Evaluation of Drugs for Elimination of Leukemic Cells From the Bone Marrow of Patients With Acute Leukemia


Relatively nonmyelotoxic drugs and drug combinations were investigated for their ability to eliminate malignant cells from human bone marrow. In vitro 90% inhibitory concentration (IC<sub>90</sub>) doses were established on granulocyte-macrophage colony-forming units (GM-CFU) in culture of bone marrow by using the GM-CFU assay for the following drugs: 4-hydroperoxycyclophosphamide (4-HC), Adriamycin, L-asparaginase, bleomycin, hydrocortisone, VP-16, spirogermanium, Taxol, and vincristine. The leukemic cell kill efficiency of these drugs at IC<sub>90</sub> doses was compared with that of 4-HC on acute lymphoid leukemia (ALL) cell lines by using the limiting-dilution assay. Under these conditions, no single drug was superior to 4-HC. To increase the in vitro effect in leukemic cell kill, combinations of vincristine with hydrocortisone, Adriamycin, VP-16, and 4-HC were investigated. Vincristine at 1 to 5 µg/mL increased the marrow cytotoxicity of hydrocortisone, Adriamycin, and VP-16, but it was protective (subadditive) with 4-HC. Vincristine and 4-HC in combination was additive to supraadditive on ALL cell lines, increased the leukemic cell kill by one to two logs above 4-HC alone at IC<sub>90</sub> doses (P < .05), and was not affected by the addition of excess marrow cells. The recommended doses for chemopurging in clinical studies are vincristine, 1 to 5 µg/mL, plus 4-HC, 5 µg/mL.

LONG-TERM, DISEASE-FREE survival and (occasionally) cure has been achieved among patients with refractory or recurrent acute leukemia who received high-dose chemotherapy and bone marrow transplantation from identical twins or HLA-compatible siblings. However, the application of this technique is limited by the availability of a suitable donor. Furthermore, the success of allogeneic bone marrow transplantation is often limited, especially in older patients, by the occurrence of graft-versus-host disease and severe infections. Therefore, autologous bone marrow transplantation (ABMT) is being used increasingly in the treatment of acute leukemia. One problem that prevents more general use of this procedure is the presence of occult leukemic cells in the remission bone marrow. Using in vitro immunologic or pharmacologic treatment of the remission marrow to eliminate occult leukemic cells—also referred to as ex vivo immunopurging or chemopurging—has been successful in animal models.

One approach involves the use of an activated derivative of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC). The Johns Hopkins Oncology Center reported their results of clinical studies of ex vivo 4-HC marrow treatment. Twenty-one patients with acute lymphoid leukemia (ALL), mostly in the second complete remission (CR), received cyclophosphamide and total-body irradiation (TBI) followed by ABMT with marrow treated ex vivo with 40 to 120 µg/mL 4-HC before ABMT. Only one patient remains in long-term CR, 18 patients relapsed, and two died of treatment toxicity. Twenty-five patients with acute myeloid leukemia (AML), mostly in second CR, received cyclophosphamide plus busulfan followed by AMBT with marrow treated ex vivo with 60 to 120 µg/mL 4-HC. Eleven patients remain in remission at a median of more than 400 days. Hematopoietic recovery time was similar to that seen with unmanipulated marrow. This and other studies suggest that ex vivo chemotherapy with 4-HC alone may be successful at least in a small number of patients with AML in second or subsequent CR. Because the results in ALL patients have been generally disappointing, further investigations are warranted to find drugs or drug combinations more effective than 4-HC. We reported the results of single-drug screening studies for antitumor effects with acceptable in vitro myelotoxicity. The goal in the present work is to identify drugs or drug combinations for ex vivo purging of the marrow of patients with ALL that have significantly better therapeutic ratios of leukemic cell kill to marrow progenitor kill than does 4-HC. This was evaluated by testing established ALL cell lines with concentrations of drugs found to cause approximately a 90% inhibition (IC<sub>90</sub>) of granulocyte-macrophage colony-forming units in culture (GM-CFU) and evaluating cell kill by limiting dilution.

MATERIALS AND METHODS

Bone marrow. Morphologically normal bone marrow cells were obtained by multiple bilateral bone marrow aspirations from normal donors, solid tumor patients without bone marrow involvement by tumor, and acute leukemia patients in remission. All gave informed consent.

ALL cell lines. The ALL cell lines (LAZ-221, KM3, REH) were provided by Dr. J. Minowada, formerly of Loyola University, Chicago. The characteristics of these cell lines have been described in detail elsewhere.

The cell suspensions were maintained in culture flasks (Corning Glass Works, catalog nos. 25120-150, 25110-75, 25100-25; Corning, New York) at approximately a 0.5 to 1.0 x 10<sup>6</sup>/mL concentration.

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and fed with fresh medium consisting of RPMI 1640 tissue culture medium (GIBCO, Grand Island, NY) and 10% fetal bovine serum (FBS) (Irving Scientific, Santa Ana, CA) three times a week.

Drugs and drug dilutions. 4-HC was kindly supplied by Dr Peter Hilgad (Astara-Werke, Bielfeld, FRG). Vinccristine sulfate (VCR, Oncovin) was supplied by Eli Lilly & Co, Indianapolis. Hydrocortisone (100-mg vial) by Elkins-Sinn, Inc, Cherry Hill, NJ; hydrocortisone (250-mg vial) by Lymphomed, Stone Mountain, GA; Adriamycin (doxorubicin) by Adria Laboratories, Inc, Columbus, OH; VP-16 (etoposide) by Bristol Laboratories, Syracuse, NY; bleomycin sulfate (Blenoxane) by Bristol Laboratories; Taxol and spirogermanium by the National Cancer Institute, Bethesda, MD; and t-asparaginase (Elspar) by Merck, Sharp and Dohme, Inc, West Point, PA. The drugs were dissolved in preservative-free normal saline solution or RPMI 1640 with 10% FBS before each experiment. Any solutions not used immediately were stored at −70°C.

The GM-CFU assay. Light-density mononuclear cells in normal bone marrow were separated by centrifugation over Ficoll-Hypaque (density, 1.077 g/μL; Ficoll from Pharmacia Fine Chemicals, Piscataway, NJ; Hypaque from Winthrop Laboratories, New York) or 40%/60% Percoll (Sigma Chemical Co, St Louis) (the cells were resuspended in RPMI 1640 with 10% FBS. Aliquots of 0.9 mL, each containing 5 × 10⁶ mononuclear cells, were incubated with 0.1 mL of drug solutions at 37°C for 60 minutes. The cells were washed three times, resuspended, and plated in a bilayer soft agar system at 100,000 cells/plate in 0.32% agar with a minimum essential medium (α-MEM; GIBCO) and 15% FBS over an underlayer of 0.5% agar containing α-MEM with 15% FBS and 20% human placental conditioned medium. Costar no. 3035 tissue culture dishes (supplied by Costar, Cambridge, MA) were used. After ten to 14 days' incubation at 37°C in a humidified atmosphere of 5% CO₂ and 12% O₂, the number of granulocyte-macrophage colonies were counted on each plate. Numbers were expressed as percent GM-CFU of the drug-free control. The IC₅₀ is the dose approximating the survival of 10% of GM-CFU.

The limiting-dilution analysis. Aliquots of 0.9 mL containing 5 × 10⁶ cells from each ALL cell line in RPMI 1640 with 10% FBS were incubated with 0.1 mL of drug solutions at 37°C for 60 minutes. The cells were then washed three times and re-suspended in 1 mL of RPMI plus 10% FBS (tube 1). Serial fivefold dilutions were made from this cell suspension. From each tube 0.2 mL is then transferred to 0.8 mL RPMI plus 10% FBS in the next tube. The concentration of the fivefold dilutions were 1 × 10⁶, 2 × 10⁶, 4 × 10⁶, 8 × 10⁶, 16 × 10⁶, 320, 64, 12.8, 2.56, and 0.512 cells/mL.

After the completion of serial dilutions, 0.1 mL of each dilution was added to six wells of a U-bottomed multwell plate (Costar no. 3799) with 0.1 mL RPMI plus 10% FBS. The final numbers of cells in each well were then 1 × 10⁶, 2 × 10⁶, 4 × 10⁶, 8 × 10⁶, 16, 32, 63, 4, 1.28, 0.256, and 0.051. The cultures were incubated for ten to 14 days at 37°C in 5% CO₂, 12% O₂, and balanced N₂. Each plate, ten rows of six wells, constitutes an experimental point. Positive wells are those containing viable cells, which are easily distinguished from the negative, fragmented, disintegrated, nonviable cells. Where the viability was in question, the contents of the wells were tested with trypan blue. This assay shows linear relationships between the number of clonogenic units per well and the logarithm of the proportion of negative wells, both when examining drug-treated and control samples. This excludes significant effects of nonviable cells on the cloning efficiency.

The number of positive wells, P, in each plate was used to estimate C, the number of clonogenic cells per unit volume in the original suspension. The Spearman estimator 23 for C is 

\[ C = \exp \left[ \frac{K - (5/2) \log_2 \left( \frac{D}{P} \right)}{W} \right] \]

where D is the natural logarithm of the dilution factor (here, D = ln [5]), W is the number of wells used for each dilution (here, W = 6), and K = 0.57722, Euler's constant. Let P, and Pₐ denote, respectively, the number of positive wells observed in experiments with treated and untreated cells. If C, and Cₐ denote the corresponding numbers of clonogens per unit volume in the original suspension, then cell survival is given by the following equations:

\[ \ln(\text{surviving fraction}) = \ln \left( \frac{C}{C_a} \right) = \left( P_a - P \right) \frac{D}{W}, \]

For D = ln 5 and W = 6, \(\ln(\text{surviving fraction}) = (P_a - P) 0.2682,\) or “log kill” = \(\log_{10} \text{(surviving fraction)} = (P_a - P) 0.1165.\)

The 95% confidence interval for C is given by:

\[ c' \exp^{-1.96 \frac{D \ln 2}{W}} < C < c' \exp^{1.96 \frac{D \ln 2}{W}} \]

Here, this corresponds to a difference of ±3.15 positive wells. Therefore, two observations will have nonoverlapping confidence intervals provided they differ by at least seven positive wells (P < 0.05). The highest cell concentration used in a dilution assay determines the maximum detectable level of log kill. With an initial concentration of 10⁶ cells/well we could detect cell kill up to 5.6 logs. The limiting-dilution analysis has been described in detail in the literature. 13, 15

Mixing experiments. Light-density cells were separated from normal bone marrow by using Ficoll-Hypaque as previously described. The light-density cells were irradiated with 4,000 cGy and incubated overnight at 37°C in humidified air (containing 5% CO₂, 12% O₂, and balanced nitrogen) in RPMI with 10% FCS at 1 × 10⁶ cells/mL.

A quantity of 0.5 × 10⁶ ALL (REH) cells was mixed with a ninefold excess (4.5 × 10⁵) irradiated light-density marrow cells and incubated with 5 μg/mL 4-HC, 5 μg/mL 4-HC plus 1 μg/mL VCR, or without drug addition (control) for one hour in a 37°C water bath with constant agitation. Also, 5 × 10⁶ ALL (REH) cells and 5 × 10⁶ irradiated (4,000 cGy) light-density bone marrow cells were treated with drugs and incubated with the same drug combinations. After incubation, the cells were washed three times in RPMI with 10% FCS, and the limiting-dilution analysis was performed as described previously. After 14 days of incubation, the results were scored in the same manner as described in the limiting-dilution assay.

Evaluation of drug combinations for synergy or antagonism. Additivity of drug kill effect was evaluated by means of the graphic isobologram analysis of Steel and Peckham. 18 Briefly, we examined the dose-response curves of drugs A and B individually and when combined in a particular experiment.

Let a and b represent concentrations or “doses” of drugs A and B, respectively.

Let kₐ and kₐ represent the percent GM-CFU kill of doses a and b, respectively.

Let fₐ and fₐ represent the dose-response curves generated such that kₐ = fₐ (s), and kₐ = fₐ (b). We then determine for drug B a theoretical dose b' for each dose a of drug A such that fₐ (b') = 90% − fₐ (a). The same is done for drug A. By definition, at the IC₅₀ dose of A, fₐ (a) = 90%, but b' may be any dose where fₐ (b) - 0% such as would occur if the dose-response curve for drug B had a “threshold” (ie, a maximum dose before an effect is noticed). When one plots b' a and a' b on the same set of a and b axes, two lines emerge with a “window” in the middle; this is called the envelope of additivity, which takes into account any “shoulder” or “plateau,” in a drug’s dose-response curve. Finally, experimentally observed doses of the combinations that actually resulted in 90% GM-CFU kill are plotted on the same graph to yield the IC₅₀ isobologram. An isobologram point lying to the right of the envelope indicates a truly subadditive response, which may imply antagonism or protection. A truly supraadditive, or synergistic, interaction would result in a point to the left of the envelope, closer to the origin.
RESULTS

Determination of myelotoxicity of single drugs on normal bone marrow. Nine single drugs with minimal in vivo myelotoxicity but with clinical effectiveness in ALL or B cell malignancies were selected for evaluation: 4-HC, VCR, hydrocortisone, Adriamycin, etoposide (VP-16), L-asparaginase, bleomycin, spirogermanium, and Taxol. Dose-response curves of these drugs were established by using the marrow GM-CFU cells as a target. Table 1 shows the IC_{50} dose of all drugs tested. An IC_{50} dose was obtained for each drug by using its dose-response curve accumulated from individual experiments. Figures 1A and 1B show the dose-response curve of GM-CFU to VCR and 4-HC that was determined from five experiments. L-Asparaginase and bleomycin reached a plateau after an initial exponential slope on the dose-response curve: their effect never achieved a 90% GM-CFU cell kill at the tested doses. Spirogermanium and Taxol precipitated before significant growth inhibition occurred and were eliminated from further testing.

Determination of leukemic cell kill efficiency of single drugs on ALL cell lines. The single drugs were tested on the ALL cell lines REH and LAZ-221 by using limiting-dilution analysis. L-Asparaginase and bleomycin were eliminated from further investigation because of minimal leukemic cell kill effect.

The leukemic cell kill (expressed as log kill) of the other drugs at their approximate IC_{50} doses was compared with that of 4-HC at its marrow IC_{50} dose on the ALL cell lines (Table 2). None of the tested single drugs was more effective against leukemic cells than 4-HC. As shown in Table 2, the other drugs at IC_{50} doses were generally equal or inferior to 4-HC (P < .05). Of note, VCR achieved a plateau effect of a 1.5- to 2.0-log kill on both ALL cell lines at only moderately myelotoxic doses well below its IC_{50}. No single drug was superior to 4-HC alone in leukemic cell kill at equal marrow-toxic doses.

Determination of myelotoxicity of drug combinations on normal bone marrow. Because no single drug was superior to 4-HC alone and because VCR offered considerable cell kill at minimally myelotoxic doses, we decided to evaluate VCR-based drug combinations by the method of Steel and Peckham.14 Combinations of VCR plus hydrocortisone (Fig 2), VCR plus Adriamycin, and VCR plus VP-16 were additive to supraadditive on normal bone marrow. On the other hand, the combination of VCR plus 4-HC was subadditive (ie, protective) of the normal bone marrow. In fact, by

| Table 1. IC_{50} Doses of Single Drugs on Human Bone Marrow |
|------------------|------------------|
| Adriamycin       | 1.7 μg/mL        |
| Bleomycin        | >100.0 U/mL      |
| 4-HC             | 6.0 μg/mL        |
| Hydrocortisone   | 12.5 mg/mL       |
| L-Asparaginase   | >20.0 U/mL       |
| Spirogermanium   | >15.0 μg/mL      |
| Taxol            | >60.0 μg/mL      |
| VCR              | 12.5 μg/mL       |
| VP-16            | 29.0 μg/mL       |

Abbreviation: >, the maximum tested dose of the drug produced less than an IC_{50} effect.
the addition of small doses of VCR, 4-HC could be given at a
dose even greater than its IC₅₀ dose before achieving and IC₄₀
effect with the combination (Fig 3A to E and Table 3).
Several representative graphs of individual patients are
supplied to underscore the variability observed among
patients.

Determination of leukemic cell kill efficiency of VCR and
4-HC combinations on ALL cell lines. Because of the
absence of additive marrow toxicity, the VCR plus 4-HC
combinations at the IC₅₀ dose were evaluated on three
lymphoid leukemia cell lines (REH, KM3, LAZ-221) and
found to be additive to supraadditive in cell kill and superior
to 4-HC alone. VCR at 1 or 5 µg/mL plus 4-HC at 5 µg/mL
(approximate IC₅₀ doses) was more effective against all three
cell lines than 4-HC at 5 µg/mL alone (P < .05) (Table 4).

Mixing experiments with excess marrow. Mixing experi-
ments were performed with a ninefold excess of irradiated
normal bone marrow to leukemic (REH) cells with the
combination of 4-HC, 5 µg/mL, plus VCR, 1 µg/mL, in
three experiments. The leukemic cell killing efficiency of the
combination was not compromised by the addition of normal
marrow to the leukemic cells (Table 4).

**DISCUSSION**

High-dose chemotherapy followed by ABMT is a rela-
tively new treatment approach for patients with acute leuke-
mia. The major drawback of this method is that occult
leukemic cells may be present in the graft remission marrow.
GM-CFU survival more quantitatively
study from the same institution suggested that even 60 to
individual variability among other drugs and drug combinations on the marrow GM-
chemotherapy, we determined the IC dose of 4-HC and
vivo chemotherapy with drugs like 4-HC, which has been
eliminated contamination.
One way to eliminate contamination of leukemic cells is ex vivo chemotherapy with drugs like 4-HC, which has been used successfully in preclinical and clinical studies.
As an important initial step of our approach to ex vivo chemotherapy, we determined the IC dose of 4-HC and other drugs and drug combinations on the marrow GM-CFU. It has been observed that successful neutrophil recovery after ABMT with or without ex vivo chemotherapy results if approximately 5% to 10% of the marrow GM-CFUs remain intact.
This is especially important, given the individual variability among patients in response to drugs in vitro. Thus, the IC represents a reasonable upper limit for ex vivo chemotherapy to allow consistent neutrophil engraftment, an important determinant of morbidity and mortality after ABMT.
A recent clinical phase I study on ex vivo chemotherapy with 4-HC from the Johns Hopkins Oncology Center reported a concentration-dependent inhibition of GM-CFU growth with virtually complete inhibition at concentrations of 80 μg/mL 4-HC or greater. This and another clinical study from the same institution suggested that even 60 to 100 μg/mL 4-HC ex vivo chemotherapy did not prevent hematopoietic reconstitution after myeloablative therapy.
However, a more recent report by Rowley et al estimated GM-CFU survival more quantitatively by accounting for RBC contamination. At even a 100-μg/mL dose of 4-HC ex vivo there were residual GM-CFUs present; the rapidity of the neutrophil recovery was related to the number of infused GM-CFUs.
We found the IC dose of 4-HC to be approximately 5 to 6 μg/mL under the conditions of in vitro incubation used in this study. The IC dose data derived from the Johns Hopkins data cannot be compared with our results because of the different experimental conditions. In the Johns Hopkins study, the in vitro incubation time was 30 minutes, and the incubated buffy coat cells were contaminated with RBCs. The drug incubation was followed by rapid cooling of the cell suspension to 4°C and only one wash before in vitro culture. We incubated light-density cells free of RBCs for 60 minutes followed by three washes at room temperature with RPMI plus FBS before culture.
Testing the chemosensitivity of fresh ALL cells is difficult because of the lack of a specific cloning system to monitor leukemic cell kill. Recently, however, assays for cloning ALL have been described. Limiting-dilution cultures using lymphocytic leukemic cell lines with high cloning efficiency can evaluate several logs of leukemic cell elimination. We used a number of ALL cell lines to test the effectiveness of our approach to prove that our specific ex vivo treatment was effective not solely on one ALL cell line.
Of the nine tested drugs, bleomycin, L-asparaginase, spirogermanium, and Taxol were eliminated from further study because of minimal leukemic cell kill or solubility problems. The drugs 4-HC, hydrocortisone, Adriamycin, VP-16, and VCR all had significant (2 to 4 log) kills on the cell lines at their IC doses. We chose to study VCR combinations because it offered considerable leukemic cell kill at minimally myelotoxic doses (ie, achieved a plateau effect of one-to-three-log kills at a moderately myelotoxic dose below its IC).
During the evaluation of the drug effects in combination, we found that VCR had a marrow-protective effect at low doses (1 to 5 μg/mL) on 4-HC myelotoxicity. With this VCR dose, 4-HC could be used at a dose greater than its IC. This finding is unique to this particular drug combination and has not been reported before. VCR, however, did not protect the marrow GM-CFUs from hydrocortisone, Adriamycin, or VP-16 myelotoxicity.
Despite its protective effect on human bone marrow, this combination showed additive effects when tested on three ALL cell lines. With 4-HC at its IC dose alone, the cell kill was approximately three logs, but on several cell lines, the cell kill with a combination approached six logs. Other efforts to eliminate ALL cells with combinations of monoclonal antibodies plus complement by using several rounds of antibody treatment, toxin-conjugated antibodies, or single drugs alone produced only three-log kills, and these approaches have been evaluated on only a limited number of cell lines. The only method that approaches the log kill of this combination-chemotherapy approach in lymphatic leukemia is the combination of activated cyclophosphamide derivatives at doses above its IC and toxin-conjugated monoclonal antibodies, which pro-

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<th>Table 3. Percent GM-CFU Survival in Single Experiments After the Ex Vivo Treatment of Normal Bone Marrow With 4-HC, VCR, and Combinations</th>
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<td>Drugs (μg/mL)</td>
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<td>4-HC (5)</td>
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<td>VCR (1) + 4-HC (5)</td>
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<td>VCR (1) + 4-HC (5) with ninefold excess marrow</td>
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*Significantly greater effect than 4-HC, 5 μg/mL, alone (P < .05).
duced a six-to seven-log kill on a Burkitt cell line. Another combination of multiple monoclonal antibodies with complement plus 4-HC tested on lymphatic leukemia cell lines was better than either approach alone, but the kill was less than six logs.

We describe here an ex vivo combination chemotherapy whose bone marrow toxicity is reproducible and no higher than 4-HC alone. At the same time, it is associated with a higher cell kill on ALL cells than that achieved with either drug alone. This approach produces results at least equivalent to what has been reported with any other ex vivo methods. Clinical studies based on the aforementioned results with VCR plus 4-HC combinations for purging second-remission acute leukemia patients’ bone marrow are currently underway. The outcome of these studies will be reported elsewhere.

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Evaluation of drugs for elimination of leukemic cells from the bone marrow of patients with acute leukemia

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