Potential of Phenylalanine Methylester as a Bone Marrow Purging Agent

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Phenylalanine methylester (PME), a lysosomotropic compound, can be used to deplete monocytes and myeloid cells from peripheral blood and bone marrow (BM). The potential of PME for purging leukemic cells from BM was investigated using U937 and HL-60 cell lines as models. Optimal purging conditions for U937 cells were determined using an MTT assay (3-4, 5-dimethylthiazol-2, 5-diphenyl tetrazolium bi- mide; Sigma). Elimination of U937 cells was time-, temperature-, and dose-dependent. PME activity was optimal at 37°C for 45 minutes. Depletion of U937 was >2.8 logs for 50 mmol/L PME. Compared with another purging agent, 100 µg/mL 4-hydroperoxycyclophosphamide had activity comparable to 40 mmol/L PME. HL-60 cells were even more sensitive to PME than U937 cells. To support observations made with the MTT assay, clonogenic assays were performed. PME, 50 mmol/L at 37°C resulted in total depletion (>5 logs) of U937 colonies. Progressive depletion of normal progenitor cells occurred when BM was incubated with PME at concentrations from 5 to 100 mmol/L. At 37°C, 50 mmol/L PME reduced colony-forming units-granulocyte-macrophage and burst-forming units-erythroid (BFU-E) recovery by 98%. Recombinant human mast cell factor augmented BFU-E after PME treatment but had no effect on HL-60 or U937. These studies suggest that PME deserves further study as an agent for ex vivo marrow purging.

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CA), and either 10% PHA (phytohemagglutinin) conditioned media (PHA-CM), 2.5 ng/mL rh granulocyte-macrophage colony-stimulating factor (rhGM-CSF), 100 U rh interleukin-3 (rhIL-3), or 100 ng/mL rhMGF. Recombinant cytokines, except EPO, were kindly provided by Immunex Corp (Seattle, WA). Triplicate cultures were incubated for 14 days in a humidified atmosphere of 5% CO2/5% O2 at 37°C. Plates were scored using an inverted microscope. Colonies were defined as aggregates of greater than 50 cells. Benzidine-positive colonies were designated erythroid burst-forming units (BFU-E) and benzidine-negative colonies as granulocyte-macrophage colony-forming units (CFU-GM).

To determine the effect of PME on U937 colonies, U937 cells were incubated under conditions shown in Results. After three washes in RPMI, cells were plated in agar-gel as previously described at concentrations ranging from 5 × 10^5 cells/mL to 1 × 10^6 cells/mL. No source of colony-stimulating activity was added to these cultures. Quadruplicate cultures were incubated for 7 days in a humidified atmosphere of 5% O2/CO2 at 37°C. Aggregates of greater than 50 cells were scored as colonies.

Statistical analysis. Data points represent the mean ± SE of three to five separate experiments percent for recovery data. For calculation of means in the MTT assays, values less than the sensitivity of the assay were arbitrarily assigned a value of 0.1. Comparisons between observations were performed using a two-sided t-test. Pearson’s correlation coefficient was used for correlation testing. A P value <.05 was considered significant.

RESULTS

Conditions affecting U937 elimination in the MTT assay. PME was tested in concentrations from 0.005 mmol/L to 100 mmol/L (for clarity, results are shown from 0.5 mmol/L to 100 mmol/L). As noted in Fig 1, increasing concentrations of PME eliminated U937 cells in a dose range from 10 to 50 mmol/L. The effects were minimal with a 10-minute exposure at room temperature and increased with longer incubation times. The greatest inhibition was seen at 37°C. Reduction of U937 cells below the sensitivity of the MTT assay was obtained in eight of nine experiments with 50 mmol/L PME at 37°C for 45 minutes.

To characterize the effect of incubation at 37°C, the MTT assay was repeated with additional time points (Fig 2). Once again, U937 elimination was enhanced by increasing the PME concentration and duration of incubation. Optimal elimination was obtained at 45 minutes.

Comparison of PME and 4HC on U937. The activity of 4HC and PME was compared in the MTT assay. Incubations were performed for 45 minutes at 37°C. At 100 μg/mL 4HC, 2.5 ± 0.2 logs of U937 cells were eliminated. The activity of 100 μg/mL 4HC was comparable with 40 mmol/L PME (Fig 3).

Effect of PME on U937 colonies. At 37°C for 45 minutes, 50 mmol/L PME eliminated greater than 5 logs of U937 cells. In contrast, when the incubation was performed at 22°C, there was less effect on U937 colonies. The activity of 50 mmol/L PME at 37°C was significantly greater than 100 mmol/L PME at 22°C (Fig 4).

Comparison of clonogenic and MTT assay. There was a high degree of correlation between the MTT and clonogenic assay at 22°C (r = .96, P < .05) and at 37°C (r = .98, P < .05).

Effect of PME on progenitor cells. Increasing PME concentrations reduced normal progenitor cell recovery. Initially, experiments were normal performed with PHA-CM plus EPO as sources of colony-stimulating activity (Fig 5).

Incubation at 37°C with 50 mmol/L PME resulted in a CFU-GM recovery of 0.23% ± 0.23% and 0.17% ± 0.12% for BFU-E. PME treatment of marrow with 100 mmol/L PME at 22°C caused less depression of progenitor cells (BFU-E was 6.4% ± 2% and 3.6% ± 1.8% for CFU-GM).

Because U937 cells were essentially eliminated by 50 mmol/L PME at 37°C, the effect of these conditions on progenitor cells was further evaluated using recombinant cytokines. Under these conditions, nucleated marrow cell recovery was 28% ± 1.4% and 87% ± 0.4% for mononuclear cells. Recovery of CFU-GM was 2.8% ± 0.9%. Recovery of BFU-E was 2.2% ± 1.4%.

The result of adding rhMGF to the progenitor cell cultures is shown in Fig 6. rhMGF significantly decreased CFU-GM in two of three experiments by a mean of 44%. In contrast, rhMGF augmented BFU-E in three of three experiments by a mean of 730%.

Evaluation of PME on other cell lines. To assure that the data with PME was not unique to U937, further experiments were performed with HL-60 cells. In the MTT assay, both PME (at 22°C and 37°C) and 4HC demonstrated dose-response elimination curves to HL-60 (Fig 7). Reduction of HL-60 cells to below the sensitivity of the MTT assay with PME was detected at 50 mmol/L at 22°C and at 37.5 mmol/L at 37°C. 4HC proved less active. At 100 μg/mL 4HC, there was a 1.5 log reduction of HL-60 that is comparable with 40 mmol/L PME at 22°C or 25 mmol/L PME.
POTENTIAL OF PME IN BONE MARROW PURGING

Fig 2. Effect of increasing PME concentrations and duration of incubation on U937 cells at 37°C. See Fig 1 for symbol explanation.

PME at 37°C. In contrast, 50 mmol/L PME (at 37°C or 22°C) decreased RPMI 8226 by less than 50%.

Protection assay. Incubation of HL-60 or U937 with rhMGF had no effect on PME toxicity compared with assays performed immediately or after overnight incubation.

DISCUSSION

Using the MTT assay, optimal conditions for U937 elimination were established. Inhibitory activity by PME was observed in concentrations from 10 to 100 mmol/L. In addition, increasing both the incubation temperature and duration enhanced U937 depletion. At 37°C for 45 minutes, greater than 2.8 logs of U937 cells were eliminated. U937 cells appear more resistant than normal myeloid cells or monocytes to PME. Under conditions that eliminate greater than 2 logs of normal cells (5 mmol/L at 22°C for 45 minutes), only 12.7% ± 3.5% U937 cells were depleted. The reason(s) for the relative PME resistance of U937 cells is unclear.

Fig 3. Comparison of U937 elimination by PME and 4HC. * P < .05 compared with previous 4HC concentration; †, P < .05 for specified 4HC concentration compared with 50 mmol/L PME. Incubations were performed at 37°C.

Fig 4. Effect of PME on U937 colony formation at various temperatures and PME concentrations. See Fig 1 for symbol explanation.

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Fig 5. Effect of PME on BM progenitor cells. EPO and PHA-CM provided colony-stimulating activity. Explanation of symbols: *, P < .05 compared with previous PME concentration for the same temperature and type of colony; †, P < .05 compared with the same type of colony and PME concentration at 22°C.
Clinically, harvested BM from patients in remission of leukemia may contain 10⁸ leukemic cells. Therefore, any "prospective" purging agent should be able to eliminate at least 5 to 6 logs of contaminating leukemic cells. Other factors, such as cryopreservation or immunologic effects, may then be able to destroy the remainder of the harvested leukemic cells. This level of sensitivity could not be obtained with the MTT assay. A clonogenic assay not only provided the additional sensitivity but also corroborated the MTT results. At 37°C for 45 minutes, 50 mmol/L PME eliminated greater than 5 logs of U937 cells. Future studies using additional cell lines and clinical specimens will be needed to examine the full spectrum of PME activity.

To put the PME findings into perspective, results from this study can be compared to reported data with 4HC on other myeloid cell lines. Most investigators have reported a 2- to 3-log reduction of clonogenic HL-60 cells with 4HC. In the MTT assay, 100 µg/mL 4HC depleted 1.5 logs of HL-60 and 2.5 logs of U937. Disparities in the observed cell line eliminations may reflect assay differences. To increase the purging efficacy of 4HC, many investigators are now exploring the use of 4HC combined with other agents. Optimal combined purging techniques are comparable to the greater than 5-log depletion that was obtained with PME as a single agent.

In vitro tests are often used to predict the engraftment potential of purged marrow. At 37°C for 45 minutes, 50 mmol/L PME decreased CFU-GM and BFU-E by 98%. To some degree, the loss of progenitor cells may be attributed to removal of accessory cells (monocytes and fibroblasts) by PME. However, progenitor cell recovery is more than would be expected by elimination of accessory cells. This would suggest that PME caused stem cell damage or that the progenitor cell assays did not reflect stem cell activity. Progenitor cell assays may not accurately reflect the presence of stem cells in purged marrow. For instance, marrows purged with 4HC engraft despite depletion of 99% CFU-GM. To determine if the standard progenitor cell assays underestimated residual stem cells after PME treatment, additional experiments were performed with rhMGF. rhMGF, or stem cell factor, is the ligand for c-kit and is synergistic with other CSFs. After incubation of marrow with 50 mmol/L PME at 37°C, rhMGF halved CFU-GM but augmented BFU-E by approximately sevenfold. Two possible explanations for these observations are that rhMGF (1) modulated colony formation in favor of BFU-E or (2) stimulated very early progenitor cells. Because other investigators have reported that rhMGF increases CFU-GM, these results probably reflect an effect of PME on marrow cells. The specificity of rhMGF for protection of progenitor cells (as compared with leukemic cell lines) suggests that rhMGF may increase the therapeutic index of PME. The effect of PME on stem cells may be further defined by the use of long-term BM cultures and by demonstrating the ability of PME purged marrow to engraft lethally irradiated animals.

The cause for the increased toxicity of PME (on both progenitor cells and leukemic cell lines) at 37°C compared with 22°C probably relates to a more rapid enzymatic hydrolysis to free amino acids at higher temperatures. Osmotic lysis of lysosomes results from concentration of the free amino acids within the lysosome.

In summary, 50 mmol/L PME eliminated greater than 5 logs of clonogenic U937 cells and 98% of progenitor cells at
37°C. Further studies in an animal model can be used to indicate whether PME retains purging properties in vitro and if stem cells are damaged by the dying leukemic cells (bystander effect).

REFERENCES

13. Mangan KF, Mullaney MT, Rosenfeld CS, Shadduck RK: In vitro evidence for disappearance of erythroid progenitor T-suppres-
sor cells following allogeneic bone marrow transplant for severe aplastic anemia. Blood 71:144,1988
14. Rosenfeld CS, Tedrow H, Boegel F, Shadduck RK: A double buffy coat method for red cell removal from ABO incompat-
18. Tamayo E, Hervé P: Preclinical studies of the combination of mafosfamide (ASTA-Z 7654) and etoposide (VP16-213) for purging leukemic autologous marrow. Exp Hematol 16:97,1988
22. Blot FJ, Dorssers L, Wagemaker G, Ldwenberg B: Stimulat-
23. Rosenfeld CS, Shadduck RK, Przepiorka D, Mangan KF, Colvin M: Autologous bone marrow transplantation with 4-hydro-
peroxycyclophosphamide purged marrows for acute nonlym-
phocytic leukemia in late remission or early relapse. Blood 74:1159,1989
25. Leung KH: Human lymphokine-activated killer (LAK) cells—II. Studies of various L-amino acid methyl esters on LAK genera-

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