Two Mechanisms of Synergy When Amphotericin B Is Used in Combination with Actinomycin D or 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea against the Human Promyelocytic Leukemia Cell Line HL-60

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ABSTRACT

The toxic effects of the combinations of amphotericin B (AmB) and actinomycin D or AmB and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea were measured against the human promyelocytic leukemia cells HL-60. The toxicities of both drug combinations were greater than the additive toxicity of each of the drugs used singly, but the exact conditions under which synergy was achieved differed for each combination. The synergism achieved by the AmB-actinomycin combination was accompanied by an AmB-induced increase in uptake of actinomycin D by the HL-60 cells, whereas the synergism of the AmB-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea combination could be linked to potentiation by 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea of AmB-induced oxidative injury. These results indicate that the synergism of these two drug combinations was caused by different mechanisms.

INTRODUCTION

It has been previously demonstrated that combinations of individual antitumor agents with the polyene macrolide AmB are more potent in curing mice of several different transplantable tumors than any of the drugs used alone. The curative action of the combinations was attributed to synergistic interactions of AmB with the second drugs, resulting in increased killing of tumor cells (2 and references in review 3). One explanation of the mechanism of the synergism was based on the action of AmB on membranes which influence the transport of antitumor agents into cancer (4, 5) and HeLa cells (6). An increased uptake of Act D by HeLa cells exposed to AmB resulted in overcoming drug resistance of the cells and in synergistic toxicity of the drug combination (6).

In this study, we investigated the synergistic interactions of AmB with Act D and CCNU in inducing toxicity to HL-60 cells. We found, in agreement with our previous findings, that AmB potentiated the uptake by cells of radioactive Act D, and we could attribute the observed synergism to this effect. In contrast, we did not observe an AmB-induced increase in the uptake of radioactive CCNU by HL-60 cells. Instead we present evidence that the synergistic interaction of AmB and CCNU was based on oxidative cell injury.

MATERIALS AND METHODS

Drugs and Chemicals. AmB as Fungizone (E. R. Squibb and Sons, Princeton, NJ) was dissolved in sterile water just before use. Sodium deoxycholate alone, in concentrations equal to that in Fungizone, had no effect on the assays. Act D dissolved in ethanol, Triton X-100, and fungal catalase (C-3515) were purchased from Sigma Chemical Company, St. Louis, MO. CCNU was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. It was dissolved in dimethyl sulfoxide and diluted in sterile water just before use. The concentration of this solvent in the sample never exceeded 0.5% and was generally 0.1% or less. Dimethyl sulfoxide had no effect on the assays at any of the concentrations used.

[3H]Actinomycin D (specific activity, 3.4 Ci/mmol) was purchased from Schwarz Mann, Orangeburg, NY. 1-(2-chloroethyl) 14C-3-cyclohexyl-1-nitrosourea (specific activity, 24.4 mCi/mmol) was obtained from the Chemistry and Life Sciences Division at the Research Triangle Institute, Research Triangle Park, NC. [methyl-3H]Thymidine (specific activity, 20 Ci/mmol) was purchased from New England Nuclear Corporation, Boston, MA.

Cells. The HL-60 cell line was obtained from Dr. Z. Bar-Shavit from the Department of Pathology of The Jewish Hospital of St. Louis, and it was maintained in RPMI-1640 medium, prepared by the Center for Basic Cancer Research, Washington University, School of Medicine, St. Louis, MO. The RPMI medium was supplemented with 20% FBS, 4 mm L-glutamine, 1% nonessential amino acids, 100 units of penicillin per ml, and 100 µg of streptomycin per ml. All of these additional medium supplements were purchased from Gibco Laboratories, Grand Island, NY. The HL-60 cells were passed twice weekly in fresh medium and maintained at 37°C in a humidified 5% CO2 atmosphere. Experiments were done in the same medium, temperature, atmosphere, and with the same supplements (unless otherwise stated) as those used in the cell cultures. Cells were harvested by centrifugation at 800 × g at 5°C and incubated in fresh medium 1 h at 37°C before the drugs were added. Cell counts were performed in a hemacytometer.

Drug Effects on Total Cell Number and Incorporation of [3H]Thymidine by Cells. On Day 0, multiple cultures of HL-60 cells were initiated at 2 × 104 cells/ml, and some were treated with AmB. CCNU or Act D was also added on Day 0 (continuous treatment) or as a 4-h pulse (pulse treatment) on each day of the experiment. Controls using each drug alone were also done. The drug effects were determined on each of the 3 subsequent days of counting cells in a hemacytometer or by exposing cells to [3H]thymidine for 2 h, starting 2 h after the addition of the drug in the pulse treatments. To measure incorporation of [3H]-thymidine into cellular DNA, 1 µCi of [3H]thymidine was added to 1 ml of the cell cultures. After incubation, cells were centrifuged, rinsed with PBS, and precipitated with 5% trichloroacetic acid. After 1 h at 4°C, the cellular precipitates were collected on glass microfiber filters (Whatman, Ltd., Maidstone, England). The filters were washed twice with 5% trichloroacetic acid, then with ethanol, and then dried in an oven at 65°C; radioactivity was measured in a liquid scintillation counter.

Cell Viability, Lysis (Loss of LDH), and Loss of K+. Cells were harvested and inoculated (1.6 × 106 cells/ml) in medium with 10% FBS, and AmB in varying concentrations was added to the samples. In some experiments, cell cultures were treated for 1 h with CCNU or Act D before the addition of AmB. After 1 h of incubation with AmB, portions of the cultures were used for clonogenic assays done as described by Doer and Koefler (7). The cloning efficiency of the cells was 20%. The highest amount of AmB transferred with the cells was 60 ng per plate, and this amount did not have any effect on the clonogenic assay.

LDH leakage from cells into the medium was measured after cen-
trifugation according to the procedure described by Sigma, St. Louis, MO (Bulletin No. 500). Total leakage was determined after cell lysis with 0.2% Triton, and nonspecific leakage measured in supernatants of untreated cells was subtracted from total and drug-induced leakage values. The concentration of LDH remaining in treated cells was calculated from these values as the percentage of the concentration found in untreated cells.

For measurements of intracellular K+ cells, were harvested by centrifugation, rinsed twice with PBS, boiled in 15 mm LiNO3 (lithium internal standard; Fisher Scientific Co., Pittsburgh, PA), and centrifuged at 2000 x g for 10 min at 4°C. Concentrations of K+ in the supernatants were measured in a flame photometer (Model 430; Cornning Instruments, Bedford, MA).

Uptake of Radioactive [3H]Act D into Cells. Because of the low specific activity of [3H]Act D and the high level of sensitivity of the HL-60 cells to this drug, the following procedure was used to measure uptake on consecutive days. Cells were harvested and inoculated in fresh medium on Day 0 with or without AmB. In experiments described in the text, the initial inoculum was 2 x 105 cells/ml; in the experiments presented in Table 1, the initial inoculation was 2 x 106 cells/ml. After 4 h, 1 or 2 days of incubation, 10-ml cultures were centrifuged; pellets were resuspended in 1 ml of fresh medium and incubated for 1 h at 37°C. [3H]Act D was then added to the cell dispersions, and cultures were incubated 4 h. Cells were harvested by centrifugation, washed twice with PBS, and filtered on glass fiber filters. The cells on the filters were then rinsed twice with PBS and dried, and the radioactivity was determined in a liquid scintillation counter.

MDA. The thiobarbituric acid test was performed according to a procedure of Buege and Aust (8). Cells (2 x 106 per ml of PBS) were incubated at 37°C for 1 h with AmB before addition of CCNU or Act D; incubations were continued for another 2 h. After heating with thiobarbituric acid under acidic conditions, the formation of a pink chromophore was measured spectrophotometrically (Model 260; Gilford, Oberlin, OH) and calibrated with MDA using an extinction coefficient of 1.56 x 10^5 M^-1 cm^-1 at 532 nm (9).

Glutathione. Total glutathione (glutathione plus glutathione disulfide) was measured by the glutathione reductase procedure according to Akerboom and Sies (10). Control glutathione values ranged from 0.9 to 1.5 μg/8 x 105 cells.

Experimental Data Analysis. To determine the type of interaction between AmB and Act D or CCNU (additive or synergistic) in inducing a decrease in cell number or in uptake of [3H]thymidine into cell cultures, we used the analysis of King et al. (11). The values obtained with each single drug were calculated as the percentage of the control values found in untreated cells. An additive effect was defined as the product of the percentage observed for each drug alone. If the observed effect was greater than the calculated additive value, we concluded that a synergistic interaction between the drugs was present. Results were compared by Student's t tests (12).

To determine the type of CCNU-AmB interaction inducing cell lysis, we constructed an isobologram according to the method of Berenbaum et al. (13).

RESULTS

Drug Effects on Incorporation of [3H]Thymidine into HL-60 Cells and on Total Cell Number. When the cells were exposed to both AmB and Act D continuously starting on Day 0, the effect on the decrease in [3H]thymidine incorporation was greater than that caused by either drug alone, and this difference increased with time of exposure (Fig. 1). On Day 1 the effect of this combination was additive; on Days 2 and 3 it was synergistic because the levels of [3H]thymidine incorporation were 40% (Day 2) and 80% (Day 3) lower than the calculated additive effect (11). Comparable results were obtained when we used total cell number as the index of drug effects (data not shown).

In cultures treated with AmB starting on Day 0 and pulsed on each of 3 subsequent days for 4 h with Act D, both indicators (cell number and [3H]thymidine uptake) showed a small synergistic effect of the drug combinations. The observed values were 20 to 40% lower than the calculated additive value, but these responses were highly variable and occurred at different points in time (data not shown).

When cells treated with AmB on Day 0 were pulsed for 4 h on subsequent days with CCNU, synergy was observed consistently. Incorporation of [3H]thymidine into DNA was 60 and 40% lower than the calculated additive values on Days 1 and 2 (Fig. 2). The decreases in cell number in the cultures exposed to AmB (5.0 μg/ml) and pulsed with CCNU (5.0 μg/ml) were 32% (Day 1) and 50% (Day 2) greater than the values calculated for an additive effect, confirming that synergy was present (data not shown). However, when the cells were exposed con-

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**Table 1. Effect of AmB on uptake of [3H]Act D by HL-60 cells**

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<th>[3H]Act D Incorporation with the following additions of [3H]Act D (μg/ml)</th>
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<tr>
<td>No AmB</td>
<td>3,511 ± 500*&lt;br&gt;6,432 ± 1,010&lt;br&gt;12,925 ± 2,450</td>
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<tr>
<td>AmB (10.0 μg/ml)</td>
<td>6,559 ± 1,010&lt;sup&gt;a&lt;/sup&gt; 13,230 ± 1,500&lt;sup&gt;c&lt;/sup&gt; 20,619 ± 3,000&lt;sup&gt;b&lt;/sup&gt;</td>
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* Results, expressed in cpm, are the mean ± SE for 6 independent experiments.
<sup>a</sup> P < 0.001 increase from the control values.
<sup>b</sup> Maximal synergistic effect (P < 0.005) in comparison to two other combinations.
continuously to the same concentrations of both drugs as those used in the pulse experiments, we observed synergistic effects only on Day 0, and these responses were variable.

Therefore synergism occurred most prominently after continuous exposure of the HL-60 cells to the combination of AmB and Act D and after pulse treatment by CCNU of cells grown in the presence of AmB.

Drug Effects on Formation of Colonies and Loss of LDH and K* by HL-60 Cells. Fig. 3 shows the AmB effects on intracellular levels of K*, LDH, and the number of colony-forming cells determined by a clonogenic assay. The decrease in the ability of the cells to form colonies (measurement of lethal effects) and to retain LDH (measurements of lytic effects) occurred at similar AmB concentrations. The levels of cell-associated K* were measured after the cells were rinsed with potassium-free buffer (PBS) and therefore reflected AmB-induced changes in cell membrane permeability and not K* levels during the incubation in medium. These changes in cell membrane permeability occurred at much lower AmB concentrations than the lethal and lytic effects. Cells incubated with 2.5 µg of AmB per ml retained 60% of control levels of K*; at 10.0 µg of AmB per ml, only about 20% of the K* remained associated with cells. In contrast, no significant decrease in either number of colony-forming cells or LDH content was seen with AmB concentrations below 10.0 µg/ml, and a 50% decrease in both indices was observed at AmB concentrations of 30.0 µg/ml (colony formation) or 60.0 µg/ml (retention of LDH). Because the dose responses of the clonogenic assays and lytic effects to AmB were similar, we used only lysis as an index of cell damage in the subsequent studies.

Treatment of cells with Act D or CCNU did not affect cell susceptibility to AmB-induced K* losses. Act D, assayed at a broad range of concentrations up to 80.0 µg/ml, did not affect the lytic action of AmB. In contrast, cells treated with CCNU and then exposed to AmB lysed to a greater extent than cells treated with AmB alone or cells incubated with CCNU in the absence of AmB. In a series of dose-response experiments, concentrations of single drugs causing lysis of 50% of the cells were determined (50.0 µg of AmB per ml and 140.0 µg of CCNU per ml) as well as concentrations of drug combinations causing the same effects. On the basis of these data, an isobologram was constructed (Fig. 4) in which doses of single drugs were treated as total doses, and doses of each drug in combination were expressed as fractions of these total doses. The line connecting the points for total doses and doses in the combinations is concave, positioned markedly below the line connecting the two total doses, which represents an additive effect.

The interaction between these drugs causing lysis of HL-60 cells is therefore synergistic (13).

Effect of AmB on Uptake of [3H]Act D or [14C]CCNU by HL-60 Cells. In the experiments with [3H]Act D, the cell suspensions which had been cultured with 10.0 µg of AmB per ml were concentrated 10-fold in fresh medium (see "Materials and Methods") and then exposed to [3H]Act D. Measurements of uptake of radioactive drug were then carried out. On Days 0, 1, and 2, the increases in the level of [3H]Act D taken up by cells exposed to AmB compared to control cells were, respectively, 20%, 40%, and 18% for 0.05 µg of [3H]Act D per ml and 42%, 58%, and 14% for 0.10 µg of [3H]Act D per ml. To determine if the differences in uptake were statistically significant, we chose Day 1 for repetitive analysis. The results are summarized in Table 1. All the differences observed between AmB-treated and the control cells were significant, and the increase in uptake induced by AmB was maximal for 0.1 µg of Act D per ml.

The uptake of [14C]CCNU by AmB-untreated cells was low (0.1% of the amount of drug present in the assay), which was in accordance with the previously reported data on the uptake of radioactive CCNU into L1210 leukemia cells (14). No increase in uptake was seen in the presence of AmB.

Involvement of Oxidative Damage in AmB-CCNU Cytotoxicity. We have recently shown that oxidative damage is involved in the antitumor action of AmB (15, 16). We reasoned that enhancement of oxidative injury may be the basis of the potentiation of the lytic effects of AmB by CCNU.

The involvement of oxidative damage in the action of the AmB-CCNU combination was suggested by the observation that addition of catalase to the medium inhibited the lysis of cells. In three experiments done in duplicate, cells exposed to 20.0 µg of CCNU per ml and 10.0 µg of AmB per ml retained 20 ± 12.0% of LDH without the presence of catalase and 78 ± 8% in the presence of 200 units of catalase per ml. This action of catalase was not due to nonspecific binding of AmB to protein, because no decrease in lysis was seen when equivalent concentrations of bovine serum albumin were added to the cultures.

A further indication that oxidative damage was involved in the cellular effects of AmB was an increase in MDA formation in HL-60 cell-AmB mixtures. Cells were incubated with several different concentrations of AmB for 1 h, and CCNU (12.5 µg/ml to 100.0 µg/ml) or Act D (0.20 µg/ml) was added to some cultures for an additional 1 h. In the cultures with increasing concentrations of AmB, proportional increases in levels of
MDA were found. Neither Act D nor CCNU caused an increase in MDA production without AmB. Fig. 5 shows that, when AmB was combined with Act D, no further increase in MDA production over that seen with AmB alone could be detected, whereas in the presence of 100.0 μg of CCNU per ml, production of MDA in mixtures of cells with 100 μg of AmB per ml increased 2-fold. Addition to cell-AmB dispersions of lower concentrations of CCNU (12.5 μg/ml, 25.0 μg/ml, and 50.0 μg/ml) resulted in proportionally lower increases in MDA production (18%, 40%, and 80%, respectively) over values found in the absence of CCNU.

The increase in MDA production caused by the CCNU-AmB combination was proportional to the increase in cell lysis, measured as a decrease in total cell number (Fig. 5, inset). Both MDA production and cell lysis were decreased by half when 500 units of catalase per ml were added to the incubation mixtures (data not shown), further suggesting that these effects were related.

Incubation of HL-60 cells with CCNU at nonlytic concentrations resulted in a dose-dependent depletion of glutathione (Fig. 6). The comparison of data presented in Fig. 4 with these in Fig. 6 shows that lysis of 50% HL-60 cells incubated with 40.0 μg of CCNU per ml (which retained 25% of glutathione level) occurred during treatment with 5.0 μg of AmB per ml, whereas in order to obtain the same extent of lysis in cells incubated with 20.0 of CCNU per ml (which retained 40% of their glutathione) AmB had to be used in a concentration of 10.0 μg/ml. The increased sensitivity to AmB-induced lysis of CCNU-treated cells may therefore be secondary to the CCNU-induced cell depletion of glutathione.

DISCUSSION

When we measured drug effects on incorporation of [3H]-thymidine or on total cell number, both AmB-Act D and AmB-CCNU drug combinations acted synergistically in inducing toxicity to HL-60 cells. However, the experimental conditions necessary to demonstrate optimal synergism were different for each drug combination. Synergism of the AmB-Act D combination was most pronounced in cultures exposed to both drugs continuously. In these conditions, the toxicity of Act D alone increased gradually, and this increase was significantly enhanced in the presence of AmB. In contrast, synergism of the AmB-CCNU drug combination was seen under conditions of pulse treatment with CCNU. When we measured cell lysis (leakage of LDH) we found that the AmB-CCNU combination was synergistic, whereas no synergism was evident in cultures exposed to the AmB-Act D drug combinations.

The potentiation of the toxic effects of Act D by AmB was correlated with an enhancement of uptake of [3H]Act D into the cells. Therefore, we concluded that the enhancement of uptake of Act D was probably the cause of the synergism. This is in agreement with previous results from our laboratory in which HeLa cells resistant to Act D were made sensitive and increased their uptake of [3H]Act D after exposure to AmB (6).

In the case of CCNU, no enhancement of uptake of [14C]-CCNU into cells by AmB could be demonstrated. Since we have shown in our laboratory that the lytic action of AmB on RBC (15) and the killing effects on Candida albicans (16) involved oxidative damage to cells, we assumed that the synergism of AmB and CCNU, two compounds that decompose with the formation of radicals (17, 18), might also be based on this mechanism. In support of our hypothesis, the addition of catalase, a well-known scavenger of H2O2, to the incubation mixture inhibited cell lysis induced by the CCNU-AmB combination. In addition, CCNU induced increased production in the cell-AmB mixture of MDA, a product of lipid peroxidation and also of oxidative decomposition of polyene antibiotics (19, 20).

It is important to note that we have not excluded the possibility that AmB-induced cell permeability is involved in the anticlellular action of the CCNU-AmB combination, because the increased uptake of [14C]CCNU or a product of its decomposition might have been below the sensitivity of our assay. What we have documented, however, is that oxidative cell damage is at least a partial explanation of the observed synergism.

We next looked for cellular events based on an oxidative mechanism that might be responsible for synergistic interactions of the AmB-CCNU combination. We considered the known effect of chloroethyl nitrosoureas on the level of cellular glutathione (21), because glutathione has been shown to play a critical role in cellular defense against a variety of injurious agents (22). One example of this is the sensitization of tumor cells to oxidative cytolysis (23, 24). Another example is the formation of blebs in a human cell line treated with 2-chloroethyl nitrosourea, an effect possibly linked to changes in cellular
thiols (25). When we compared the effects of CCNU on the level of glutathione in HL-60 cells and sensitization of the cells to AmB-induced lysis, we found that both actions occurred in parallel. Thus, we assume that the depletion of cellular glutathione by CCNU or products of its decomposition made cells more vulnerable to the oxidative action of AmB.

Previously we could not find evidence for an oxidative mechanism in the AmB-induced increase in permeability of RBC (15) or C. albicans cells (16) to K⁺. In this study we did not observe any effect of CCNU on AmB-induced K⁺ leakage. These observations agree with the assumption that the lytic and permeabilizing actions of AmB on cells may have separate mechanisms (24).

The notion, that synergistic effects of AmB-drug combinations against tumor cells may occur through two different mechanisms, is in line with previous findings on the variable levels of potentiation of different antitumor agents observed on in vivo murine cancer models (2, 26). It will be important to test the effects of AmB in combination with other agents which are known to act through an oxidative mechanism, such as radiation sensitizers and prooxidants. These combinations might result in a marked increase in cytotoxicity and broaden the spectrum of drugs which could be used with AmB to kill tumor cells.

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REFERENCES


