Ether lipids (EL) and hyperthermia have been shown to possess a relatively selective cytotoxicity to leukemic cells. In this study, the combined effects of ET-18-OCH₃, ET-16-NHOCH₃, or BM 41.440 and hyperthermia on the growth of hematopoietic progenitors, myeloid leukemic cell lines, and leukemic cells obtained from patients with acute myeloid leukemia (AML) were examined to determine if this combination resulted in a greater selective killing of leukemic cells than that achieved by either EL or heat alone. When the cells were treated simultaneously with EL (50 μg/mL) and hyperthermia (42°C) for one hour, the killing of leukemic cell line cells was enhanced considerably. Among the three EL, however, the combination of ET-18-OCH₃ and heat seemed to be the most cytotoxic to leukemic cell line cells with no effect on the growth of hematopoietic progenitors. An increase in the duration of treatment with ET-18-OCH₃ to four hours with heat added during the last hour resulted in a further reduction of leukemic cell line cells while sparing 50% of hematopoietic progenitors after cryopreservation. The combined treatment with ET-18-OCH₃ and heat also inhibited the growth of leukemic progenitors obtained from AML patients by 97% to 100%. These data indicate that the combined treatment with EL and hyperthermia might offer an efficient means to eliminate myeloid leukemic cells in vitro.

The use of autologous bone marrow transplantation permits the administration of intensive myeloablative antileukemic therapy circumventing the restrictions in allografts. However, autografts have resulted in a higher incidence of leukemic relapse than in allografts. One possible explanation for the higher leukemic relapse rate in autograft is that the marrow sample obtained during remission might contain residual, clonogenic, leukemic cells.

To increase the efficacy of autologous marrow grafts for therapy, preclinical and clinical attempts have been made to eliminate residual leukemic cells from the autograft by in vitro procedures. Although the efficacy of purging marrow cells of residual leukemic cells has not been proven yet, some encouraging data have been reported in clinical trials.

Ether lipids (EL), analogues of the naturally occurring lysophosphatidylcholine, display cytotoxicity to leukemic cells by causing direct injury to the cell membrane. This action has been reported to be relatively selective for leukemic cells in comparison with normal cells, and this selectivity was thought to be due to the relative lack of an alkyl-cleavage enzyme to metabolize this compound in malignant cells. However, the presence of a tetrahydropteridine-dependent cleavage enzyme in leukemic cells was recently reported and other mechanisms might play a role.

Hyperthermia has also been shown to inhibit the proliferation of leukemic cells that are more sensitive to hyperthermic killing than their normal counterparts. Therefore, hyperthermia or EL could be used to purge leukemic cells from remission marrow in acute leukemia.

The combination of hyperthermia and EL might be even more selective in eliminating leukemic cells from remission marrow. In this study, we have investigated the combined effect of three different ether lipids plus heat on the growth of hematopoietic progenitors, myeloid leukemic cell line cells, and leukemic cells obtained from patients with acute myeloid leukemia (AML) to determine if the combination resulted in a greater selective killing of leukemic cells than that achieved by either singular treatment.

MATERIALS AND METHODS

Leukemic cells. Four myeloid leukemic cell lines were used in this study. HL60 human myelocytic cell line was obtained from Dr Robert Gallo, National Institutes of Health (NIH), Bethesda, MD. The K562 cell line, established from a patient with a blast crisis of chronic myelogenous leukemia, was obtained from Dr Bismark Lozzo, University of Tennessee, Knoxville, TN. The KG-1a human myeloblastic leukemic cell line was kindly supplied by Dr Philip Koehler. University of California, Los Angeles. The doxorubicin-resistant HL60 human myelocytic leukemia cell line (HL60/DXR) was a generous gift from Dr Steven Grant, NIH. All cell lines were continuously cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (FBS) except HL60/DXR, which was cultured in the same media plus 1 μmol/L doxorubicin (Adria Laboratories, Dublin, OH). Peripheral blood specimens were also obtained from three patients with AML at diagnosis after obtaining the appropriate informed consent. All samples contained more than 90% leukemic cells.

Bone marrow cells. Histologically normal bone marrow cells were obtained from patients with acute leukemia or malignant lymphoma in complete remission on the occasion of bone marrow harvest for subsequent autologous bone marrow transplantation. In a few instances, hematologically normal bone marrow cells were obtained by crushing bone marrow fragments provided from orthopedic surgery. In all instances, informed consent was obtained.

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for the use of a bone marrow specimen for research purposes. Bone marrow mononuclear cells were separated by the Ficoll-Hypaque method (Histopaque-1077, Sigma Chemical Co, St Louis).

**Ether lipids.** The 1-octadecyl-2-0-methyl-rac-glycero-3-phosphocholine (ET-18-OCH₃) was purchased from Medmark Chemicals (Munich, Federal Republic of Germany), and 1-hexadecylthio-2-methoxymethyl-rac-glycero-3-phosphocholine (BM 41.440) has been supplied from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany). The 1-0-hexadecyl-2-acetamide-sn-glycero-3-phosphocholine (ET-16-NHCOCH₃) was a generous gift of Dr J. Hajdu, California State University, Northridge, CA. All solutions were sterilized by micropore filtration (0.22 µm, Gelman Science, Inc, Ann Arbor, Mich) and stored at −20°C until use. We compared the cytotoxic effects of these compounds on µg/mL concentration basis, as these compounds have similar molecular weights (mol wts).

**Treatment of cells with EL and/or hyperthermia.** Cell suspensions containing 5 x 10⁶ cells/mL and various concentrations of EL in a volume of 1 mL were placed in 15-mL, sealable, plastic tubes (Fisher Scientific, Pittsburgh), left very lightly capped at 37°C, and stored at −20°C until use. We compared the cytotoxic effects of these compounds on µg/mL concentration basis, as these compounds have similar molecular weights (mol wts).

**Assay for hematopoietic progenitors.** Mixed hematopoietic colonies (CFU-GEMM), erythroid colonies (CFU-E), erythroblast bursts (BFU-E), granulocyte-macrophage colonies (CFU-GM), and clusters were assayed according to a modification of the method of Fauser and Messner. Briefly, the bone marrow cells were cultured in triplicate in medium containing 0.8% methylcellulose, 20% FBS, 0.3% bovine serum albumin, 5 x 10⁻³ mol/L mercaptoethanol, 5.6 x 10⁻⁴ mol/L selenium, 10% human serum, and the following stimulators: 5% phytohemagglutinin stimulated leucocyte conditioned medium (PHA-LCM), 10% human placental conditioned medium (HPCM), and 1 U/mL erythropoietin (step III, Connaught, Swiftwater, PA). One-milliliter aliquots containing 1 x 10⁶ cells were cultured in duplicate in 35-mm grid culture dishes (Lux, Miles Scientific, Naperville, IL) at 37°C in a 5% CO₂ and 95% air atmosphere. The growth of colonies on each plate was scored on day 14 of culture. The growth of colonies in a mixture of normal bone marrow cells and HL60 cells was also assayed in this medium. The total hematopoietic progenitors was defined as the sum of CFU-GEMM, BFU-E, CFU-E, CFU-C, and clusters.

**Assay for leukemic progenitors from the patients.** Leukemic progenitors were assayed according to a modification of the method of Hoang and McCulloch. Briefly, mononuclear cells were obtained from the peripheral blood of AML patients by centrifugation through Ficoll-Hypaque density gradient. The mononuclear cells were cultured in duplicate or triplicate in petri dishes at a cell concentration of 2 x 10⁶/mL in α-minimum essential medium (Flow Laboratories, Inc, MacLean, VA) containing 0.8% methylcellulose, 20% FBS, and 10% of the supernatant of HTB-9 human bladder carcinoma cell line cells. The HTB-9 cell line was a generous gift from Dr E.A. McCulloch, Ontario Cancer Institute, Toronto. After five to seven days, cell aggregations containing >20 cells were counted as leukemic colonies. Their blast-like phenotype was verified by subjecting randomly removed colonies to further analysis by Wright-Giemsa stain.

**Assay for leukemic cell line cells.** Leukemic cell line cells were cultured with growth media and 5 x 10⁶ cells/dish were cultured in triplicate in the medium used for the assay of hematopoietic progenitors minus stimulators except for the growth of KG-1a where 10% HPCM was added to the culture. Cell aggregations of more than 40 cells were scored as colonies on day 7 of culture (HL60, HL60/DXR, and K562) or on day 14 of culture (KG-1a). In those experiments where there was increased killing of leukemic cells expected, the plating density of cells was increased up to 5 x 10⁶ cells/dish in order to provide enough colonies to evaluate. This assay can measure the elimination of almost five log quantities of leukemic cells.

**Freezing and thawing of cells treated with EL and hyperthermia.** Cells were suspended in RPMI 1640 medium containing 10% FBS and 10% dimethylsulfoxide (Sigma), frozen at a controlled rate of −1°C/min to −80°C, and stored in the vapor phase of liquid nitrogen. Freezing was performed in freezing vials (Nunc, Denmark) containing 1 mL with a cell concentration of 3 to 5 x 10⁶/mL. Thawing of cells was performed rapidly by placing the vials in a 37°C water bath. After thawing, the dimethylsulfoxide concentration was reduced by rapid dilution in medium to a concentration below 0.1%. Deoxyribonuclease (type I, Sigma) at a final concentration of 100 µg/mL was used when there was cell clumping.

**Statistical methods.** For comparison of means, the unpaired two-tailed t test was used. A P value of <.01 was considered significant.

| Table 1. Effect of Ether Lipids and Hyperthermia on the Growth of Hematopoietic Progenitors |
|------------------------------------------|-----------------|----------------|----------------|----------------|
| **Temperature** | **EL** | **CFU-C + Cluster** | **BFU-E + CFU-E** | **CFU-GEMM** |
| 37°C | | | | |
| O | 1.00 | 1.00 | 1.00 | 1.00 |
| ET-18-OCH₃ (13) | 0.91±0.06 | 1.07±0.06 | 0.96±0.22 | 1.00±0.03 |
| ET-16-NHCOCH₃ (5) | 1.09±0.08 | 0.86±0.11 | 0.85±0.27 | 1.01±0.04 |
| BM 41.440 (5) | 0.92±0.12 | 0.86±0.12 | 0.79±0.10 | 0.89±0.08 |
| 42°C | | | | |
| O (4) | 0.96±0.03 | 1.02±0.07 | 0.99±0.06 | 0.97±0.03 |
| ET-18-OCH₃ (5) | 1.01±0.09 | 0.90±0.07 | 0.80±0.13 | 0.92±0.05 |
| ET-16-NHCOCH₃ (4) | 0.91±0.09 | 0.68±0.10* | 0.60±0.15 | 0.81±0.05 |
| BM 41.440 (4) | 0.61±0.13† | 0.50±0.14† | 0.17±0.10† | 0.54±0.07† |

Cells were treated with 50 µg/mL of various EL for one hour at either 37°C or 42°C. The mean of control growth (± SE, n = 13) was CFU-C + cluster, 107 ± 25; BFU-E + CFU-E, 138 ± 44; CFU-GEMM, 4 ± 1; and total progenitors, 249 ± 68/10⁶ marrow cells. The numbers in parentheses represent the number of separate experiments.

*Varied significantly from the value of control at 42°C.
†Varied significantly from the value of ET-18-OCH₃ at 42°C.
PURGING LEUKEMIA CELLS WITH EL AND HEAT

RESULTS

To assess the effect of the sequence of heat and EL exposure, HL60 cells were treated with ET-18-OCH₃ (50 μg/mL) and heat (42°C) for one hour in various sequential combinations. The killing of HL60 cells was significantly better when the cells were treated with ET-18-OCH₃ during or immediately before heat exposure (data not shown). Therefore, the simultaneous treatment sequence was chosen for the following experiments.

The one-hour treatment with either EL (50 μg/mL) or heat (42°C) had little effect on the growth of hematopoietic progenitors as shown in Table 1. However, when the cells were simultaneously treated with EL and heat for one hour, the treatment with BM 41.440 resulted in a smaller survival of hematopoietic progenitors than that achieved with ET-18-OCH₃ or ET-16-NHCOCH₃ (Table 1).

The one-hour treatment of leukemic cell lines with EL (50 μg/mL) at 37°C inhibited their growth as shown in Table 2. The cytotoxicity of ET-18-OCH₃ was similar to that of BM 41.440 and greater than that of ET-16-NHCOCH₃ for all leukemic cell lines except K562. Hyperthermia alone (42°C for one hour) showed similar relative cytotoxicity to the leukemic cell line cells (Table 2). When the cells were exposed simultaneously to EL (50 μg/mL) and heat (42°C) for one hour, further reduction of the surviving fraction was observed for all leukemic cell lines (Table 2). Again, the cytotoxic effect of ET-18-OCH₃ plus hyperthermia was similar to that of BM 41.440 plus hyperthermia for all cell lines and more than that of ET-16-NHCOCH₃ plus hyperthermia on HL60/DXR and KG-1α (Table 2).

To assess the efficacy of the combination of ET-18-OCH₃ and heat in clinical autologous bone marrow transplantation, remission bone marrow was simulated by mixing HL60 cells with irradiated normal marrow cells at a ratio of 1:20 plus RBCs at a hematocrit of 10%, and treated with 50 μg/mL ET-18-OCH₃ for one hour at 42°C. As shown in Table 3, this combined treatment resulted in killing of more than a three-log quantity of clonogenic HL60 cells in this cell mixture, which was comparable to the amount killed in the absence of marrow cells and RBCs.

An increase in the duration of treatment with 50 μg/mL ET-18-OCH₃ from one hour to four hours, where heat (42°C) was added in the final hour, also had little effect on the hematopoietic progenitors before freezing (Table 4). After thawing, the surviving fraction of hematopoietic progenitors treated with this sequence became smaller than that of the surviving fraction in the control or the surviving fraction after ET-18-OCH₃ or heat treatment alone, yet, more than 50% of progenitors were spared (Table 4). However, by comparing the data shown in Table 5, a greater reduction of clonogenic leukemic cells than that achieved by one-hour incubation with ET-18-OCH₃ at 42°C was observed. The cytotoxic effect due to the combined treatment with ET-18-OCH₃ and heat was enhanced in all leukemic cell lines compared with either treatment alone. After thawing, more than a five-log reduction in the quantity of HL60 and HL60/DXR, a three-log reduction in the quantity of KG-1α, and a two-log reduction in the quantity of K562 were achieved (Table 5). This combination treatment with ET-18-OCH₃ (50 μg/mL for four hours) and heat (42°C for one

### Table 2. Effect of Ether Lipids and Hyperthermia on the Growth of Leukemic Cell Lines

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Treatment</th>
<th>EL</th>
<th>Surviving Fraction (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>ET-18-OCH₃</td>
<td>0.12 ± 0.02 (14)*</td>
<td>0.13 ± 0.03 (6)*</td>
</tr>
<tr>
<td></td>
<td>ET-16-NHCOCH₃</td>
<td>0.27 ± 0.06 (8)**</td>
<td>0.85 ± 0.13 (4)**</td>
</tr>
<tr>
<td></td>
<td>BM 41.440</td>
<td>0.18 ± 0.06 (6)*</td>
<td>0.36 ± 0.12 (5)*</td>
</tr>
<tr>
<td>42°C</td>
<td>ET-18-OCH₃</td>
<td>0.0030 ± 0.0009 (5)t</td>
<td>0.033 ± 0.008 (3)**</td>
</tr>
<tr>
<td></td>
<td>ET-16-NHCOCH₃</td>
<td>0.0062 ± 0.0002 (3)t</td>
<td>0.11 ± 0.001 (3)t</td>
</tr>
<tr>
<td></td>
<td>BM 41.440</td>
<td>0.0036 ± 0.0004 (4)t</td>
<td>0.030 ± 0.0016 (3)t</td>
</tr>
</tbody>
</table>

The cells were treated with 50 μg/mL of various EL for one hour at either 37°C or 42°C. Calculation of P value based on absolute number of colonies.

*Varied significantly from the value of control at 37°C.
†Varied significantly from the value of ET-18-OCH₃ at 37°C.
‡Varied significantly from the value of control at 42°C.
§Varied significantly from the value of ET-18-OCH₃ at 42°C.

### Table 3. Combined Effect of ET-18-OCH₃ and Heat on HL60 in the Presence of Irradiated Marrow Cells and Red Blood Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature</th>
<th>ET-18-OCH₃ (μg/mL)</th>
<th>Clonogenic HL60 cells/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>0</td>
<td>5,240 ± 465* (1.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>643 ± 55 (0.12)</td>
<td></td>
</tr>
<tr>
<td>42°C</td>
<td>0</td>
<td>1,247 ± 165 (0.24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0 ± 0 (0.00)</td>
<td></td>
</tr>
</tbody>
</table>

One x 10⁶ HL60 cells were mixed with 2 x 10⁷ irradiated normal marrow cells (5,000 rad) and RBCs (HCT = 10%), then treated with 50 μg/mL ET-18-OCH₃ at 37°C or 42°C for one hour.

*Mean number of colonies for three separate determinations ± SE.
†Surviving fraction.
‡Less than 0.001.
The mean control growth (±SE) was HL6O.516 ± 0.25 (Table 4). Simultaneous treatment did inhibit the growth of hematopoietic progenitors by the treatment with a higher concentration of ET-18-OCH3 (75 μg/mL) for four hours, data not shown). In HL6O, the therapeutic ratio value was 1433.3 with combined treatment and 11.2 with single treatment. In K562, the therapeutic ratio values were 5 with combined treatment and 2.82 with the single treatment.

Higher concentrations of ET-18-OCH3 (up to 125 μg/mL) were tested. However, we were unable to evaluate the efficacy of higher doses because of cell clumping.

An increase in the duration of the simultaneous treatment with both ET-18-OCH3 (50 μg/mL) and hyperthermia (42°C) for up to three hours resulted in a one-log quantity greater reduction of all clonogenic leukemic cells than that achieved by the sequence of 50 μg/mL ET-18-OCH3 for four hours with 42°C heat during the last hour. However, this simultaneous treatment did inhibit the growth of hematopoietic progenitors by 75% (data not shown).

The treatment of leukemic cells obtained from AML patients with ET-18-OCH3 and heat also resulted in a 97% to 100% reduction of leukemic progenitors, which was greater than that achieved by either ET-18-OCH3 or heat alone (Table 6).

**DISCUSSION**

Hyperthermia (42°C) has been combined with EL in this in vitro study to determine if this approach might be more efficacious than treatment with EL alone in ex vivo purging of bone marrow for autologous transplantation. An increase in leukemic cell kill resulted from this approach with an enhancement of the therapeutic ratio necessary to successful purging. This combined approach also successfully eliminated leukemic cells from a simulated remission marrow containing 5% leukemic cells, the accepted upper limit in defining a complete remission.

The cell membrane is an important target of both heat and ET-18-OCH3 toxicity, and the adsorption of ET-18-OCH3 to the cell membrane has been reported to increase at higher temperatures. It has been demonstrated that one action of ET-18-OCH3 is an effect on cellular lipid metabolism. It has also been demonstrated that changes in membrane fatty acid composition can influence the sensitivity of neoplastic cells to heat. Although the mechanism of the

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**Table 4. Effect of ET-18-OCH3 and Hyperthermia on the Growth of Hematopoietic Progenitors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature</th>
<th>ET-18-OCH3 (μg/mL)</th>
<th>CFU-C + Cluster</th>
<th>BFU-E + CFU-E</th>
<th>CFU-GEMM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 B*</td>
<td>1.00</td>
<td>0.74 ± 0.05</td>
<td>0.78 ± 0.25</td>
<td>0.79 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A†</td>
<td>1.00</td>
<td>0.84 ± 0.11</td>
<td>0.88 ± 0.08</td>
<td>0.97 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 B</td>
<td>0.69 ± 0.03</td>
<td>0.70 ± 0.07</td>
<td>0.75 ± 0.25</td>
<td>0.67 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 B*</td>
<td>1.00</td>
<td>0.75 ± 0.07</td>
<td>0.86 ± 0.03</td>
<td>0.86 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A†</td>
<td>1.00</td>
<td>0.57 ± 0.09</td>
<td>0.50 ± 0.27</td>
<td>0.54 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

Normal marrow cells were treated with 50 μg/mL ET-18-OCH3 for three hours at 37°C plus one hour at 42°C. The mean control growth (±SE, n = 11) was CFU-C + cluster, 92 ± 15.6; BFU-E + CFU-E, 109 ± 28; CFU-GEMM, 6 ± 1; and total progenitors, 207 ± 43/10^5 marrow cells. The numbers in the parentheses represent the number of separate experiments.

*Before freezing.
†After thawing.
‡Varyed significantly from control after thawing.

---

**Table 5. Effect of ET-18-OCH3 and Hyperthermia on the Growth of Leukemic Cell Lines**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature</th>
<th>ET-18-OCH3 (μg/mL)</th>
<th>Surviving Fraction (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
<td></td>
<td>HL60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HL60/ADR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KG-1a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>K562</td>
</tr>
<tr>
<td></td>
<td>0 B*</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>A†</td>
<td>0.46 ± 0.07 (6)</td>
<td>0.73 ± 0.11 (3)</td>
</tr>
<tr>
<td></td>
<td>50 B</td>
<td>0.02 ± 0.007 (10)</td>
<td>0.04 ± 0.002 (3)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.001 ± 0.0005 (6)</td>
<td>0.011 (1)</td>
</tr>
<tr>
<td></td>
<td>42°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 B*</td>
<td>0.20 ± 0.06 (5)</td>
<td>0.28 ± 0.08 (3)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.02 ± 0.002 (1)</td>
<td>0.11 ± 0.02 (3)</td>
</tr>
<tr>
<td></td>
<td>50 B</td>
<td>0.000006 ± 0.00004 (5)</td>
<td>0.0004 ± 0.0001 (3)</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0.000000 ± 0.00000 (4)</td>
<td>0.0000 ± 0.0003 (3)</td>
</tr>
</tbody>
</table>

Leukemic cell line cells were treated with 50 μg/mL ET-18-OCH3 for three hours at 37°C then further treated with ET-18-OCH3 for one hour at 42°C. The mean control growth (±SE) was HL60, 516 ± 122 (n = 10); HL60/ADR, 317 ± 64 (n = 3); KG-1a, 50 ± 10 (n = 6); and K562, 536 ± 95 (n = 7) per 10^5 cells. The numbers in the parentheses represent the number of separate experiments.

*Before freezing.
†After thawing.
combined effect of drugs and heat is complicated, it is possible to speculate that with the accelerated uptake of ET-18-OCH₃ at higher temperatures, the lipid metabolism also might be altered more quickly in such a way as to influence the sensitivity to thermal damage. Therefore, each independent metabolic consequence of the presence of ET-18-OCH₃ or heat might increase the chances for the metabolic consequences of the other to occur, the result being the enhancement of toxicity due to the combined application of heat and ET-18-OCH₃.

Previously, Dulisch et al reported that ET-18-OCH₃ was more cytotoxic to HL60 cells than to normal hematopoietic progenitors. However, they failed to show a further increase in selective cytotoxicity of ET-18-OCH₃ by changing the incubation temperature. Our data clearly showed the advantage of combining heat and ET-18-OCH₃ on its selective cytotoxicity. The reason for this difference in results is not clear at this point; however, it might be explained by the difference in the dose of ET-18-OCH₃ and incubation time. They treated cells with ET-18-OCH₃ at a lower dose (4 to 16 μg/mL) and longer incubation time (18 hours).

The hematopoietic progenitor assay used in this study does not measure "true" pluripotent stem cells, which are necessary for successful marrow transplantation. Unfortunately, no assays for human pluripotent stem cells are currently available. The question as to what is the effect of the combined treatment with ET-18-OCH₃ and heat on pluripotent stem cells can only be answered by the hematopoietic recovery in clinical autologous marrow transplantation. However, there is considerable evidence suggesting that the hematopoietic progenitors and pluripotent stem cells parallel each other with respect to quantity, and a correlation has been observed between the number of progenitors infused and hematopoietic recovery in autologous marrow transplantation in humans. Although there was considerable reduction in the number of hematopoietic progenitors observed using our purging method, the number of CFU-C demonstrated was adequate to indicate inferentially that the number of pluripotent stem cells was sufficient to allow hematopoietic recovery; however, a delayed recovery of hematopoiesis might result especially in the simultaneous treatment with ET-18-OCH₃ and heat, as has been observed in clinical trials of other drugs.

Because of the lack of the specific antigens for AML cells, purging of AML cells by pharmacologic methods has been more extensively investigated than purging by immunologic methods. Combined methods for purging have been also examined preclinically and have been shown to be more effective in the eradication of leukemic cells than a single treatment. Because of differences in the cell lines, the number of cells treated, and the methods for evaluating the killing of leukemic cells, a direct comparison of these methods is not possible. However, in terms of the killing of HL60 cells, the efficacy of our new combined treatment was comparable to other methods and better than that of our previous method. However, in terms of killing K562, which was resistant to both ET-18-OCH₃ and heat, our current method was less effective than that of others.

In clinical marrow transplantation, it is possible that fresh leukemic cells might be encountered that are resistant to both ET-18-OCH₃ and heat, as is the case with the leukemic cell line K562. However, our previous data showed that most leukemic cells obtained from AML patients were more sensitive to the cytotoxic effect of ET-18-OCH₃ than was K562, and our preliminary data seem to indicate that this combined treatment has at least the same degree of cytotoxic effect on leukemic cells from the patients as on KG-1a leukemic cell line.

From these data, we conclude that this combined treatment with ET-18-OCH₃ and hyperthermia could be applied effectively for the elimination of residual myeloid leukemic cells in autologous remission marrow grafts.

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