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In vitro synergism of 4-hydroperoxycyclophosphamide and cisplatin: relevance for bone marrow purging*

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Summary. Autologous bone marrow transplantation with 4-hydroperoxycyclophosphamide (4-HC)-purged bone marrow gives long-term remission in almost half of relapsed acute nonlymphocytic leukemia and non-Hodgkin's lymphoma patients, but relapse of disease is the main cause of failure, suggesting ineffective purging in some cases. Cisplatin (CP) has activity against a variety of human tumors and is not commonly used for initial therapy of leukemia and lymphoma. Using established human leukemia cell lines, combinations of 4-HC and CP were investigated as a potential regimen for improving the ex vivo removal of leukemia cells from bone marrow. The cell lines (K-562 and Raji) were incubated for 1 (4-HC) or 4 h (CP), washed, and assayed for inhibition of colony formation in semisolid media. In both cell lines, CP (4 h) was more potent than 4-HC (1 h). Combinations of the drugs in various molar ratios were studied after the cells were sequentially incubated with 4-HC and CP. The effects of the drugs were analyzed using the multiple drug-effect analysis of Chou and Talalay [6]. Analysis of data on in vitro inhibition of colony formation suggested that all combinations studied were synergistic in both cell lines, with the greatest synergism being found in the Raji cell line. In addition, for K-562 cells we could detect at least a 4.6 log reduction in cloning with the CP:4-HC combination (1:10 molar ratio). We conclude that CP is a potential candidate in drug combinations for ex vivo bone marrow purging because of its high potency against human leukemia cell lines, its synergistic activity in combination with 4-HC, and its ability to reduce a high tumor burden when combined with 4-HC.

Introduction

One alternative treatment for patients with relapsed acute leukemia and non-Hodgkin's lymphoma is autologous bone marrow transplantation, which involves selectively killing or removing the malignant cells from the marrow graft ex vivo and reinjecting the treated marrow back into the patients after they have received extensive radiation therapy and/or chemotherapy [29]. Various techniques (pharmacological, immunological, and physical) are used to "purge" selectively the marrow graft of contaminating tumor cells [12, 18, 24–26, 29, 32, 34].

Yeager et al. [34] have reported 45% disease-free survival in patients with acute nonlymphocytic leukemia who were transplanted in second and third remission with 4-hydroperoxycyclophosphamide (4-HC)-purged marrow grafts. However, the same purging technique has been less successful in treating adult lymphoblastic leukemia in which the actuarial relapse rate has exceeded 85% (Santos, G. W., personal communication). These results emphasize the need for improved purging techniques. We elected to study combinations of cisplatin (CP) with 4-HC as a potential purging regimen because (a) cisplatin is effective against a variety of animal and human tumors [20, 28]; (b) unlike cyclophosphamide or VP-16, CP is not usually part of the standard treatment of human lymphomas or leukemias; (c) CP probably has a different mode of action than cyclophosphamide, that of the former being associated with intrastrand [8, 15, 27] rather than interstrand DNA cross-linking [7, 15]; (d) CP has been shown to be synergistic with cyclophosphamide in mouse models [1, 13, 14, 30, 33]; (e) cells resistant to cyclophosphamide are not necessarily resistant to CP [28]; and (f) there are multiple platinum coordination complexes to which CP-resistant cells may be sensitive [1, 27].

The goals of our work were (a) to study the in vitro sensitivities of human leukemia cell lines to 4-HC, CP, and various combinations of the two; (b) to analyze the interactions of these drugs using the multiple drug-effect analysis of Chou and Talalay [6]; and (c) to demonstrate that the combination of the drugs can effectively and significantly reduce a high tumor load.

Materials and methods

Cell lines. The K-562 (blastic chronic myelogenous leukemia) [22] and Raji (acute lymphoblastic leukemia) cell lines [9] were obtained from the American Type Culture Collection (ATCC, Rockville, Md). To avoid possible changes in properties of the cells as they were passaged, aliquots from the original sample were routinely thawed every 2 months. The culture medium for both cell lines

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was RPMI 1640 (GIBCO, Madison, Wis) with 10% (v/v) fetal calf serum (FCS) (Hyclone Laboratory, Inc., Logan, Utah), 1% (v/v) penicillin:streptomycin (P/S) antibiotics (GIBCO), and 1% (v/v) L-glutamine (GIBCO). All cells were kept at 37° C in an incubator (NAPCO 6300, Portland, Ore) in a humidified 7.5% CO_2 atmosphere. In preparation for the drug exposure experiments, the cells were maintained in exponential growth, and their viability (dye exclusion) was at least 90% prior to and immediately after drug treatment.

Chemotherapeutic agents. 4-HC was synthesized by Dr. O. M. Colvin (The Johns Hopkins Oncology Center, Baltimore, Md), and CP was obtained from Bristol Laboratories (Syracuse, NY). 4-HC was dissolved in a few microliters of dimethylsulfoxide (ATCC), and CP was reconstituted with distilled water (to yield a solution containing 1 mg/ml CP in 0.9% NaCl) immediately before the experiments; all subsequent dilutions were made in RPMI 1640.

Drug-effect assays. The cells were incubated in RPMI 1640 + 10% FCS with various drug concentrations at 37° C for 1 h (4-HC) and/or 4 h (CP); the incubation cell concentration was 1×10^5 cells/ml as determined by a Coulter counter, model Zf (Coulter Electronics, Hialeah, Fla). For the "log kill" experiments (Fig. 6), the incubation cell concentration was 1×10^7 cells/ml. For the drug combinations, the cells were first incubated with 4-HC, washed once with RPMI 1640, then incubated with CP and again washed with RPMI 1640. Finally, the cells were resuspended with RPMI 1640 + 10% FCS. The viability of the cells was determined by trypan blue exclusion. The cells were plated in quadruplicate in 35-mm Lux plates (Miles Laboratories, Inc., Napeville, Ill). For K-562 cells, the culture medium consisted of 0.3% agar (bacteriological grade, GIBCO) in McCoy 5A (GIBCO) with 15% FCS and 1% P/S; for Raji cells, it consisted of 1.4% methylcellulose 1500 centipoise (Fisher, Fair Lawn, NJ), 10% FCS, 10% bovine serum albumin (Sigma, St Louis, Mo), Alpha MEM (GIBCO), and 50 μM mercaptoethanol (Sigma). All dishes were cultured in an incubator at 37° C in a humidified 7.5% CO_2 atmosphere. Plates were scored for colony formation (clusters of more than 40 cells) 7 days after plating.

Data analysis. Dose-effect relationships were analyzed by the median-effect equation derived by Chou [3]:

$$fa/fu = (C/IC_{50})^m, \quad (1)$$

where fa is the fraction of affected cells, fu is the fraction of unaffected cells, C is the concentration of drug giving the observed effect (fa), IC_{50} is the concentration of drug required for 50% cloning inhibition, and m is the Hill-type coefficient signifying the degree of sigmoidicity [6]. The logarithmic form of Eq. (1) gives the basis for the median-effect plot [4]: Log(fa/fu) vs Log(C) with slope (m) and X-intercept [Log(IC_{50})]. The IC_{50} can be calculated from the antilog of the X-intercept. The synergism, summation, or antagonism of drug effects were quantitatively analyzed by the multiple drug-effect analysis of Chou and Talalay [5, 6]. Slopes (m) and IC_{50} values for 4-HC, CP, and their molar mixtures can be determined by the median-effect plot. Interaction of the effects of these two drugs is quantitatively determined by the combination index (CI), which is defined by [5, 6]:

$$CI = \frac{C_{4HC}}{C_{4HCx}} + \frac{C_{CP}}{C_{CPx}} + \frac{@ \cdot C_{4HC} \cdot C_{CP}}{C_{4HCx} \cdot C_{CPx}}, \quad (2)$$

where C_{4HCx} and C_{CPx} are the concentrations of each drug required to produce x% effect when the drug is used alone, and C_{4HC} and C_{CP} are the concentrations of each drug required to produce x% effect when used in combination. If the drugs are mutually exclusive [excluding one another from their binding site(s)], @ = 0; if the drugs are mutually nonexclusive [not excluding one another from their binding site(s)] @ = 1. Interestingly, Eq. 2 is the same as the equation used to calculate isobols [21] when @ = 0 [6]. All of the factors in Eq. 2 can be determined from the median-effect plot parameters m and IC_{50} and Eq. 1. This analysis generates the combination effect as: when CI = 1, summation is indicated; when CI < 1, synergism is indicated; when CI > 1, antagonism is indicated. A computer program [2] based on the above equations was used in the present study for automated analysis of the dose-effect data with an IBM-compatible microcomputer.

Statistical analysis. Regression analysis data for the median-effect plots of the K-562 and Raji cells (Figs. 3 and 4) were generated by the computer program written by Chou and Chou [2]. For the log kill data (Fig. 6), regression lines were calculated by least-squares analysis using the Microstat computer software (Ecosoft, Inc., Indianapolis, Ind) on an IBM-compatible microcomputer.

Results

Incubation time

Preliminary studies with 4-HC (data not shown) indicated that incubating the cells with the drug for 1 h resulted in additional inhibition of cloning compared with a 30-min exposure; we thus chose to incubate the cells with 4-HC for 1 h. For CP, survival curves for Raji and K-562 cells were constructed after incubating the cells for 1, 2, 4, 6, and 8 h with graded concentrations of the drug (0–20 μM for Raji cells; 0–50 μM for K-562 cells). The concentration survival curves for the Raji cell line are presented in Fig. 1. A similar graph was obtained for the K-562 cell line (data not shown). The data generated by the concentration-survival plots were analyzed using median-effect

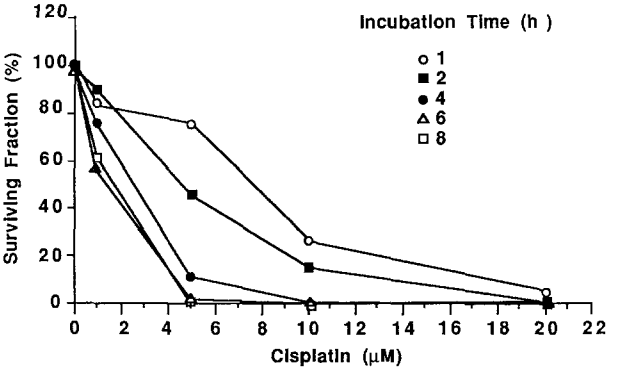

Fig. 1. Concentration-survival curves for clonogenic Raji cells. The cells were exposed to graded concentrations of CP (0–20 μM) for 1, 2, 4, 6, and 8 h and assayed for colony formation. Errors were < 5% of the means and were omitted

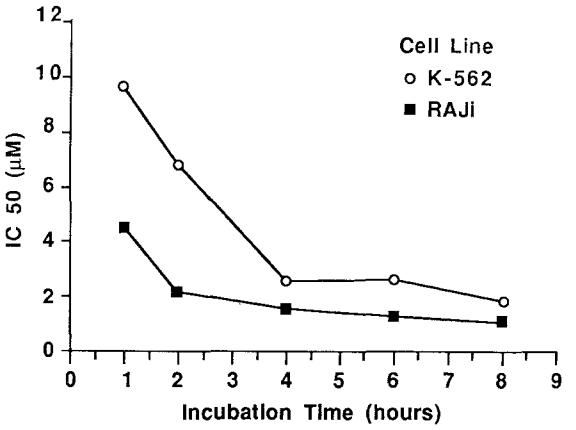

Fig. 2 Effect of incubation time on CP potency. The IC_{50}s (the concentration of drug resulting in 50% cloning inhibition) were determined using the median-effect plot

Table 1. Sensitivity of human leukemia cell lines to 4-HC and CP as measured by the colony formation assay

Cell line	4-HC[a]		CP[b]	
	IC_{50}[c] (μM)	X-int[d]	IC_{50}[c] (μM)	X-int[d]
K-562	9.1	0.96 ± 0.165 (16)	1.78	0.251 ± 0.37 (23)
Raji	4.1	0.61 ± 0.45 (14)	1.0	0.011 ± 0.356 (25)

[a] 1-h incubation
[b] 4-h incubation
[c] The IC_{50} is obtained by taking the antilog of the X-intercept of the median-effect plot
[d] X-int, X-intercept of the median-effect plot ± 1 SD. The X-int is the point where log(fa/fu) = 0, i.e., fa = fu (see *Materials and methods*)
Numbers in parentheses represent the number of separate data points used for the median-effect plots

analysis [4], and the IC_{50}s for each incubation time were determined from the antilog of the X-intercept of median-effect plots (see *Materials and methods*). Figure 2 shows the influence of incubation time on the IC_{50} of CP in both cell lines. The observed inhibitory effect of the drug increased from 1 to 4 h but beyond 4 h there was no significant change; thus, we decided to incubate the cells with CP for 4 h.

Inhibitory activity of the drugs

Table 1 lists the IC_{50}s (derived from the median-effect plots) of the two drugs for the two cell lines. For both cell lines, CP (4 h) was more potent than 4-HC (1 h).

Median-effect plot

The median-effect plots of 4-HC, CP, and various molar ratios of both drugs (CP:4-HC = 1:1 and 1:10 for K-562 cells; 1:3 and 1:10 for Raji cells) are shown in Fig. 3 (K-562) and Fig. 4 (Raji). Adequacy of the median-effect equation is generally defined as regression coefficients > 0.9 [6]. All of the regression lines in Figs. 3 and 4 meet this requirement.

Combination index plot

The results of computer analysis of combination effects using the combination index plot (see *Data analysis*) for K-562 and Raji cells are shown in Fig. 5. For K-562 cells, the 1:1 combination appeared to be more synergistic than the 1:10 combination, whereas for Raji cells, the 1:3 and 1:10 combinations seemed to generate the same amount of synergism. For both cell lines, all ratios showed synergism at high fractional cloning inhibition. In addition, the drugs seemed to be more synergistic in the Raji than in the K-562 cell line.

Log kill

Preliminary studies had shown that the presence of a high number of treated cells (4×10^5 cells/dish) did not affect the cloning of a small number of nontreated cells (data not shown). To determine the maximal detectable cloning inhibition (log kill) of K-562 cells, the latter were incubated at a higher cell density (1×10^7 cells/ml) and plated at various cell concentrations of up to 4×10^5 cells/dish to maximize the detection of surviving clonable cells. Figure 6 demonstrates at least a 4.6 log reduction in cloning for the

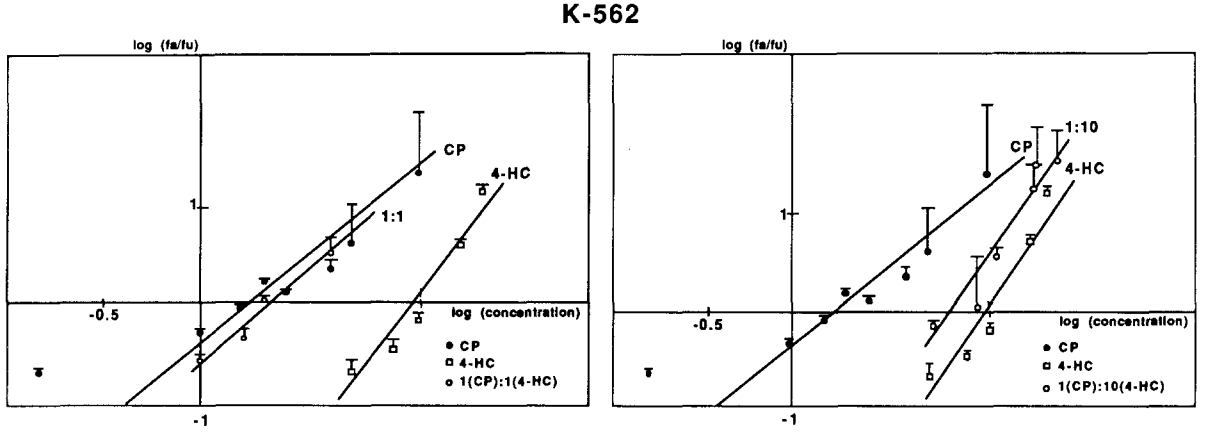

Fig. 3. Dose-effect relationship of CP and 4-HC alone and in combination on the K-562 cell line. Data were analyzed by linear regression. Combinations are expressed as molar ratios (CP: 4-HC). CP (cisplatin alone) $r = 0.91$, $n = 23$; 4-HC (4-HC alone) $r = 0.96$, $n = 16$; 1:1 (1[CP]:1[4-HC]) $r = 0.94$, $n = 11$; 1:10 (1[CP]:10[4-HC]) $r = 0.92$, $n = 14$. Each point represents the mean of two to five experiments. The bars represent the SEM

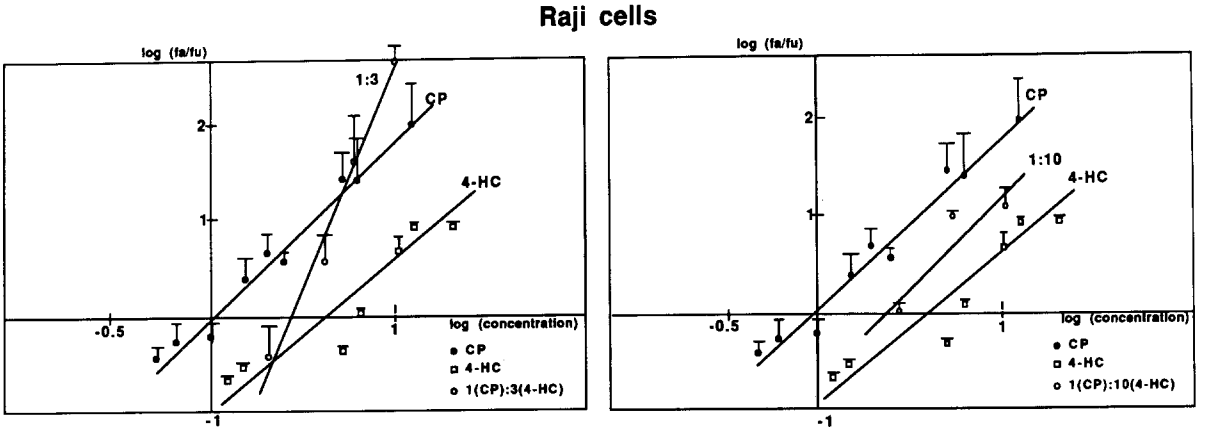

Fig. 4. Dose-effect relationship of CP and 4-HC alone and in combination on the Raji cell line. Data were analyzed by linear regression. Combinations are expressed as molar ratios (CP:4-HC). CP (cisplatin alone) $r = 0.93$, $n = 25$; 4-HC (4-HC alone) $r = 0.92$, $n = 14$; 1:3 (1[CP]:3[4-HC]) $r = 0.95$, $n = 9$; 1:10 (1[CP]:10[4-HC]) $r = 0.92$, $n = 8$. Each point represents the mean of two to four experiments. The bars represent the SEM

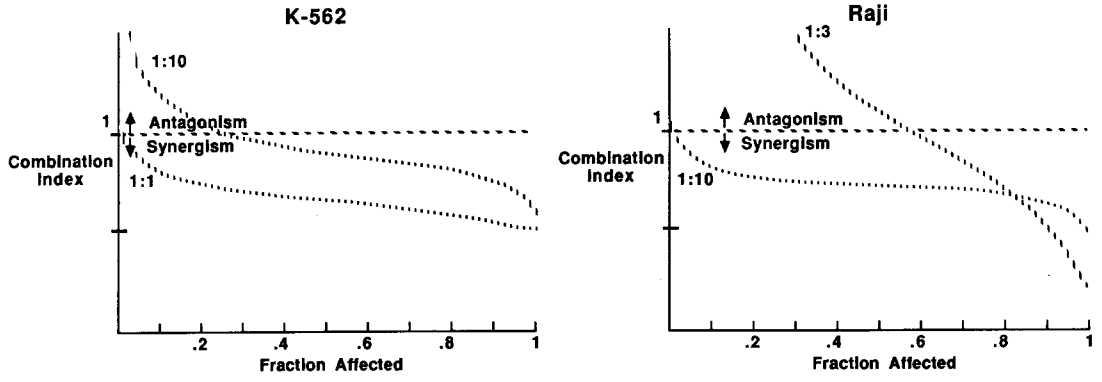

Fig. 5. Combination effect of CP and 4-HC on the K-562 and Raji cell lines at various molar ratios (CP:4-HC)

1:10 combination (CP = 5 μ*M*; 4-HC = 50 μ*M*), whereas CP alone (5 μ*M*) achieved a 1.6 log reduction and 4-HC alone (50 μ*M*) achieved a 2.4 log reduction in cloning.

Discussion

4-HC is a congener of cyclophosphamide but, unlike the latter, does not require biological activation to exhibit its activity [11, 31]. In aqueous solution it is an active alkylating agent [31] that interacts with DNA, RNA, and proteins. DNA cross-links are the most frequent cytotoxic lesions, and it is widely accepted that the antitumor action of the drug lies in its ability to cause interstrand cross-links [15, 27].

CP is also an anticancer agent that interacts with DNA, RNA, and proteins [17, 36]. DNA is its major intracellular target [35]. Rosenberg [27] has reviewed the postulated modes of action of CP as well as the evidence of others [8, 15] pointing at intrastrand DNA cross-linking as the lesion responsible for the antitumor effect of the drug. CP is not currently part of the standard treatment of human lymphomas and leukemias because renal toxicity is excessive at doses that produce significant myelotoxicity [20, 27]. For ex vivo purging, however, one is not limited by renal toxicity; thus, the use of CP for in vitro treatment of hematologic malignancies by purging autologous marrow is conceivable.

Howle and Gale [17] showed that increasing the incubation time up to 4 h with CP increased the inhibition of DNA synthesis of Ehrlich ascites tumor cells. Moreover, Eastman [8] has shown that the majority of DNA platination occurs in the first 4 h of incubation with the drug. In Fig. 1 and 2, we also demonstrate that, if the maximal effect of the drug is to be studied, cells should be incubated in vitro for at least 4 h instead of 1 or 2 h as has been done in other studies [10, 16, 23, 36].

The combination index plots (Fig. 5) show that the two drugs are synergistic at concentrations causing high cloning inhibition. These results are encouraging because for bone marrow purging it is essential to operate at very high cloning inhibition to achieve complete eradication of tumor cells. Hence, the apparent antagonism at concentrations causing low cloning inhibition is of little relevance in the setting of bone marrow purging. In addition, we studied the effect of the drugs on the Raji cell line, a lymphoblastic leukemia cell line, because clinical trials in acute lymphoblastic leukemia have had little success (85% actuarial relapse rate) when 4-HC alone was used for purging (G. W. Santos, personal communication). It is very encouraging that combinations of CP and 4-HC are highly synergistic in the Raji cell line.

Finally, although it is important for two drugs to be at least additive when used in combination, their mixture

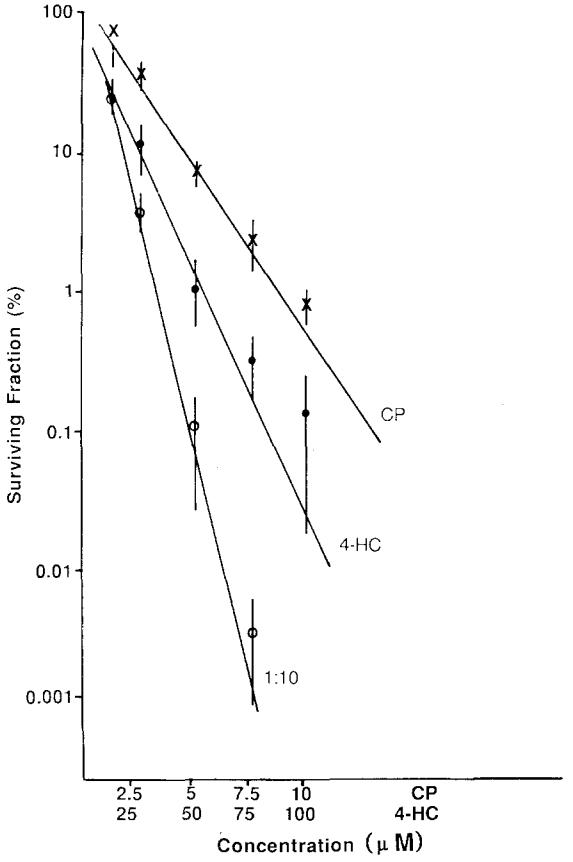

Fig. 6. Survival of clonogenic K-562 cells with CP, 4-HC, and the 1:10 combination (CP: 4-HC). The cells were incubated with the drugs at 1×10^7 cells/ml. Regression lines were calculated by least-squares analysis. Each point represents the mean of three to six experiments. The bars represent the SEM. ×, CP ($r = -0.93$); ●, 4-HC ($r = -0.92$); ○, 1:10 ($r = -0.97$)

should also be able to eradicate a significant tumor load if they are to be considered as potential candidates for bone marrow purging. Figure 6 shows that, with K-562 cells, there was at least a 4.6 log reduction in cloning with the CP:4-HC combination (1:10) at drug concentrations that individually resulted in 1.6 (CP) and 2.4 (4-HC) log reductions, respectively. These results demonstrate that the two drugs in combination can eradicate a high tumor load. Since the plating efficiency of the Raji cell line is lower (15%–25%) than that of the K-562 cell line (35%–50%) and the colonies formed by the Raji cells tend to spread in semisolid medium, we could not accurately measure the maximal detectable cloning inhibition of Raji cells.

Agents that can selectively eliminate residual leukemic cells in remission marrow with no effect on normal bone-marrow stem cells would be ideal, but clinical experience with 4-HC demonstrates that even myelotoxic drugs can be useful in actual practice [19]. Work is under way in our laboratories to test the in vitro toxicity of combinations of 4-HC and CP on normal marrow cells.

In conclusion, this study suggests that CP is a potential candidate for combination treatment of autologous marrow grafts because of its high potency against the human leukemic cell lines tested, its synergistic interaction with 4-HC, and its ability to achieve a high reduction in cloning when used with 4-HC.

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