL-60 cells induced by short-term exposure to EG-6 appeared to be less subject to the cytotoxic effect of EG-6 than the differentiated cells induced by continuous treatment with EG-6 (data not shown). It will be interesting to examine the therapeutic effect of EG-6 on myeloid leukemia by means of *in vivo* studies on induction of differentiation of leukemia cells.

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