Elimination of Leukemic Cells by the Combined Use of Ether Lipids in Vitro

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ABSTRACT

Two ether lipids, CP-46,665-1 (4-aminoethyl-1-2,3-(di-n-decyl oxy)-n-propyl-4-phenylperidine) and ET-18-OCH₃ (racemic 1-O-decacyl-2-O-methylglycero-3-phosphocholine) have been shown to possess antileukemic activity in vitro. To explore the possible use of these compounds for purging remission bone marrow cells of leukemic cells, we examined the cytotoxic effect of these compounds on normal hematopoietic progenitor cells and leukemic cell line cells (HL-60, K-562, KG-1α, KG-1, and Daudi) by using the clonogenic assay.

When cells were treated with CP-46,665-1 or ET-18-OCH₃ (50 μg/ml for 1 h), these compounds did not inhibit the growth of normal progenitors, whereas the growth of the clonogenic leukemic cells was inhibited with differences in their sensitivities to the cytotoxic effect of CP-46,665-1 and ET-18-OCH₃. Incubation of leukemic cells (HL-60 and Daudi cells) with both CP-46,665-1 (50 μg/ml) and ET-18-OCH₃ (50 μg/ml) for 1 h resulted in a greater reduction of clonogenic leukemic cells than treated with each compound alone. Approximately a 3 log killing of clonogenic HL-60 cells and a 5 log killing of Daudi cells was achieved; however, the combined treatment of normal bone marrow cells with CP-46,665-1 and ET-18-OCH₃ did not alter the growth of normal progenitors. This combined treatment also selectively eliminated the leukemic cells (HL-60 and Daudi cells) from a mixture (1000:1) of normal bone marrow cells and leukemic cells. It is conceivable that the pronounced difference in sensitivity to this combined treatment can be exploited for the elimination of residual leukemic cells in autologous remission marrow grafts.

INTRODUCTION

Certain ether lipids have been reported to possess antileukemic activity in vitro. This activity is mediated partially by enhancing the cytotoxic properties of macrophages and by a direct effect on leukemic cells (1–3). Among them, ET-18-OCH₃ has been reported to be selectively toxic to leukemic cells (4, 5). This selectivity is due presumably to low or absent levels of O-alkyl-cleavage enzyme in neoplastic cells but not in normal cells (4, 6). A lack of this enzyme resulted in the accumulation of this compound in neoplastic cells causing injury to the cell membrane by disturbing phospholipid metabolism. We have demonstrated that this compound selectively eliminated leukemic cells from a mixture of leukemic cells and normal bone marrow cells in both animal and human systems (7, 8), and we have explored the possible use of this compound for purging leukemic cells in autologous marrow transplantation in acute leukemia. However, we have seen some heterogeneity in the sensitivity of leukemic cells to ET-18-OCH₃. To overcome the heterogeneity of leukemic cells and effectively purge marrow cells of leukemic cells, a combination of drugs or other purging methods may be required.

Revised 2/12/87; accepted 2/16/87. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/20/86; revised 2/12/87; accepted 2/16/87.

The abbreviations used are: ET-18-OCH₃, racemic 1-O-decacyl-2-O-methylglycero-3-phosphocholine; CP-46,665-1, 4-aminoethyl-1-2,3-(di-n-decyl oxy)-n-propyl-4-phenylperidine; FBS, fetal bovine serum; HPDM; human placental conditioned medium; CFU-E, erythroid colonies; BFU-E, erythroid bursts; GM, granulocyte-macrophage; GM-CFC, granulocyte-macrophage colonies; CFU-GEMM, mixed hematopoietic colonies.

CP-46,665-1 is an alkyl-linked lipid amine (9) which also has a direct antileukemic activity by injury to the cell membrane (10). This activity was thought to be considerably greater than that of ET-18-OCH₃ (10).

Therefore, this compound might also be used for purging leukemic cells. However, its effect on the growth of normal hematopoietic progenitors had not been previously tested. Furthermore, the direct cytotoxic effect of CP-46,665-1 on leukemic cells was demonstrated when cells were treated with this compound continuously for more than 24 h at lower concentrations (10). This setting might be unrealistic in the clinical autologous bone marrow transplantation because of loss of viable hematopoietic progenitors.

One aim of this study was to determine whether CP-46,665-1 has selective cytotoxicity to leukemic cells. Using the clonogenic assay, we examined the effect of short-term treatment with higher concentrations of CP-46,665-1 on the growth of normal human hematopoietic progenitors and several human leukemic cell lines. A further aim was to explore the possible combined use of CP-46,665-1 and ET-18-OCH₃ for purging bone marrow to achieve complete killing of residual leukemic cells without altering the viability of normal hematopoietic progenitors.

MATERIALS AND METHODS

Antileukemic Compounds. CP-46,665-1 was kindly supplied by Dr. K. E. Jensen, Central Research, Pfizer, Inc. ET-18-OCH₃ was a kind gift of Dr. W. E. Berdel, Technical University, Munich, Germany. The drugs were dissolved in RPMI 1640 medium containing 10% FBS (GIBCO, Grand Island, NY), the solutions were sterilized by micropore filtration (0.22 μm; Gelman Sciences, Inc., Ann Arbor, MI) and stored at −20°C until used.

Cells. Normal bone marrow cells: Histologically normal bone marrow cells were obtained by direct aspiration from a patient with acute leukemia in remission or by crushing bone marrow fragments provided from orthopedic surgery. Bone marrow mononuclear cells were separated by Ficoll-Hypaque (Histopaque-1077, Sigma Chemical Co., St. Louis, MO). In all instances, informed consent was obtained to donate a bone marrow specimen for research purposes.

Leukemic Cells. Five leukemic cell lines were used in this study. The HL-60 human promyelocytic cell line was obtained from Dr. Robert Gallo, NIH, Bethesda, MD (11). The K-562 cell line, established from a patient with a blast crisis of chronic myelogenous leukemia, was obtained from Dr. Bismark Lozzio, University of Tennessee, Knoxville, TN (12). The Daudi human B-cell leukemic cell line was obtained through American Type Culture Collection, Rockville, MD (13). The KG-1 and KG-1a human myeloblastic leukemic cell lines were kindly supplied by Dr. H. Phillip Koeffler, University of California, Los Angeles, CA (14, 15). All cell lines were continuously cultured at 37°C in a 5% CO₂ atmosphere in RPMI 1640 containing 10% heat-inactivated FBS except the KG-1 cell line which was cultured in RPMI 1640 medium supplemented by 20% FBS. All studies were done in log-phase growth.

Treatment of Cells with Ether Lipids. Cells were incubated at 37°C in 5% CO₂ for 1 and 4 h with various concentrations of CP-46,665-1 and/or ET-18-OCH₃. Incubation was performed in a plastic culture tube in RPMI 1640 medium containing 10% FBS and 5 × 10⁶ cells in a volume of 1 ml. After the incubation, cells were washed twice with RPMI 1640 medium and viability and clonogenicity were examined. In some experiments, incubation was performed in the RPMI 1640 me-
ELIMINATION OF LEUKEMIC CELLS BY ETHER LIPIDS

RESULTS

Cytotoxic Effect of CP-46,665-1 on the Normal Hematopoietic Progenitors. To assess the feasibility of using CP-46,665-1 for the purging of leukemic cells from remission marrow, we examined the effect of a short-term exposure to CP-46,665-1 and freezing and thawing on the growth of normal hematopoietic progenitors. When bone marrow cells obtained from four separate occasions were incubated with 50 µg/ml of CP-46,665-1 for 1 h, the growth of normal progenitors was not significantly altered. But CP-46,665-1 significantly reduced the number of colonies when the marrow cells were treated with 100 µg/ml of CP-46,665-1 for 1 h (Table 1). There was no selective loss of granulocyte, erythroid, and mixed colonies in any sample (Table 2). Incubation of the marrow cells for 4 h with more than 50 µg/ml concentration of CP-46,665-1 completely inhibited the growth of normal progenitors (data not shown). When the marrow cells exposed to 50 µg/ml of CP-46,665-1 for 1 h were cryopreserved before culture for hematopoietic progenitors, about 50% of progenitors was lost; however, the loss of progenitors was not significantly different from the loss in the untreated cultures (Table 1). On the other hand, when the cells were treated with 100 µg/ml of CP-46,665-1 for 1 h and then cryopreserved, loss of progenitors was greater than that of untreated cells. As far as the composition of progenitors is concerned, cryopreservation of marrow cells treated with CP-46,665-1 resulted in a smaller proportion of CFU-GEMM and viability of leukemic cell lines and compared the effect with that of ET-18-OCH3. Because these compounds have similar molecular weight, we compared the two compounds at a µg/ml level. Five leukemic cell lines (four myeloid leukemic cell lines and one lymphoid leukemic cell line) were examined. The growth of all leukemic cell line cells assayed by colony formation was inhibited by CP-46,665-1 (range, 99.0–42.5%) (Table 3). This cytotoxic effect was greater than that of ET-18-OCH3 on Daudi, and K-562, equal to that of ET-18-OCH3 on KG-1, KG-1a, and less than that of ET-18-OCH3 on HL-60. The viability of cells treated with CP-46,665-1 also decreased in a similar relationship as that observed in the colony assay (Table 4); however, in the case of Daudi cells, the differences between CP-46,665-1 and ET-18-OCH3 were not significant.

Effect of Temperature and Serum Concentration in the Incubation System on the Cytotoxic Effect of CP-46,665-1. To determine some of the conditions of incubation with CP-46,665-1
which might alter the selective cytotoxicity of CP-46,665-1 between normal and leukemic cells, we assessed the effect of temperature and of FBS concentration on the growth of normal progenitors and clonogenic HL-60 cells. When the cells were treated with 50 and 100 μg/ml CP-46,665-1 for 1 h, CP-46,665-1 showed little difference in cytotoxicity at 25 or 37°C for either normal or leukemic cells. On the other hand, changing serum concentration in the incubation system did have an effect upon the survival of colonies. As shown in Fig. 1, increasing the serum concentration in the incubation system up to 50% improved the percentage of the survival of colonies. As shown in Fig. 1, increasing the mean ± SE of % survival of colonies). Whereas this condition significantly improved the growth of normal progenitors (40.8 ± 8.6 to 91.5 ± 8.7, mean ± SE of % survival of total progenitors, P < 0.02).

Effect of Combined Treatment with CP-46,665-1 and ET-18-OCH3 on the Growth of Normal Progenitors and Leukemic Cells. We determined if the combined use of CP-46,665-1 and ET-18-OCH3 could result in the further reduction of clonogenic leukemic cells without affecting the growth of normal progenitors. Two leukemic cell lines, HL-60 (myeloid leukemic cell line) and Daudi (lymphoid leukemic cell line), were examined in this experiment. Results are shown in Table 5. ET-18-OCH3 did not alter the growth of normal progenitors at 50 μg/ml after 1 h incubation as observed previously (8). When the four different normal marrow cells were treated with 50 μg/ml concentration of both compounds for 1 h, no significant reduction of normal progenitors was observed. On the other hand, when the HL-60 and Daudi cells were treated with both compounds, greater reduction of clonogenic leukemic cells was observed than when treated with each compound alone (approximately 3 log killing of HL-60 cells and 5 log killing of Daudi cells). Freezing and thawing of cells treated with both compounds resulted in further reduction of clonogenic leukemic cells (about a 5 log reduction of HL-60 cells and more than a 6 log reduction of Daudi cells) with some effect upon the recovery of approximately 3 log killing of HL-60 cells and 5 log killing of Daudi cells). Freezing and thawing of cells treated with both compounds resulted in further reduction of clonogenic leukemic cells with some effect upon the recovery.

Table 3 Effect of CP-46,665-1 and ET-18-OCH3 on the viability of leukemic cell line cells

<table>
<thead>
<tr>
<th>Concentration of drug (μg/ml)</th>
<th>Drug</th>
<th>Normal progenitors (N = 100)</th>
<th>HL-60 (N = 10)</th>
<th>Daudi (N = 10)</th>
<th>K-562 (N = 10)</th>
<th>KG-1a (N = 5)</th>
<th>KG-1 (N = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>CP-46,665-1</td>
<td>100</td>
<td>95.9 ± 2.1</td>
<td>92.2 ± 1.7</td>
<td>97.7 ± 1.7</td>
<td>97.1 ± 1.7</td>
<td>97.2 ± 1.7</td>
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<tr>
<td>25</td>
<td></td>
<td>57.4 ± 2.0</td>
<td>56.1 ± 2.2</td>
<td>58.9 ± 2.8</td>
<td>57.6 ± 2.8</td>
<td>57.3 ± 2.8</td>
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<tr>
<td>50</td>
<td></td>
<td>35.3 ± 3.8</td>
<td>34.6 ± 4.1</td>
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</tr>
</tbody>
</table>

* 4.5 ± 5.9 (for FBS 50%) per 106 HL-60 cells.

Table 4 Effect of CP-46,665-1 and ET-18-OCH3 on the viability of leukemic cell line cells

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* 4.5 ± 5.9 (for FBS 50%) per 106 HL-60 cells.

Table 5 Effect of combined treatment with CP-46,665-1 and ET-18-OCH3 on the viability of leukemic cell line cells

<table>
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<tr>
<th>Concentration of drug (μg/ml)</th>
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<th>Normal progenitors (N = 100)</th>
<th>HL-60 (N = 10)</th>
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* 4.5 ± 5.9 (for FBS 50%) per 106 HL-60 cells.

** Table 3 Effect of CP-46,665-1 and ET-18-OCH3 on the viability of leukemic cell line cells

Results are expressed as percentage of control. Mean ± SE of viable cells as (%) in control culture is shown in parentheses.

<table>
<thead>
<tr>
<th>Drug (μg/ml)</th>
<th>Normal progenitors (N = 100)</th>
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</thead>
<tbody>
<tr>
<td>CP-46,665-1</td>
<td>100</td>
</tr>
<tr>
<td>ET-18-OCH3</td>
<td>98.8 ± 2.8</td>
</tr>
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</table>

* P value compared to CP-46,665-1 and ET-18-OCH3.

* NS, not significant.
of total normal progenitors (37.1 ± 1.8% of total progenitors were recovered).

We then examined whether the combined treatment also effectively killed the leukemic cells in the presence of a large amount of normal marrow cells. Normal marrow cells were mixed with HL-60 cells or Daudi cells at a ratio of 1000:1. These mixtures were treated with the two ether lipids simultaneously. HL-60 or Daudi cell colonies could easily be distinguished from normal granulocytic colonies by their distinctive morphology. However, to confirm that the colonies counted as such were of leukemic cell origin, morphological analysis of cells in the colonies were performed. Furthermore, in the mixture of marrow cells derived from a female donor and Daudi cells, which were derived from a male patient, the presence of Y-chromatin in cells from individual colonies was examined. As shown in Fig. 2, when the cell mixtures were treated with CP-46,665-1 or ET-18-OCH₃, these compounds inhibited the growth of leukemic colonies without reducing the normal colonies, and combined treatment with 50 μg/ml concentrations of both compounds completely eradicated observable leukemic cells from both cell mixtures while sparing the normal progenitors. Complete eradication of leukemic cells was also confirmed by culturing the cell mixture which had been treated with both compounds in a liquid media (5 × 10⁶ cell mixture cells/10 ml RPMI 1640 containing 10% FBS). No leukemic cells regrew after 14 days in culture.

DISCUSSION

In this study, we demonstrated that CP-46,665-1 was selectively cytotoxic to several leukemic cell lines when the cells were exposed to 50 μg/ml concentration of this compound for 1 h. In comparing its cytotoxicity toward leukemic cells with that of ET-18-OCH₃, CP-46,665-1 did not uniformly show greater cytotoxicity; also, various leukemic cell lines have some differences in their sensitivities to these ether lipids. Furthermore, the cytotoxicity of CP-46,665-1 was independent of the temperature of the incubation system. This finding is quite different from that for ET-18-OCH₃ which has been shown to be highly temperature-dependent in its cytotoxic action (19). As far as their effects on hematopoietic progenitors are concerned, CP-46,665-1 seems to be more toxic than ET-18-OCH₃ which did not alter the growth of hematopoietic progenitors in up to 4 h incubation at 50 μg/ml concentration (8). These observations may indicate that these two ether lipids have different mechanisms for their direct cytotoxicity.

Unlike many other chemotherapeutic agents, the cell membrane appears to be the major target of ether lipids (3, 10, 20). This uniqueness may have a benefit since these compounds would be effective in both cycling and resting cells. However, the low serum concentrations needed for the cytotoxic activity of CP-46,665-1 and ET-18-OCH₃ (10) might be an obstacle in its clinical application. Therefore, in vitro use of these compounds may be the more effective approach for eliminating leukemic cells.

For purging leukemic cells from remission marrow for autologous marrow transplantation, a single drug that would eliminate all the leukemic cells but leave enough cells to restore total lymphohematopoietic recovery would be quite satisfactory. However, we have previously observed that ET-18-OCH₃ was not uniformly cytotoxic to leukemic cells from the patients with acute nonlymphocytic leukemia (21). The same variation of its sensitivity has been reported about Asta Z 7557 (22). Therefore, combined use of drugs or combined use of other purging methods would be necessary to overcome this tumor heterogeneity toward cytotoxic drugs (23). To address this issue, possible combined use of these two ether lipids for purging the leukemic cells was explored in this study. Treating the cells with two ether lipids at a concentration and incubation time which did not have an effect upon the growth of hematopoietic progenitors provided further increase of the selective killing of leukemic cells in vitro. Augmentation of this cytotoxic effect through the process of freezing and thawing was also observed. This treatment was equally effective for both myeloid and lymphoid leukemic cell lines and the results of killing leukemic cells were comparable to that achieved by using monoclonal antibodies (24, 25). In further experiments, it will be necessary to examine this combined use of ether lipids by using cells from leukemic patients rather than cultured cells.

Agents which can selective eliminate residual leukemic cells in remission marrows with no effect on normal bone marrow stem cells would be ideal. Glasser et al. observed that ET-18-OCH₃ was much more toxic to murine leukemic cells than hematopoietic stem cells as assayed in a spleen colony assay (7). Unfortunately, in humans, assays of the pluripotent hematopoietic stem cell do not exist. A correlation has been observed between the number of progenitor cells infused and hematopoietic recovery in autologous transplantation in humans (26, 27). This inferentially suggests that agents which spare progenitor cells may spare the pluripotent stem cells as well. However, Kaizer et al. found that marrows exposed to doses of 4-hydroperoxycyclophosphamide which eliminated GM-CFCs were capable of restoring hematopoiesis in vivo (28). Thus the in vitro assay systems leave a lot to be desired but are the only in vitro methods available for developing effective purging techniques.

ACKNOWLEDGMENTS

The authors very much appreciate the excellent technical assistance of Sheree Benner and other members of the Leukemia Research Laboratory at Emory University.

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