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Relationship of cell survival, drug dose, and drug uptake after 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine treatment

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Abstract. The mechanisms that govern the activity and the factors that control the anticancer activity of synthetic ether lipids have not been fully elucidated. In this study, three factors were studied in relationship to cell survival after treatment with 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3): (1) exposure dose, (2) drug uptake, and (3) cell density and cell-cycle distribution. In BG-1 human ovarian carcinoma cells, cell survival was an exponential function of exposure dose and was dependent on drug concentration. Drug uptake was dependent on the concentration of ET-18-OCH_3, whereas the reduction in cell survival was directly related to the uptake of drug only in the first decade of cell kill. When the quantity of cells per flask was tripled from 4 to 12×10^6 cells, ET-18-OCH_3 failed to induce a G_2 block. Furthermore, the cell kill induced by a 72-h exposure to 2 μM ET-18-OCH_3 was decreased by a factor of 2 when the cell density increased. Therefore, exposure dose and cell density are important parameters in determining the cell kill induced by ET-18-OCH_3.

Key words: Ether lipid – ET-18-OCH_3 – Cell kill – Drug dose

Introduction

Synthetic ether lipids such as 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) have been shown to be active anticancer agents both in vitro and in vivo [2, 3]. Some of the ether lipids are showing promise as a new class of clinical cancer chemotherapeutic drugs [4, 8, 13, 15] and also in clinical trial as bone-marrow-purging agents [4, 20].

The factors that regulate cell killing and cytostasis induced by synthetic ether lipids have not been fully determined; in fact, the exact mechanisms of their anticancer properties are not known. Some of the effects that these compounds have on tumor cells are inhibition of phosphatidylcholine biosynthesis, incorporation of fatty acids into lysophospholipids, activity of Na- and K-adenosine triphosphatase(ATPase) and the sodium pump, and activity of protein kinase C and of phospholipase C [5, 6, 14, 16, 22]. Ether lipids are also known to increase the fluidity of the membranes [14] and alter the calcium content [19] within the cells. In the experiments described herein, the following questions were addressed:

1. How do the exposure time and the ET-18-OCH_3 concentration interrelate to determine the cell kill?
2. Is the cell kill directly related to the uptake of ET-18-OCH_3?
3. Does the cell density and/or cell-cycle distribution influence the response of BG-1 cells to ET-18-OCH_3?

Materials and methods

Cells. BG-1 cells derived from a human ovarian carcinoma cell line were used [9, 11]. The cells were grown exponentially in McCoy's Medium 5A supplemented with 10% bovine serum albumin, 0.05% L-glutamine, 1% BME nonessential amino acids, 100 U penicillin G/ml, 100 µg streptomycin sulfate/ml and 0.1 U semilente insulin/ml. The cell line was rejuvenated from frozen stock every 6 months.

Ether lipid treatment. ET-18-OCH_3 (provided by Dr. R. Nordström, Medmark Pharma GmbH, Munich, Germany) was dissolved in ethanol and diluted to 10 times the desired concentration with phosphate-buffered saline. The maximal concentration of ethanol (0.05%) in the medium had no effect on colony formation. All treatments were carried out at 37° C in an incubator containing 7% CO_2 in air.

Clonogenic cell survival. Cell survival was measured using a standard colony assay on plastic. Briefly, exponentially growing BG-1 cells

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Abbreviations: EL, ether lipid; CFE, colony-forming efficiency

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(day 3) were trypsinized, resuspended, and plated. Five petri dishes at each of three cell dilutions per treatment were used. The cells were incubated in an atmosphere containing 7.0% CO_2 at 37° C for 12–16 h to allow the cells to attach to the dishes and to recover from any damage from trypsinization. The multiplicity of the cells at plating was <1.1. The drug was then added at the desired concentrations. The drug was removed after a designated time, and the cells were rinsed with drug-free medium and then incubated for colony formation for an additional 14 days to determine the cytotoxicity of ET-18-OCH_3 [21], thus correcting for the cytostasis induced by the ether lipid. Colonies containing >50 cells were counted, and the surviving fraction was determined by dividing the mean colony-forming efficiency (CFE) obtained at three different cell dilutions for the treated cells by that obtained for untreated cells. Since the cells were plated prior to the treatment, there was no reduction in cell number caused by treatment. Survival lines were fit by linear least-squares regression analysis, and each experiment was replicated a minimum of three times.

ET-18-OCH_3 incorporation studies. Uptake of ET-18-OCH_3 was measured by the incorporation of [^{3}H]-L-ET-18-OCH_3 [23] by BG-1 cells. Exponentially growing BG-1 cells (day 3), which were originally plated at 3×10^6 cells/flask and whose density at the time of treatment was approximately 1.2×10^7 cells/flask, were incubated with 1 μCi [^{3}H]-L-ET-18-OCH_3 for the indicated periods in medium containing 8, 14, or 20 μ*M* cold ET-18-OCH_3. Cells in supernatant were spun down and combined with trypsinized cells. The lipids were extracted from the cells and the radiolabeled lipid was quantitated by scintillation counting. The phosphorus content of the phospholipid in each sample was quantitated by the method of Rouser et al. [18]. The data are expressed as mole-percent values relative to the total cellular phospholipid content.

Cell-density experiments. BG-1 cells were plated at either 1 or 3×10^6 cells/75-cm^2 flask and incubated for 72 h. The cells were then treated with 2μ*M* ET-18-OCH_3 for 24, 48, 72, or 120 h. The cell density at the start of the drug exposure was approximately 4.0×10^6 or 1.2×10^7 cells/flask. The drug was removed at designated times and the cells were fixed with 70% ethanol for cell-cycle analysis. The fixed cells were treated with RNase A (1 mg/ml) for 1 h and then stained with 35 μg propidium iodide/ml to determine the DNA content. The stained cells were held overnight at 4° C. Analysis was performed on a FACStar^{plus} flow cytometer (Becton-Dickinson) with a 488-nm excitation beam. Propidium iodide fluorescence was monitored using a 630 ± 22-nm band filter.

The cells treated with ET-18-OCH_3 for 72 h and untreated BG-1 cells were trypsinized and plated for colony formation. Five 60-mm petri dishes at each of three cell dilutions per treatment were used for the assay. The cells were incubated in an atmosphere containing 7% CO_2 at 37° C for 14 days. The colonies were fixed and stained with 1.25% crystal violet in methanol. Colonies containing >50 cells were counted and the CFE was calculated.

Results

Cell-survival experiments

When BG-1 cells were exposed to ET-18-OCH_3 at 2, 8, 14, or 20 μ*M*, the surviving fraction decreased exponentially as a function of exposure time. The CFE of untreated BG-1 cells was $63.5\%\pm1.1\%$. The degree of the cell kill was dependent on the concentration of ET-18-OCH_3 (Fig. 1). When the surviving fraction of BG-1 cells was expressed as a function of the exposure dose (concentration × time) of ET-18-OCH_3 the cell survival was directly related to the exposure dose as shown in Fig. 2.

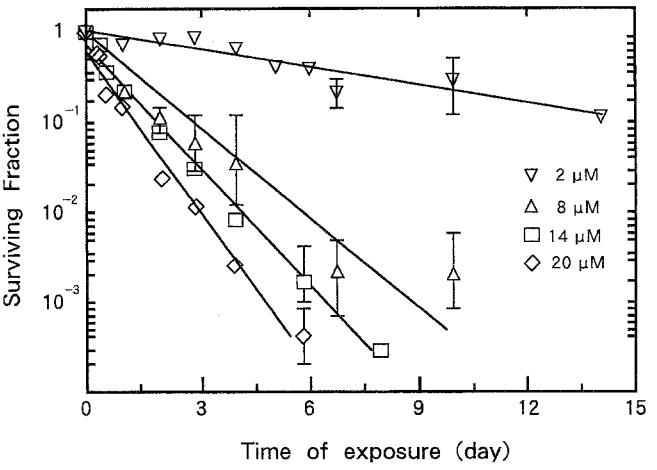

Fig. 1. Cell survival as a function of the duration of exposure to ET-18-OCH_3 at either 2, 8, 14, or 20 μ*M*. Cell survival is decreased as a function of exposure time, and the degree of the decay is dependent on the concentration of ET-18-OCH_3

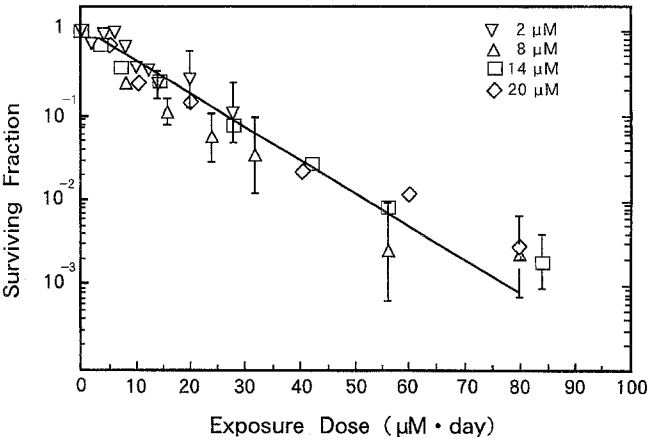

Fig. 2. Cell survival as a function of the exposure dose (concentration × time in days) of ET-18-OCH_3. Cell survival is directly related to the exposure dose and is independent of the concentration of the drug. Data points represent geometric mean values for 3 independent experiments

Uptake studies

Incorporation of ET-18-OCH_3 by BG-1 cells reached a plateau within 1 day, and the uptake was dependent on the concentration (upper panel, Fig. 3). However, there was no relationship between drug uptake and the exposure dose of ET-18-OCH_3 once the uptake reached the plateau level, although a positive correlation was observed while the incorporation was in progress (lower panel, Fig. 3). Therefore, the survival of BG-1 cells was not directly related to uptake of the drug after the incorporation of ET-18-OCH_3 reached plateau levels. However, the assay would be strongly influenced at that point by the $>90\%$ content of dead cells.

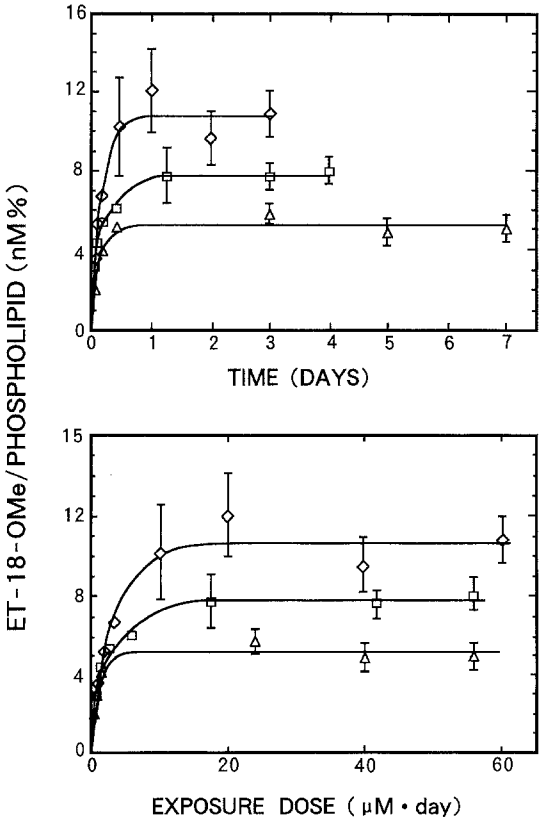

Fig. 3. *Upper panel:* Uptake of ET-18-OCH_3 as a function of exposure time after treatment of cells with 8 (△), 14 (□), or 20 (◇) μ*M*. *Lower panel:* Uptake of drug as a function of exposure dose. Although uptake was dependent on the concentration of the drug, cell survival was not directly related to uptake of the drug

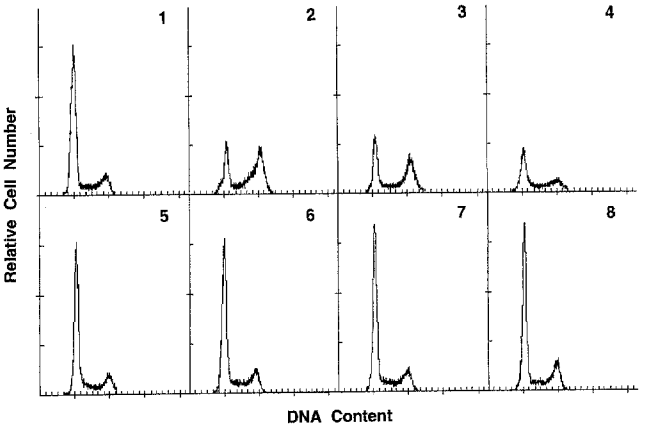

Fig. 4. Cell-cycle distribution of untreated cells (*1*); cells treated at a density of 4×10^6/flask with 2 μ*M* ET-18-OCH_3 for 24 (*2*), 48 (*3*), or 72 h (*4*); or cells treated at a density of 1.2×10^7/flask with 2 μ*M* ET-18-OCH_3 for 24 (*5*), 48 (*6*), 72 (*7*), or 120 h (*8*). At the lower cell density, a pronounced G_2 block was observed in the 24- and 48-h samples. No perturbation was observed in similarly treated cells at the higher density

Cell-density experiments

DNA analysis (Fig. 4) showed a pronounced G_2 block in BG-1 cells plated at the lower density (plated at 1×10^6 and treated at 4×10^6) and treated with ET-18-OCH_3 at 2 μ*M* for 24, 48, and 72 h. However, no perturbation was observed in similarly treated cells at the higher density (plated at 3×10^6 and treated at 1.2×10^7), although a G_2 block had been observed in this population when the cells were treated with 8 μ*M* ET-18-OCH_3 for 24 and 48 h in our previous experiments [10].

The CFE of BG-1 cells treated with ET-18-OCH_3 (2 μ*M* for 72 h) at the higher cell density was 58.3% ± 2.2%, and that of cells treated at the lower cell density was 30.9% ± 2.3%. The surviving fraction was 92% and 49%, respectively. These results indicated that the effect of ET-18-OCH_3 on cell-cycle perturbation and cell kill was dependent on the dose/cell and the growth conditions.

Discussion

In this study, we measured the relationship between the cytotoxicity of BG-1 cells and the exposure dose (concentration × time). As shown in Fig. 2, they are exponentially related. This result suggested that the amount of uptake of ET-18-OCH_3 might play an important role in determining the cytotoxicity of ET-18-OCH_3. This assumption was well supported by the results of the experiment that tested the effects of cell density on the cell kill and cell-cycle perturbations induced by ET-18-OCH_3 (Fig. 4). The results of these experiments suggested that the number of drug molecules per cell must be the important determinant of the cytotoxicity of ET-18-OCH_3. The cell density may determine the "effective" exposure dose in that the drug is diluted among more cells.

However, when we tested uptake of ET-18-OCH_3 by BG-1 cells using [^{3}H]-ET-18-OCH_3, the level of ET-18-OCH_3 in BG-1 cells was saturated within 1 day (Fig. 3) and the plateau level of intracellular ET-18-OCH_3 was dependent on the concentration of ET-18-OCH_3 in the medium. There was no increase in [^{3}H]-ET-18-OCH_3 uptake after the 2nd day of exposure. Although there was a positive correlation between the incorporation of ET-18-OCH_3 and exposure doses <20 μ*M* × day, no such correlation was found between the incorporation of [^{3}H]-ET-18-OCH_3 and the exposure dose of ET-18-OCH_3 for exposure doses of >20 μ*M* × day (Fig. 3, lower panel). Therefore, there may be other cytotoxic mechanisms working beyond the point at which the incorporation of ET-18-OCH_3 reaches a plateau, since increasing cytotoxicity is seen progressively to day 14 (Fig. 1). It may be that once a cytotoxic level has been reached, further incorporation of drug may take place but be without effect. Another consideration is that short exposure periods are sufficient for cytotoxicity (Fig. 1) and that the plateau seen in Fig. 3 is due to the release of drug from dead cells, with cytotoxicity continuing for the small fraction of surviving cells and further uptake by these surviving cells being unmeasurable.

Although the difference in cellular incorporation of the drug seems to be one of the most important factors determining the response of sensitive and resistant leukemic cells to ET-18-OCH_3 [12], the amount of ET-18-OCH_3 in-

corporation could not explain the cytotoxicity of ET-18-OCH_3 to BG-1 cells once the incorporation reached the plateau as shown in the present study (Fig. 3). Bazill and Dexter [1] have shown that endocytosis is a major route for uptake of ET-18-OCH_3 by demonstrating that inhibition of endocytosis protects cells from the cell-killing effect of ET-18-OCH_3. Endocytosis can explain that part of our data in which the uptake of ET-18-OCH_3 (at exposure doses of up to 20 $\mu M \times$ day) was dependent on the concentration of ET-18-OCH_3 in the medium. However, other factors must be considered to explain why lower-level exposure doses of ET-18-OCH_3 (concentration $\times$ time) determine the cytotoxicity of ET-18-OCH_3, whereas higher-level exposure doses do not.

In the cell-density experiments, a G_2 block was observed in the cells treated at lower density. Similar results have been observed by Principe et al. [17], who demonstrated that ET-18-OCH_3 induced a G_2 block as well as a G_1 block in HT29 cells. The mechanism of these cell-cycle perturbations induced by ether lipid is under investigation. However, the G_2 arrest is a manifestation of the cytostasis induced by the ether lipid and may serve as a protective mechanism. The cell-cycle specificity of ET-18-OCH_3 in inducing cell kill and cytostasis will need to be elucidated to provide a full understanding of the cell-density experiments shown in Fig. 4.

Since previous studies regarding the cellular uptake of ether lipid used shorter exposure periods ranging from 3 to 48 h [1, 12, 22], we cannot make a simple comparison between our data (exposure times of up to 15 days) and these results. However, we can assume that there may be some other mechanisms in addition to drug uptake that govern ether lipid cytotoxicity. For example, binding of the drug to serum albumin and modulation of its cytotoxicity by cholesterol are other possible factors [7]. Metabolism of the ether lipid is another possible mechanism. However, as very little metabolism of ether lipid occurs for up to 48 h in cell culture [5], metabolism seems unlikely as an explanation. In our study, metabolism could have occurred to a somewhat greater extent during the longer exposure periods of up to 15 days, but we did not investigate this point. These questions require further investigation.

Although the data obtained in this study are not yet clearly explained, these results have important implications for the administration of ET-18-OCH_3 in clinical trials. For example, the most recent clinical studies [13, 15], showing some partial responses in phase I trials, involved prolonged continuous infusion of ether lipids. Since the cell kill induced by ET-18-OCH_3 correlates directly with the exposure dose, clinicians should compare the clinical response according to careful calculations of the area under the curve after parenteral drug administration by several different routes and schedules. Also, since cell density influences the cell-cycle perturbation and cell kill induced by ET-18-OCH_3, patients with minimal residual disease should be primarily selected for treatment and patients with bulky disease may require escalated doses.

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