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Comparative Cytotoxicity of Various Drug Combinations for Human Leukemic Cells and Normal Hematopoietic Precursors[1]

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ABSTRACT

The development of suitable methods for purging the malignant cells contaminating the bone marrow of patients with cancer may offer a better chance of success for autologous bone marrow transplantation. In this paper, we further describe our efforts at purging acute myelogenous leukemia cells. HL-60, a promyelocytic leukemia cell line, was used as a model. 4-Hydroperoxycyclophosphamide (4-HC), VP-16-213 (VP-16), and Adriamycin were used alone or in combination to develop the best method to purge HL-60 cells. The cytotoxicity of 29.2 μg/ml (100 μM) of 4-HC was 99.8 ± 0.12% (SD) on HL-60 cells and 82.5% on colony forming units-granulocyte, macrophage. Ninety-nine % of HL-60 cells and 72.7% of colony forming units-granulocyte, macrophage were inhibited by VP-16 at a concentration of 25 μg/ml (42.5 μM). The cytotoxicity of 1.5 μg/ml (2.76 μM) of Adriamycin on HL-60 cells was 98.6 ± 0.8% and inhibited colony forming units-granulocyte, macrophage by 50.8%. The cytotoxicity and interactions of any two drug combinations at different combination ratios and the different effect levels were quantitatively determined by median effect plot and the multiple drug effect equation (T-C. Chou and P. Talalay. Adv. Enzyme Regul. *22:* 27–55, 1984). The combination of 4-HC and VP-16 at a 4-HC:VP-16 drug ratio of 1:0.342 was found to be the best for selective toxicity towards HL-60 cells and was superior to the 4-HC-Adriamycin or VP-16 Adriamycin combination for usefulness in purging bone marrow.

INTRODUCTION

Combination chemotherapy for AML[4] results in a significant complete remission rate, but most patients relapse (1–4). Myeloablative chemotherapy requiring rescue with allogeneic or autologous bone marrow has provided encouraging results, especially when the procedure is performed while the leukemia is in remission (5–16). Although success using unpurged autologous bone marrow transplant has been reported (5–9), it is likely that the methods to purge the leukemic cells from the BM will allow wider application of autologous bone marrow transplant. Thus, drugs active against AML cells *in vivo* were examined for their usefulness in purging BM of patients with AML *in vitro*. 4-HC is an alkylating agent (17) that interacts with preformed DNA, RNA, and protein. The mode of action for VP-16 is not well defined, but it is thought to affect cells in the mitotic and G_2 phases (18–20). ADR, an anthracycline antibiotic, is thought to inhibit DNA replication by intercalation of base pairs. Two active drugs with different modes of action sometimes have enhanced therapeutic efficiency. The combination of 4-HC and VP-16 appears to have a synergistic cytotoxic effect on HL-60 (21) and lymphoma (22) cell lines. The purpose of this paper is to investigate the efficiency of 4-HC, VP-16, and ADR by combining these drugs in pairs at two different combination ratios in order to determine the best combination and dosage levels for purging myelogenous leukemia cells.

MATERIALS AND METHODS

Cell Line and Bone Marrow. HL-60 cells (a human acute promyelocytic leukemia cell line) was chosen for this study (23, 24). The cell line was maintained in RPMI 1640 (GIBCO, Grand Island, NY), with 10% (v/v) fetal calf serum (Sterile Systems, Inc., Logan, UT), 1% (v/v) pencillin-streptomycin-neomycin antibiotics (GIBCO), and 1% L-glutamine (GIBCO) and kept at 37°C in a humidified atmosphere of 5% CO_2. The cells were maintained in exponential growth with cell concentrations ranging from 0.5 to 1.0 × 10^6 cells/ml. The viability of the HL-60 cells was 90% or higher and the cells were devoid of *Mycoplasma* or bacterial infection. Viable cells were stained with Wright-Giemsa stain and exhibited no evidence of morphological changes before or after the drug treatment (25–27).

BM specimens were obtained after informed consent from normal volunteers. The bone marrow aspirations were done under local anesthesia (1% xylocaine without adrenaline) and specimens were obtained from the posterior iliac crests with plastic syringes containing preservative free heparin.

Chemotherapeutic Agents. 4-HC was kindly provided by Dr. M. Colvin (The Johns Hopkins University, Baltimore, MD). VP-16 was obtained from Bristol Laboratories, Syracuse, NY, and Adriamycin was obtained from Adria Laboratories, Columbus, OH. All the drug solutions were prepared immediately before the experiments and diluted with phosphate buffered saline without calcium or magnesium.

Drug Effect Assays. Twenty million HL-60 cells and 10 × 10^6 cells of buffy coat preparations of normal bone marrow were incubated separately with different drug concentrations at 37°C for 1 h. The final cell concentration for incubation was 10 × 10^6/ml. After incubation, the cells were washed twice with RPMI 1640 and the supernatant was removed after centrifugation at 1800 rpm for 10 min at room temperature. After the cell washing, the HL-60 cells were resuspended in 2 ml of media (10% FCS in RPMI) from which the cell count and viability were determined by trypan blue exclusion. After incubation, 1.8 ml of the cells were diluted with 25 ml of medium for long term liquid culture. Daily cell counts and viability tests were performed. Bone marrow cells after incubation were washed, resuspended in FCS, and plated in quadruplicate onto 35- × 10-mm Lux plates (Miles Laboratories, Inc., Naperville, IL) at the following concentration for CFU-GM: 100,000 cells, 10% FCS, 1% penicillin-streptomycin-neomycin, 1.07% (w/v) methylcellulose in McCoy's media; in a total volume of 1 ml which was placed on 1- to 7-day-old underlayers containing 1 × 10^6 peripheral blood mononuclear cells in 0.5% agar. Colonies (cellular aggregates of greater than 40 cells) were counted on day 10.

Data Analysis. The combined effects of the two drugs in terms of synergism, summation, or antagonism were quantitatively analyzed by the median effect plot and multiple drug equation derived by Chou and Talalay (28–31). If the observed effect of the two drugs acting simultaneously is larger or smaller than that calculated from the product expression, it was assumed that synergism or antagonism, respectively, had occurred. The computer program was developed by Chou and Chou

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[4] The abbreviations used are: AML, acute myelogenous leukemia; 4-HC, 4-hydroperoxycyclophosphamide; VP-16, etoposide; CFU-GM, colony forming units-granulocyte, macrophage; FCS, fetal calf serum; ADR, Adriamycin; GIBCO, Grand Island Biological Co.; CFU-GEMM, colony forming units-granulocyte, erythroid, macrophage, megakaryocyte; BM, bone marrow; CI, combination index.

(32) and was used for automated data analysis with an Apple microcomputer (IBM-PC compatible program is also available). Interaction of the two drugs is quantitatively determined by the CI which is described by

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{\alpha(D)_1,(D)_2}{(D_x)_1,(D_x)_2}$$

where D_x is the dose that is required to produce x% cytotoxic effect. The relationship between D_x and x% can be determined by the median effect plot parameters m, D_m, and the median effect equation as described previously (28–33). The slope of the median effect plot is represented by m. The magnitude of the m value is a measure of the sigmoidicity of the dose-effect curve and is analogous to the Hill coefficient. In the analysis of enzyme action, it represents the cooperativity coefficient of "binding" or the (minimum) number of binding site(s) on the enzyme molecule. When $m = 1$, a Michaelis-Menten type of hyperbolic dose-effect curve is observed (28–32). This analysis generates the combination effect below:

When CI = 1, summation is indicated
CI <1, synergism is indicated
CI >1, antagonism is indicated

When the regression lines of the median effect plot for each drug alone are parallel and the combination has the same slope, then a similar mode of action of the two drugs is indicated (*i.e.*, $\alpha = 0$; the effects of the two drugs are mutually exclusive). If the median effect plot of the combination has a higher slope than individual drugs, then a dissimilar mode of action of the two drugs is indicated (*i.e.*, $\alpha = 1$; the effects of the two drugs are mutually nonexclusive). When the exclusiveness of the two drugs is difficult to evaluate, then it is suggested that the CI values be calculated by mutually exclusive ($\alpha = 0$) and mutually nonexclusive ($\alpha = 1$) conditions.

RESULTS

HL-60 cells were incubated with 4-HC, VP-16, and ADR (used alone or in combination; the two drug combinations were tested at two different drug ratios) at various concentrations at 37°C for 1 h. After incubation the cells were washed and then diluted with fresh media to provide logarithmic growth condition, and cell counts were done at 1 h and then every 24 h (data not shown). The results at 72 h of long term culture were more consistent for evaluating cytotoxicity (21) and were used for all the calculations in this paper. The cytotoxicity of these three drugs (used alone and in combination) upon HL-60 cells and CFU-GM of normal bone marrow is shown in Fig. 1. The computer analyses for the interactions of the two drug combinations are shown in Fig. 2. Due to the necessity of at least a 3–4 $\log_{10}$ leukemic cell cytotoxicity for marrow purging, only the results in higher cytotoxic effect levels have been detailed. Mixing experiments were performed where fresh HL-60 cells were mixed with irradiated normal BM, but these experiments were hard to interpret because of the high amount of cellular debris.

4-HC and VP-16. As shown in Fig. 1*A*, the cytotoxicity of 4-HC for both HL-60 cells and CFU-GM was more prominent than that of VP-16. 4-HC at a dose of 29.2 μg/ml (100 μM) can eliminate 99.8 ± 0.12% (SD) of HL-60 cells and inhibit CFU-GM by 82.5%. The cytotoxicity of 25 μg/ml VP-16 was 99% for HL-60 cells and 72.7% for CFU-GM. These results can be improved by combining the two drugs at a ratio of 1:0.342 and 1:0.856. However, the results with a 4-HC:VP-16 ratio of 1:0.342 is much better. The combination with a ratio of 1:0.342 gave a synergistic effect for HL-60 cells when the fractional cytotoxicity was greater than 73% (Fig. 2A_1) and gave an antagonistic effect on CFU-GM when the fractional cytotoxicity was greater than 85% (Fig. 2A_2). A mixture containing 29.2 μg/ml of 4-HC and 10 μg/ml of VP-16 can reduce the HL-60 cells to immeasurable amounts and inhibits CFU-GM by only 87.2% (Fig. 1*A*). The cytotoxic effect upon HL-60 for the two drug combinations tested was higher than the toxicity from either drug alone, and 4-HC:VP-16 drug ratio of 1:0.342 showed higher synergism (especially at higher dose levels) of cytotoxicity than the drug ratio of 1:856.

4-HC and ADR. The cytotoxicity of 4-HC used alone or in combination with ADR was much more prominent than ADR used alone (Fig. 1*B*). Adriamycin at a dose of 1.5 μg/ml can eliminate only 98.63 ± 0.8% of HL-60 cells and inhibits 50.6% of CFU-GM. 4-HC combined with a lower dose of ADR at a ratio (4-HC:ADR) of 1:0.034 had a synergistic effect for both

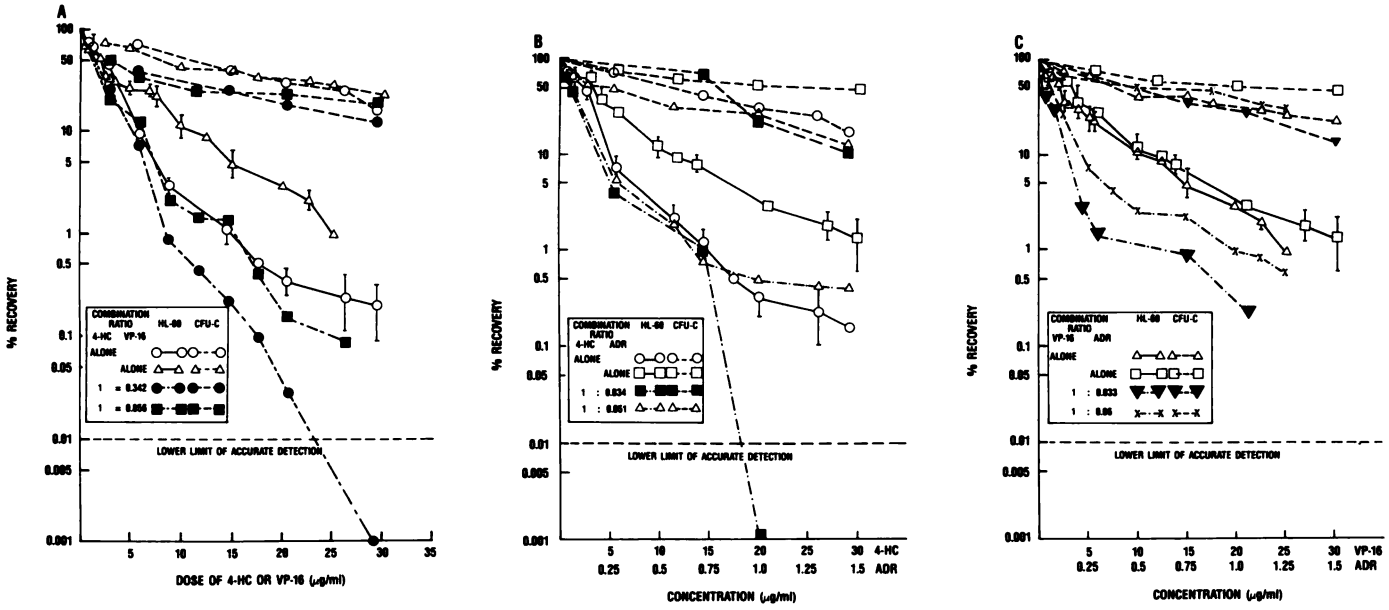

Fig. 1. Percentage of recovery of HL-60 cells or normal BM CFU-GM. The cells were treated with various drug combinations for 1 h, washed, and then allowed to grow. Seventy-two-h data of long term liquid culture for HL-60 cells and the day 10 colony count for treated normal BM CFU-GM are depicted. *A* was published elsewhere (21) and is reproduced by permission from the editorial board of *Cancer Research*. *A*, effect of 4-HC:VP-16: *B*, effect of 4-HC:ADR; *C*, VP-16: ADR. *CFU-C*, colony forming units-cell.

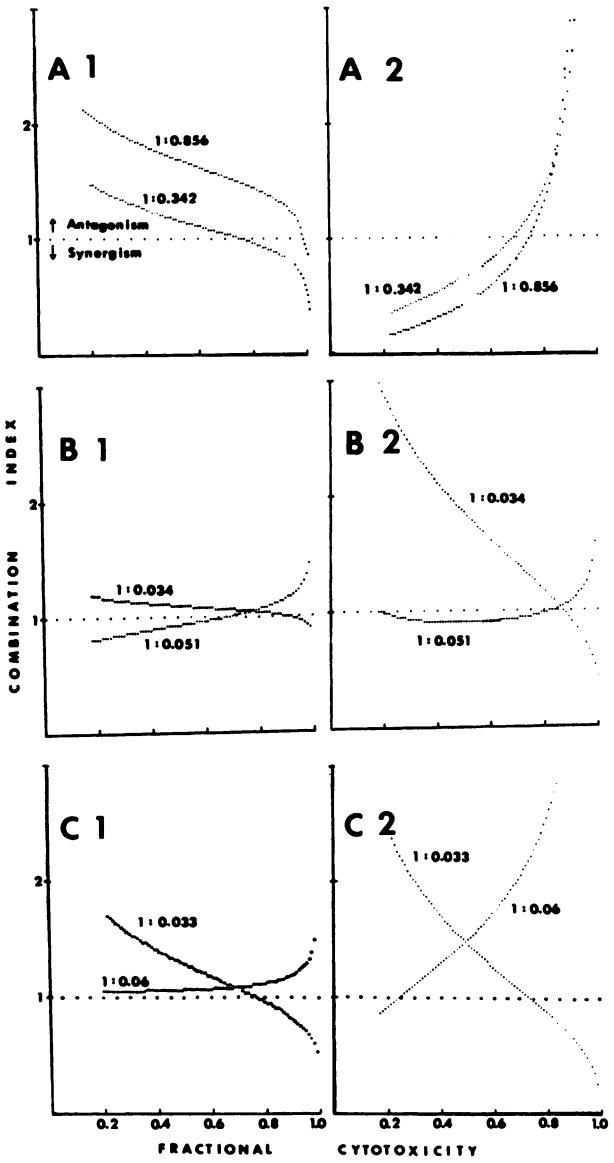

Fig. 2. Combination effect of various drug combinations upon HL-60 cell line (A_1, B_1, and C_1) and normal BM CFU-GM (A_2, B_2, and C_2). *A*, 4-HC:VP-16; *B*, 4-HC:ADR. *C*, VP-16:ADR. *Data points* above the *horizontal dotted line*, antagonism; *data points* below the *horizontal dotted line*, synergism.

HL-60 cells (Fig. 2B_1) and CFU-GM (Fig. 2B_2). The fractional cytotoxicity was greater than 90 and 88%, respectively. The inhibitory effect of the drug dose consisting of 29.2 μg/ml of 4-HC and 0.7 μg/ml of ADR was 89.1% (Fig. 1*B*). The dosage with a combination ratio of 1:0.051 was less toxic than the drug ratio of 1:0.034 or when ADR was used alone; these conditions exhibited an antagonistic effect for both HL-60 cells (Fig. 2B_1) and CFU-GM (Fig. 2B_2). 4-HC at 29.2 μg/ml and ADR at 1.5 μg/ml can eliminate only 99.6% of HL-60 cells (Fig. 1*B*).

VP-16 and ADR. The cytotoxicity of various concentrations of VP-16 and ADR on HL-60 cells and normal BM CFU-GM is shown in Fig. 1*C*. In the dose range used, the toxicity of VP-16 on CFU-GM was also more prominent than ADR (Fig. 1*C*). The combination of these two drugs at a ratio (VP-16:ADR) of 1:0.033 had a synergistic effect for both HL-60 cells and CFU-GM at higher fractional cytotoxic levels (Fig. 2, C_1 and C_2), but the cytotoxicity for HL-60 cells was still less than 3 $\log_{10}$ at a higher dosage level (Fig. 1*C*). A combination ratio of 1:0.06 was antagonistic for both HL-60 cells and CFU-GM. (Fig. 2, C_1 and C_2). The dose containing 25 μg/ml of VP-16 and 1.5 μg/ml of ADR inhibits HL-60 cells by 99.4% (Fig. 1*C*).

DISCUSSION

The ability of 4-HC to purge murine leukemia cells has been demonstrated by Sharkis *et al.* (34). The feasibility of using 4-HC for purging human leukemia and lymphoma cells is being explored and clinical phase I trials are in progress (16). VP-16 has also been reported to be an effective agent for purging murine leukemia cells by Stiff *et al.* (35). The combination of 4-HC and VP-16 has been shown to have a synergistic cytotoxic effect on human B-cell lymphoma cell lines SK DHL-2 (22) and HL-60 (21). ADR is recognized as an effective drug for treating patients with AML, but the use of ADR for purging leukemia cells from the marrow *in vitro* has not been investigated. It would be preferable to evaluate drug toxicities for fresh leukemia and/or lymphoma cells, but fresh leukemia and lymphoma cells are difficult to grow consistently, although occasional successful studies have been reported (36). The purpose of this study is to compare the efficiency of 4-HC, VP-16, and ADR in various combinations in order to develop the best method for purging human BM of acute myeloblastic leukemia cells.

VP-16 when combined with ADR at a combination ratio of either 1:0.033 or 1:0.06 resulted in less than a 3 $\log_{10}$ decrease in HL-60 cells; as a result, it is probably not an effective drug combination for marrow purging. The combination of 4-HC and ADR when used at a drug ratio of 1:0.051 exhibited an antagonistic effect on HL-60 and this was also thought to be unsuitable for purging human bone marrow. The 4-HC:ADR combination at a drug ratio of 1:0.034 produced a synergistic cytotoxic effect on HL-60 cells and reduced the HL-60 cells to an immeasurable amount, but the enhanced toxicity for CFU-GM was also significant. The combination of 4-HC and VP-16 was synergistic for toxicity to the HL-60 cells but had an antagonistic effect on CFU-GM. This phenomenon was more prominent at higher drug concentrations (Fig. 2, A_1 and A_2), especially when the ratio of 4-HC to VP-16 was 1:0.342. Of the drug combination ratios tested, 4-HC and VP-16 were found to be most suitable for purging human bone marrow of SK DHL-2 lymphoma cells (22) and of HL-60 leukemic cells, especially at a ratio of 1:0.342.

The present method for analyzing interaction of multiple drug effects has several important features: (*a*) it utilizes small numbers of data points; (*b*) it quantitates synergism, summation, or antagonism at different effect levels; (*c*) it provides information about relative potency of each drug and their mixtures; (*d*) it determines the sigmoidicity of the dose-effect curve and the exclusivity of drug effects and includes these parameters into overall calculations. Many of these features are not available by the isobologram or the fractional product methods (31). Furthermore, the availability of computer software of this method (32) greatly facilitates the present studies.

Several parameters including cell dose, viability of cells by trypan blue dye exclusion, CFU-GM, blast forming units-erythroid, and CFU-GEMM assays are being investigated for predicting the ability of purged marrows to engraft, but completely reliable methods for predicting bone marrow engraftment are still not available (16, 37–41). Purged marrows have been reported to result in full hematopoietic reconstitution even when no measurable CFU-GM were present (16). Several papers have reported the clinical usefulness of unpurged autologous stem cell transplants for patients with AML (5–9), but

longer follow-up of these patients will be necessary to determine the true frequency of relapse. Purging the bone marrow of residual leukemic cells may result in a lower rate of relapse. Our data show that two active drugs, with different modes of action, can be combined to cause a marked increase in cytotoxicity to HL-60 cells with minimal increase in toxicity to normal bone marrow CFU-GM. The toxicity of 4-HC and VP-16 toward other hematopoietic progenitors (blast forming unit-cell, CFU-GEMM, etc.) must be established. Our results suggest that the combination of 4-HC and VP-16 at a ratio of 1:0.342 may be clinically useful for purging acute myeloblastic leukemia cells contaminating the bone marrow. Further work will be required to establish if other AML cell lines will show similar results for cytotoxicity to various drug combinations. There is also the need to evaluate the relevance of the above data to clinical trials.

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