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Glucocorticoids and Polyamine Inhibitors Synergize to Kill Human Leukemic CEM Cells[1]

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Abstract

Glucocorticoids are well-known apoptotic agents in certain classes of lymphoid cell malignancies. Reduction of intracellular polyamine levels by use of inhibitors that block polyamine synthesis slows or inhibits growth of many cells *in vitro*. Several such inhibitors have shown efficacy in clinical trials, though the toxicity of some compounds has limited their usefulness. We have tested the effects of combinations of the glucocorticoid dexamethasone (Dex) and two polyamine inhibitors, difluoromethylornithine (DFMO) and methyl glyoxal bis guanylhydrazone (MGBG), on the clonal line of human acute lymphoblastic leukemia cells, CEM-C7-14. Dex alone kills these cells, though only after a delay of at least 24 hours. We also evaluated a partially glucocorticoid-resistant c-Myc–expressing CEM-C7-14 clone. We show that Dex downregulates ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis. Pretreatment with the ODC inhibitor DFMO, followed by addition of Dex, enhances steroid-evoked kill slightly. The combination of pretreatment with sublethal concentrations of both DFMO and the inhibitor of *S*-adenosylmethionine decarboxylase, MGBG, followed by addition of Dex, results in strong synergistic cell kill. Both the rapidity and extent of cell kill are enhanced compared to the effects of Dex alone. These results suggest that use of such combinations *in vivo* may result in apoptosis of malignant cells with lower overall toxicity.

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Keywords: apoptosis, glucocorticoids, inhibitors, polyamines, leukemia.

Introduction

The three physiological polyamines — putrescine, spermidine, and spermine — are all positively charged polycations at cellular pH and presumably function as stabilizing agents of RNA, DNA, or negatively charged proteins. Cells regulate polyamine pool sizes through synthesis, active transport, degradation, and interconversions [1,2]. The active transport system permits exogenous polyamines to be recruited as needed by the cell [3]. Polyamines are required for cells to cycle, and these complicated, tightly regulated mechanisms allow cells to adjust polyamine levels constantly as growth dictates [4]. The rate-limiting enzyme in the polyamine synthetic pathway is ornithine decarboxylase (ODC), which is responsible for putrescine synthesis. Spermidine and spermine are produced from putrescine by the actions of *S*-adenosylmethionine decarboxylase (AdoMetDC) and spermidine/spermine synthase. ODC activity is required for cells to proceed into S-phase of the cell cycle [5] and is under multilevel, strict regulation [6,7]. AdoMetDC contributes to the production of spermidine and spermine by facilitating the formation of decarboxylated *S*-adenosylmethionine, the amino propyl donor for spermidine and spermine synthases. Methyl glyoxal bis guanylhydrazone (MGBG), a structural analog of spermidine, is a potent reversible inhibitor of AdoMetDC. Alone, MGBG effectively lowers spermidine and spermine levels while causing an accumulation of putrescine by several mechanisms [8].

Because of their importance in many cellular functions, polyamines have long been the focus of research as potential chemotherapeutic agents [8]. Combinations of difluoromethylornithine (DFMO) and MGBG have proven to be more effective at halting cell growth than either drug alone. Synergism between the two has been reported [8-10,11-13] when cells were incubated for a period of time with combinations of the inhibitors which allow polyamine pools to drop. Although *in vivo* outcomes with the combination have sometimes shown heightened toxicity [14], several studies have found the polyamine inhibitor drug combination effective in treatment of cancers in mice and humans [9-11,12,13].

The ability of glucocorticoids to affect cell growth has generated much interest to cancer investigators over the years [15-17,18]. We have shown that transcriptional downregulation of the proto-oncogene c-*myc* is a critical signal in the pathway of the glucocorticoid-evoked apoptosis of CEM cells [19,20]. The *odc* gene contains E box binding

Abbreviations: AdoMetDC, *S*-adenosylmethionine decarboxylase; Dex, dexamethasone; DFMO, difluoromethylornithine; FBS, fetal bovine serum; MGBG, methyl glyoxal bis guanylhydrazone; ODC, ornithine decarboxylase; PBS, isotonic phosphate-buffered saline pH 7.4; PI, propidium iodide.

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sites for c-Myc and is a c-Myc transcriptional target [5,21]. We demonstrate here that c-Myc levels are suppressed by glucocorticoids, as well as cellular *odc* mRNA, protein levels, and ODC activity. Historically, it has been shown that direct inhibition of ODC through the use of DFMO, a specific, irreversible inhibitor of the enzyme, slows cell growth [22]. In several systems, DFMO reduces putrescine and spermidine levels dramatically through irreversible inhibition of ODC; however, spermine levels show an increase. Spermine has been implicated as a possible salvage polyamine, convertible to the precursor polyamines as the need arises [8].

There has been only limited data published on the combination of these inhibitors with glucocorticoids in malignant cells [23]. This study has centered on the ability of polyamine inhibitors to influence glucocorticoid-induced apoptosis in human lymphoblastic leukemic CEM cells. We report here that the synthetic glucocorticoid dexamethasone (Dex) causes a reduction in ODC activity, mRNA, and protein in CEM cells. Treatment of these cells with a combination of low nonlethal concentrations of DFMO and MGBG, followed by Dex, speeds the onset of apoptosis and increases its extent.

Materials and Methods

Cell Culture

The human CCRF-CEM cell line was grown from a patient with acute lymphoblastic leukemia [24]. The glucocorticoid-sensitive CEM-C7-14 clone was subcloned from the original, sensitive clone CEM-C7 [25]. To obtain c-Myc–expressing cells, CEM-C7-14 cells were transfected with pBpuroMycER, which contains the human c-Myc protein fused to the modified ligand-binding domain of the murine estrogen receptor α [26]. Selection in puromycin gave rise to a mass culture of cells partially resistant to Dex; clone CEM-MycER-22 was chosen for use herein. All cells were grown in RPMI 1640 medium (Cellgro; Media Tech, Herndon, VA), pH 7.4, with 5% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Subculturing at regular intervals insured logarithmic growth. Viable cell numbers were determined by Trypan blue dye (Sigma-Aldrich, St. Louis, MO) exclusion using a hemacytometer, averaging triplicate counts at each data point. All experiments were initiated at 1×10^5 cells/ml. All reagents, unless otherwise identified, were from Sigma-Aldrich.

Extensive preliminary experiments (not shown) indicated that addition of the polyamine pathway inhibitors simultaneously with Dex was less effective than pretreatment with DFMO (Merrell Dow Pharmaceuticals, Cincinnati, OH) and MGBG followed by Dex. To find optimum concentrations of the polyamine pathway inhibitors, DFMO was tested between 0.1 and 1.0 mM, and MGBG between 50 nM and 25 μM. The most effective and least toxic concentrations proved to be 0.5 mM DFMO and 0.5 μM MGBG dissolved in isotonic phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}, pH 7.4. For all the experiments herein, we employed the following standard protocol, unless noted otherwise. Cells were pretreated for 24 hours with both inhibitors before Dex was added to a final concentration of 10^{-7} M and $\leq$0.1% ethanol. Control cultures all received ethanol and PBS equivalent to the volume used to introduce DFMO, MGBG, and Dex.

ODC Activity

A total of 5×10^6 cells were collected by centrifugation at 250*g* for 10 minutes at 22°C. The pellet was washed once by resuspension in 22°C PBS and repelleted. The cells were suspended in 500 μl of lysis cocktail [0.05 M sodium–potassium phosphate buffer, pH 7.2, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.4 mM pyridoxal phosphate (Calbiochem, San Diego, CA)]. After brief probe sonication, to disrupt cell membranes, samples were distributed in 200-μl aliquots to glass tubes fitted with stoppers from which filter papers saturated with 1 M hyamine hydroxide were suspended. An equivalent volume of lysis cocktail was used as a reagent blank. A 20-μl aliquot from a stock solution containing 31 μCi/ml of L-^{14}C-labeled ornithine (Amersham Pharmacia Biotech, Piscataway, NJ) and 5 mM unlabeled ornithine in deionized water was added to each tube. After incubating at 37°C for 60 minutes with agitation, a 500-μl aliquot of 1 M citric acid was added to stop the reaction. The tube was further incubated at 22°C for 90 minutes, and radioactivity on the filters was estimated in an organic scintillation cocktail and counted on a LS5801 liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

Isolation and Analysis of RNA

Total cellular RNA was isolated from cell pellets of approximately 8×10^7 cells, washed as above with sterile, 4°C PBS. To each pellet, 3 ml of TRIzol reagent (Gibco/BRL, Grand Island, NY) was added, and after mixing, the sample was processed according to the manufacturer's instructions, with minor modifications. The resulting RNA was dissolved in RNAse-free water. The RNA (20 μg/lane) was separated in agarose–formaldehyde gels, transferred to a PROTRAN nitrocellulose membrane (Intermountain Scientific, Kaysville, UT), and hybridized to a ^{32}P-labeled (ICN, Costa Mesa, CA) *odc* DNA fragment in QuikHyb hybridization solution (Stratagene, La Jolla, CA). The gel was dried and exposed to a Phosphorimager screen (Molecular Dynamics, Sunnydale, CA) and the densities of the *odc* mRNA bands quantified relative to the 18*S* and 28*S* ribosomal RNA bands using Image Quant software (version 3.3) from Molecular Dynamics.

Immunoblot Analysis

From cultures initially in log growth, 1×10^7 CEM-C7-14 cells were harvested by centrifugation at 250*g* for 10 minutes, 4°C, at 0, 24, and 48 hours after treatment with 1 μM Dex. The cells were resuspended in 4°C PBS, repelleted, and taken up in lysis buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 20 μM leupeptin, and 400 μM 4-[2-aminoethy]benzensulfonyl fluoride. The cellular lysates were centrifuged at 30,000 rpm

for 30 minutes at 4°C using a Beckman TL-100 ultracen trifuge (Beckman Instruments). The supernatants were removed to fresh 1.5-ml microcentrifuge tubes and a Bio-Rad (Hercules, CA) protein assay kit was used to estimate the protein concentration of the extract. Before electrophoresis, 2-mercaptoethanol (Bio-Rad) was added to all samples to a final concentration of 5%. Samples of the whole cell extracts containing 50 μg of protein per lane were electrophoresed in 10% polyacrylamide minigels (Bio-Rad) containing SDS and transferred to nylon membranes (Bio-Rad) using a semidry electroblotter (Integrated Separation Systems, Hyde Park, MA). After incubation in 5% nonfat dry milk powder in PBS for 2 hours at 22°C, the membranes were incubated with gentle rocking for 16 hours at 4°C in a solution of 5% powdered nonfat milk in PBS plus a monoclonal ODC antibody derived from ODC-29 hybridoma (Sigma-Aldrich). After six 10-minute washes with PBS at 22°C, the membranes were incubated for 2 hours at 22°C with a horseradish peroxidase goat/anti–mouse secondary antibody (Bio-Rad). After washing the membrane as above, it was saturated with the horseradish peroxide substrate ECL (Amersham Pharmacia Biotech) and exposed to photographic film (Eastman Kodak, Rochester, MY) using various times of exposure to assure that evaluations for quantification of the signals were within the linear response range. The densitometric analysis of ODC protein was performed using an Image Analyzer (Applied Imaging, Santa Clara, CA).

Determination of Putrescine, Spermidine, and Spermine Concentration by HPLC

The HPLC procedure was modified from those of Merali and Clarkson [27] and of Weiss et al. [28]. Briefly, standards were dissolved in 0.2 M borate buffer, pH 8.8 (0.2 M sodium hydroxide, 0.2 M boric acid, and 1 mM EDTA), at a concentration of 1×10^{-5} M, fluorescence tagged with AccQFluor reagent (Waters, Milford, MA), and fractionated on a C_8 Microsorb-MV (150×4.6 mm id) column with a 100-Å pore size (Varian Analytical Instruments, Walnut Creek, CA). The fractions were evaluated on a Hewlett Packard HPLC, Series II 1090, AminoQuant with a programmable fluorescence detector, 1046A using ChemStation software, Dos series (Hewlett Packard, Palo Alto, CA). Unknowns were quantified by comparison to known standards, and experimental repeatability was determined after five injec-

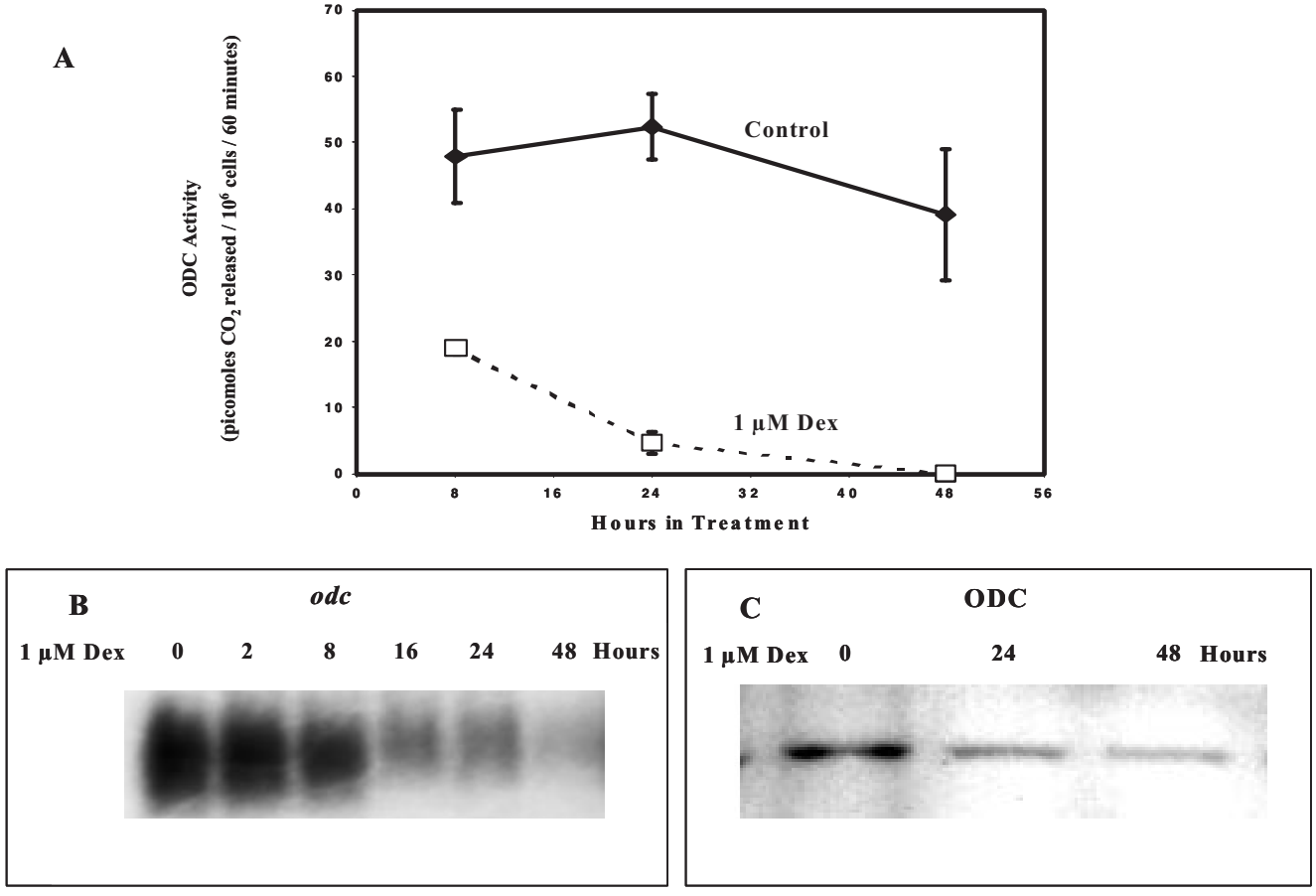


Figure 1. *Dex regulation of ornithine decarboxylase. Panel A: ODC activity. CEM-C7 cells were cultured in RPMI 1640 with 5% FBS, plus 0.1% ethanol only — Control cells (◆) — or with 1 μM Dex delivered in ethanol (□) for 48 hours. ODC activity was determined at various times. The error bars show standard deviations, n=3. Where no bars are seen, the standard deviation fell within the dimension of the symbol. Panel B: Northern blot showing specific odc mRNA bands. There were no significant variations in the corresponding 18S and 28S ribosomal RNA bands on the same filter. Panel C: Immunoblots showing the reaction with a monoclonal antibody to ODC in CEM-C7-14 cell lysates from cells cultured in RPMI 1640 with 5% FBS and treated for 0, 24, and 48 hours with 1 μM Dex. Data shown in panels B and C were reproduced in several experiments.*

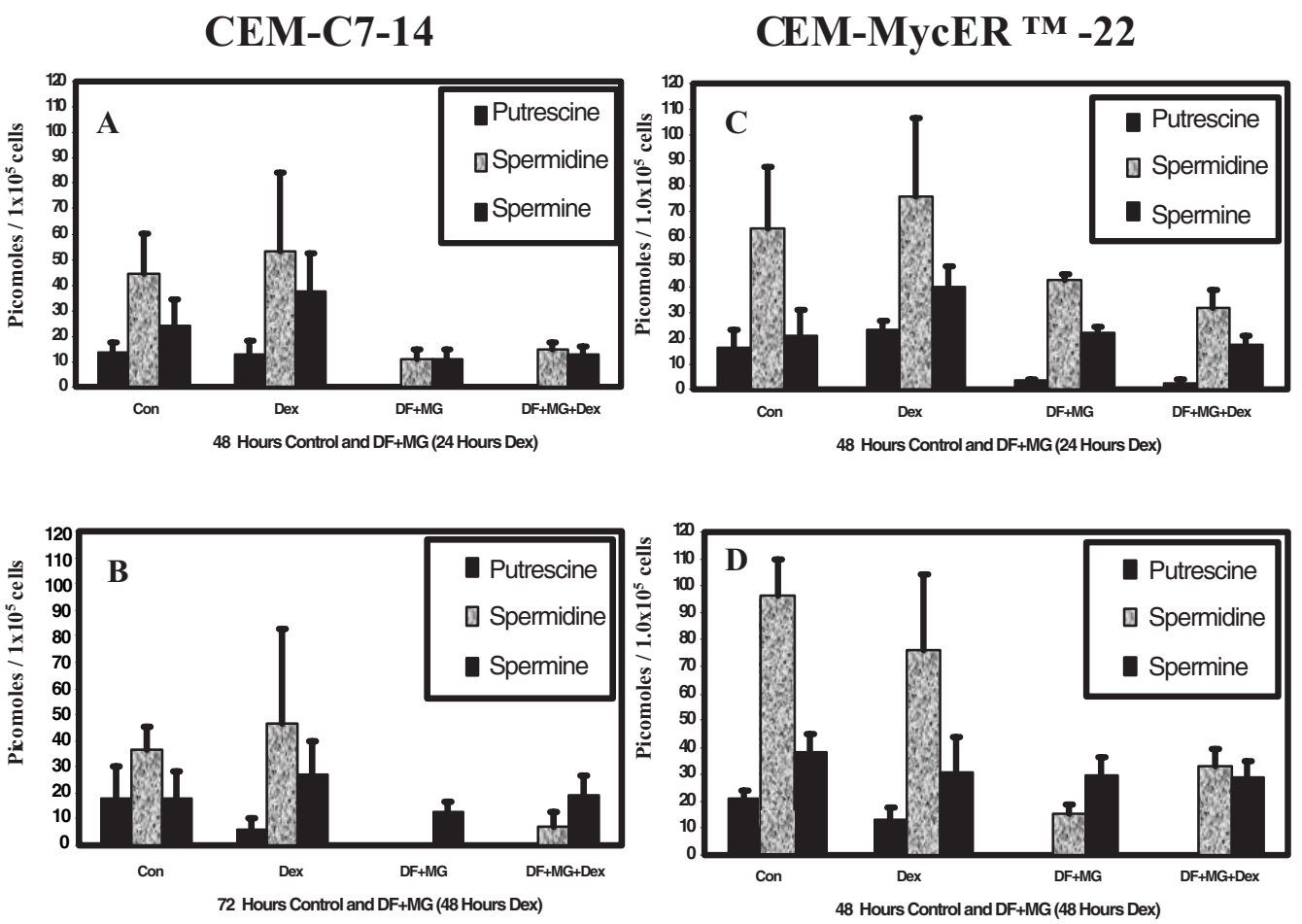


Figure 2. *Polyamine levels after treatment with polyamine inhibitors, Dex alone, or both in combination. CEM-C7-14 (A,B) and CEM-MycER-22 (C,D) cells were cultured in RPMI 1640 plus 5% FBS with 100 nM Dex for 48 hours (A,C) and 72 hours (B,D). As a control, the cultured cells were treated with vehicle only. In each Dex plus inhibitor-treated sample, 0.5 mM DFMO and 0.5 μM MGBG were added 24 hours before Dex. Cell lysates of 1.0× 10^5 cells were evaluated by HPLC and normalized with the internal standard 1,7-diaminoheptane. In each experiment, each time point was evaluated with a minimum of duplicate independent samples, n=4–6. Statistical analyses were done using a t-test, and P values of less than .05 were considered to be significant.*

tions. To prepare cell lysates, 6.4×10^6 cells were pelleted at 250*g* for 10 minutes, 4°C, resuspended once in 4°C PBS, repelleted, and suspended in 100 μl of 50 mM borate buffer, pH 7.4 (50 mM sodium hydroxide, 50 mM boric acid, and 1 mM EDTA). The samples were lysed by three freeze–thaw cycles of −80°C and then 37°C, and debris was pelleted at 12,000 rpm for 10 minutes at 4°C in a Beckman Microfuge Lite centrifuge. Protein was removed by precipitation with trichloroacetic acid and centrifugation. The clarified samples were then filtered through a 0.22-μm syringe filter (Millipore, Bedford, MA). For analysis, HPLC sample tubes (Supelco, Bellefonte, PA) were set up with a mixture of 30 μl of 0.2 M borate buffer, pH 9.3, and 10 μl of 0.2 M borate buffer, pH 8.8. A 10-μl aliquot of the internal standard (1×10^{-5} M 1,7-diaminoheptane) and a 10-μl aliquot of experimental cell lysate were added, followed by a 20-μl aliquot of AccQFluor reagent. The samples were incubated at 55°C for 10 minutes before HPLC analysis. A sample volume of 20 μl was injected onto the column, and the polyamine profile was characterized using a flow rate of 1 ml/min at 37°C, excitation=250 nm and emission=395 nm, eluting with AccQTag Eluent A/acetonitrile (Waters; Burdick and Jackson, Muskegon, MI) as a linear gradient. All samples were analyzed within 24 hours of derivation. Determination of statistical significance was done using a *t*-test, and *P* values of less than .05 were considered significant.

Phosphatidylserine Membrane Eversion

Cell pellets were prepared following the instructions of the Annexin-V-FITC kit from PharMingen (San Diego, CA). A 5-μl aliquot of Annexin-V-FITC and 10 μl of 7-AAD stain were added to 1×10^5 cells and incubated in the dark at 25°C for 15 minutes. A 400-μl aliquot of binding buffer was added and 20,000 cells were analyzed for Annexin-V (yellow/green) and 7-AAD (red) positive cells by flow cytometry using a 488-nm excitation and a 515-nm bandpass filter for fluorescein detection and a filter >600 nm for 7-AAD detection on a FACScan (Becton Dickinson, San Jose, CA). Doublets and cell aggregates were gated out and only the singlet cell population was analyzed.

JC-1 Staining of Depolarized Mitochondrial Membranes

CEM-C7-14 and CEM-MycER-22 cells were stained following the protocol of Bradberry et al. [29] using 5,5′,6,6′-

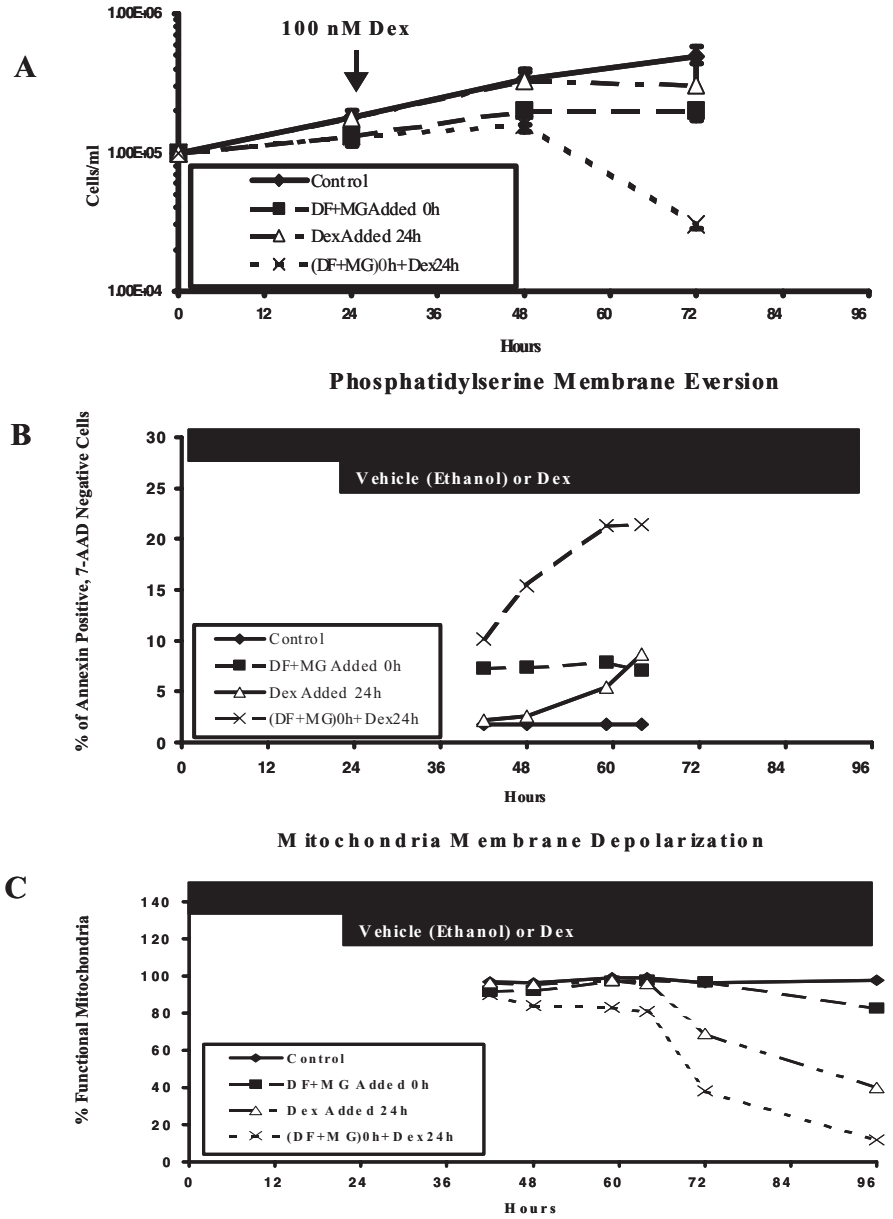
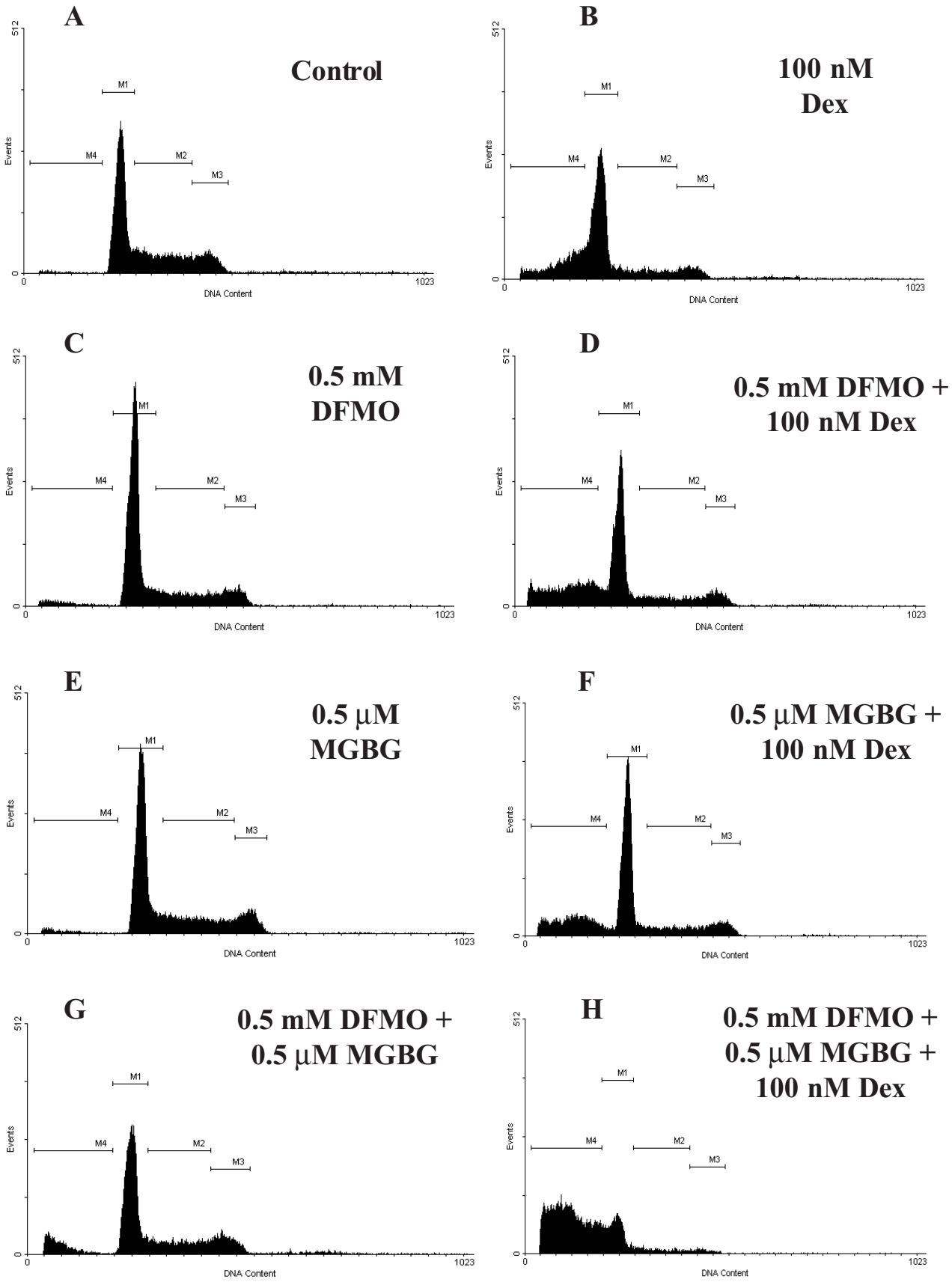


Figure 3. *Apoptotic events after three-drug treatment, cell loss, phosphatidyl serine membrane eversion, and loss of mitochondrial function. CEM-C7-14 cells were treated according to the standard protocol. Panel A: The number of viable cells was determined by counting cells that excluded trypan blue dye exposure to vehicle only (◆), DFMO plus MGBG added at 0 hours (■), Dex added at 24 hours (△), DFMO plus MGBG added at 0 hours plus Dex added at 24 hours (×), n=3. Error bars show 1 SD. Where no bars are seen, 1 SD fell within the symbols. The data in (B) and (C) were obtained from cells treated by the same protocol as in (A), but data collection began at 42 hours, 18 hours after Dex treatment. In panel B after the same protocol, cells were evaluated by Annexin-V-FITC/7-AAD staining at 42, 48, 59, and 64 hours to determine the enversion of the phosphatidylserine portions of the cell membrane. In panel C, cells were evaluated by JC-1 staining at 42, 48, 59, 64, 72, and 96 hours. Data for panels B and C were obtained by FACScan analyses.*

tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) from Molecular Probes (Eugene, OR). Briefly, 1×10^6 cells were suspended in 130 μl, 37°C RPMI 1640 plus 5% FBS. A working solution of 250 μl of JC-1 (1 mg/ml diluted 1/50 in PBS) was added and the cells were incubated at 37°C in an air/CO_2 incubator for 30 minutes. After one 22°C PBS wash, 20,000 cells were analyzed for intact mitochondria by flow cytometry using a FACScan. Green

Figure 4. *Cell cycle distribution and accumulation of subdiploid cells. CEM-C7-14 cells were treated according to the standard protocol and the phases of the cell cycle were determined by FACScan analyses after PI staining of DNA; $M_1=G_1/G_0$ (gap 1, interval between mitosis and DNA replication); $M_2=S$ (synthesis of DNA); $M_3=G_2/M$ (gap 2, preparation for cell division); M_4=subdiploid, apoptotic cells or DNA-containing, countable subcellular particles. (A) Control cells treated with vehicle only; (B) cells incubated with 100 nM Dex for the final 48 hours; (C) cells were cultured for 72 hours with 0.5 mM DFMO; (D) cells were cultured for 72 hours with 0.5 mM DFMO plus 100 nM Dex for the final 48 hours; (E) cells were cultured for 72 hours with 0.5 μM MGBG; (F) cells were cultured for 72 hours with 0.5 μM MGBG plus 100 nM Dex for the final 48 hours; (G) cells were cultured for 72 hours with 0.5 mM DFMO plus 0.5 μM MGBG; and (H) cells were cultured for 72 hours with 0.5 mM DFMO plus 0.5 μM MGBG plus 100 nM Dex for the final 48 hours. All data were collected after 72 hours in culture. The data shown are from one experiment. Reproducible results were obtained for each variable in two to three experiments. The percentages of cells in each compartment are presented in Table 1.*


A
Control
B
100 nM
Dex
C
0.5 mM
DFMO
D
0.5 mM DFMO +
100 nM Dex
E
0.5 μM
MGBG
F
0.5 μM MGBG +
100 nM Dex
G
0.5 mM DFMO +
0.5 μM MGBG
H
0.5 mM DFMO +
0.5 μM MGBG +
100 nM Dex
Events
DNA Content
M1
M2
M3
M4
0
512
1023

cells with impaired depolarized mitochondrial membranes were detected using a 530-nm filter and viable red cells with a 585-nm filter.

Cell Cycle Evaluation by Propidium Iodide (PI) Staining

Cells (1×10^6) were pelleted at 250*g* for 10 minutes at 4°C and resuspended one time in 1 ml of 4°C PBS/0.1% sodium azide solution and transferred to 1.5-ml microtubes. The cells were collected at 250*g* for 5 minutes at 4°C and supernatants removed, and then were suspended in low salt stain [3% polyethylene glycol 8000, 50 μg/ml PI, 4 mM sodium citrate (Mallinckrodt, Hazelwood, MO), plus 0.1% Triton X-100 (Bio-Rad) and 180 U of RNAse A (Worthington Biochemicals, Lakewood, NJ)]. They were then incu-

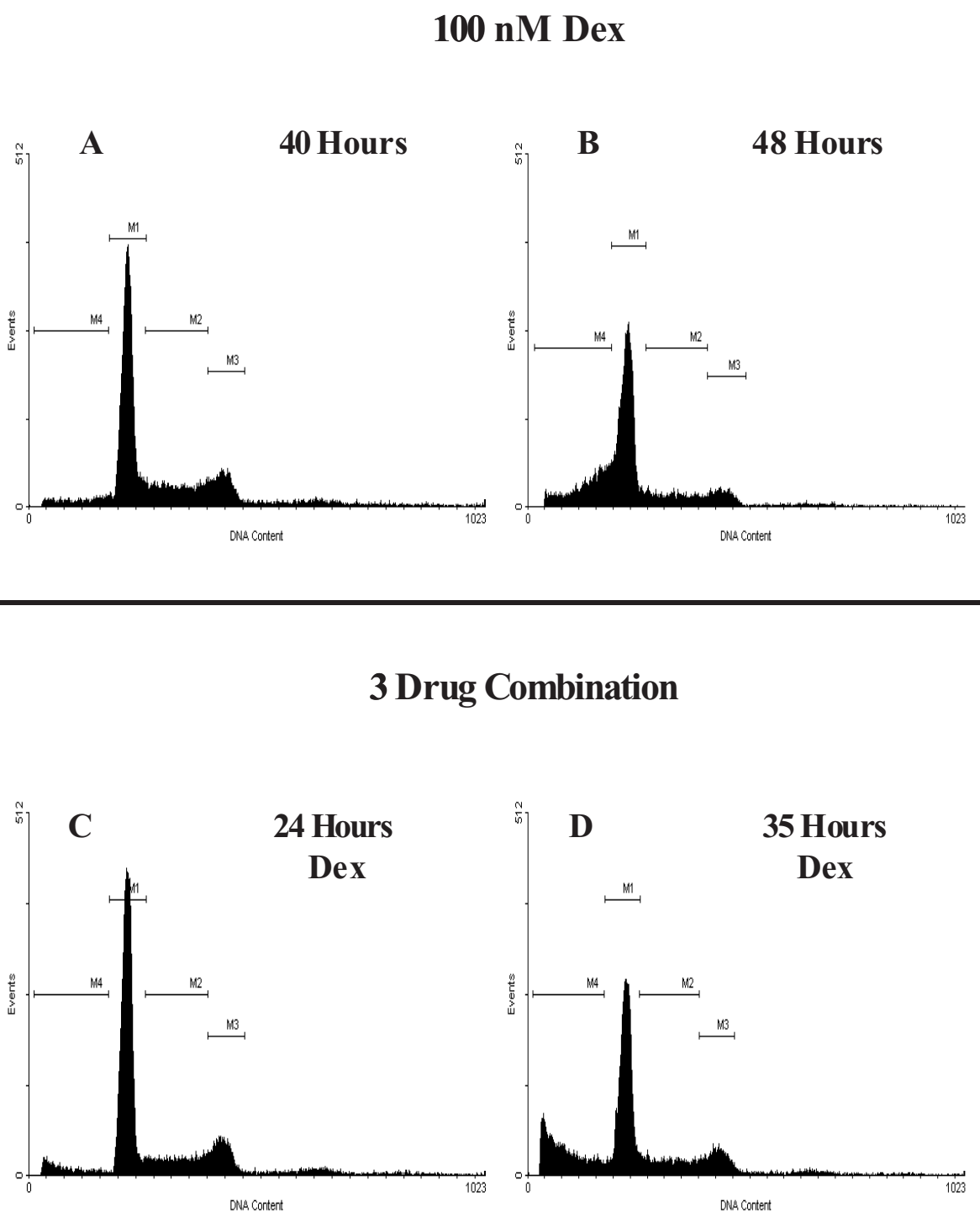


Figure 5. *Acceleration of apoptosis following sequential treatment with DFMO plus MGBG followed by Dex. CEM-C7-14 cells were treated according to the standard protocol and the cellular DNA determined by PI staining and FACScan analyses. (A,B) Forty and 48 hours of 100 nM Dex treatment. (C,D) Twenty-four hours of polyamine pathway inhibitors and 24 or 35 additional hours with 100 nM Dex added. The percentages of cells in each compartment are presented in Table 2.*

bated in a 37°C water bath in the dark for 20 minutes. An equal volume of high salt stain (3% polyethylene glycol 8000, 50 μg/ml PI, 400 mM sodium chloride, 0.1% Triton X-100) was added. The samples were covered and placed at 4°C for at least 1 hour in the dark. Sample lots of 20,000 cells were analyzed for DNA content by flow cytometry. Red (PI) was detected using a 585-nm filter. Cells containing subdiploid DNA were not used for determination of percentages in G_1/G_0, S, or M phase.

Results

Dex Suppresses ODC

In earlier studies, we had demonstrated that by 4 days of exposure, 1 μM Dex substantially kills most CEM-C7 cells [30], with DNA fragmentation appearing between 36 and 48 hours [31]. Due to repression of c-*myc* transcription, levels of c-*myc* mRNA begin to be suppressed as early as 1 hour after addition of Dex [19,20]. We tested the effect of 1 μM Dex alone on ODC levels in CEM-C7 cells over 48 hours. The data of Figure 1, *A* – *C* demonstrate that ODC activity (A), mRNA (B), and protein (C) were all suppressed, whereas ODC activity remained relatively constant throughout the measured time course in control cells (Figure 1*A*). ODC mRNA was reduced by 8 hours and strongly so by 16 hours and beyond (Figure 1*B*). ODC protein levels were clearly reduced as well (Figure 1*C*).

Effects of Dex and Inhibitors of Polyamine Synthesis on Polyamine Levels in CEM Cells

In preliminary control experiments (not shown), we established in detail that the levels of each of the three polyamines assayed at 24-hour intervals remained relatively constant in untreated CEM cells. Over the course of 72 hours, putrescine and spermine fluctuated within the range of 10 to 25 pmol/10^5 cells, spermidine in the range of 35 to 45 pmol/10^5 cells. For example, Figure 2, panels A *and* B) show data for control CEM-C7-14 cells in logarithmic growth at 48 and 72 hours after addition of vehicle to the cultures. Further preliminary experiments following the effects of adding DFMO or MGBG singly revealed that DFMO alone reduced putrescine to undetectable levels by 24 hours and spermidine to undetectable levels by 48 hours (not shown); MGBG alone dramatically elevated putrescine levels by 48 hours with a marked reduction in spermidine and spermine levels (not shown). The combination of both inhibitors, however, effectively lowered the levels of all three polyamines (Figure 2, *A* and *B*). Although this slowed cell growth considerably, it caused little cell death (Figures 3*A* and 4*G*). We therefore tested the inhibitors in combination with Dex. After preliminary studies comparing the effects of adding the inhibitors and Dex simultaneously or sequentially, we chose the following protocol, which was used for the data in Figures 2 – 7). Cultures were divided and treated at time zero with vehicle only (control) or 0.5 mM DFMO plus 0.5 μM MGBG. Twenty-four hours later, subsets of control and inhibitor-treated cells received 100 nM Dex. Samples for polyamine analysis or viability were taken at several times thereafter. Polyamine data from such experiments on CEM-C7-14 cells are shown in Figure 2, *A* and *B*), demonstrating that after 48 hours of Dex treatment alone, putrescine levels were significantly lowered (P=.045), but there was no statistically significant effect on spermidine or spermine concentrations. Adding Dex 24 hours after both inhibitors caused insignificant effects in addition to those caused by DFMO plus MGBG for 48 hours (Figure 2*A*). By 72 hours in the inhibitors plus 48 hours in Dex, putrescine levels remained undetectable; spermidine, however, was elevated well over the undetectable levels found after inhibitors alone for the same total time of 72 hours, whereas spermine was increased but not to a significant extent (Figure 2*B*).

CEM-MycER-22 is a clone of CEM-C7-14 that has been stably transfected with an expression vector for c-*myc*. In these cells, the hybrid MycER protein, which conveys c-Myc functions, is constitutively expressed [32]. These cells exhibit a marked resistance to cell death after 48 hours in 100 nM Dex, compared to the parental CEM-C7-14 clone (Ref. [32], also Figure 6). To test the dependence of polyamine levels on c-Myc in the face of the drug combinations, we carried out the standard protocol on CEM-MycER-22 cells. The putrescine pool was not elevated significantly in the CEM-MycER-22 cells, compared to the CEM-C7-14 cells under the control conditions, but the remaining downstream polyamines were elevated (compare controls, Figure 2, *A* and *B* with *C* and *D*). DFMO and MGBG reduced polyamine levels, but not as dramatically as in the CEM-C7-14 cells. In the CEM-MycER-22 cells, Dex alone for 48 hours lowered putrescine significantly (Figure 2*D*), P=.02 but not the other two polyamines, compared to time-matched controls. Comparison of polyamine levels in CEM-MycER-22 cells exposed to inhibitors only with those exposed to inhibitors plus Dex revealed a significant difference in spermidine levels (Figure 2*D*),

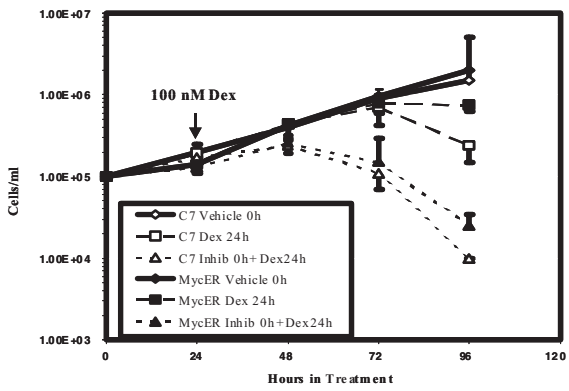
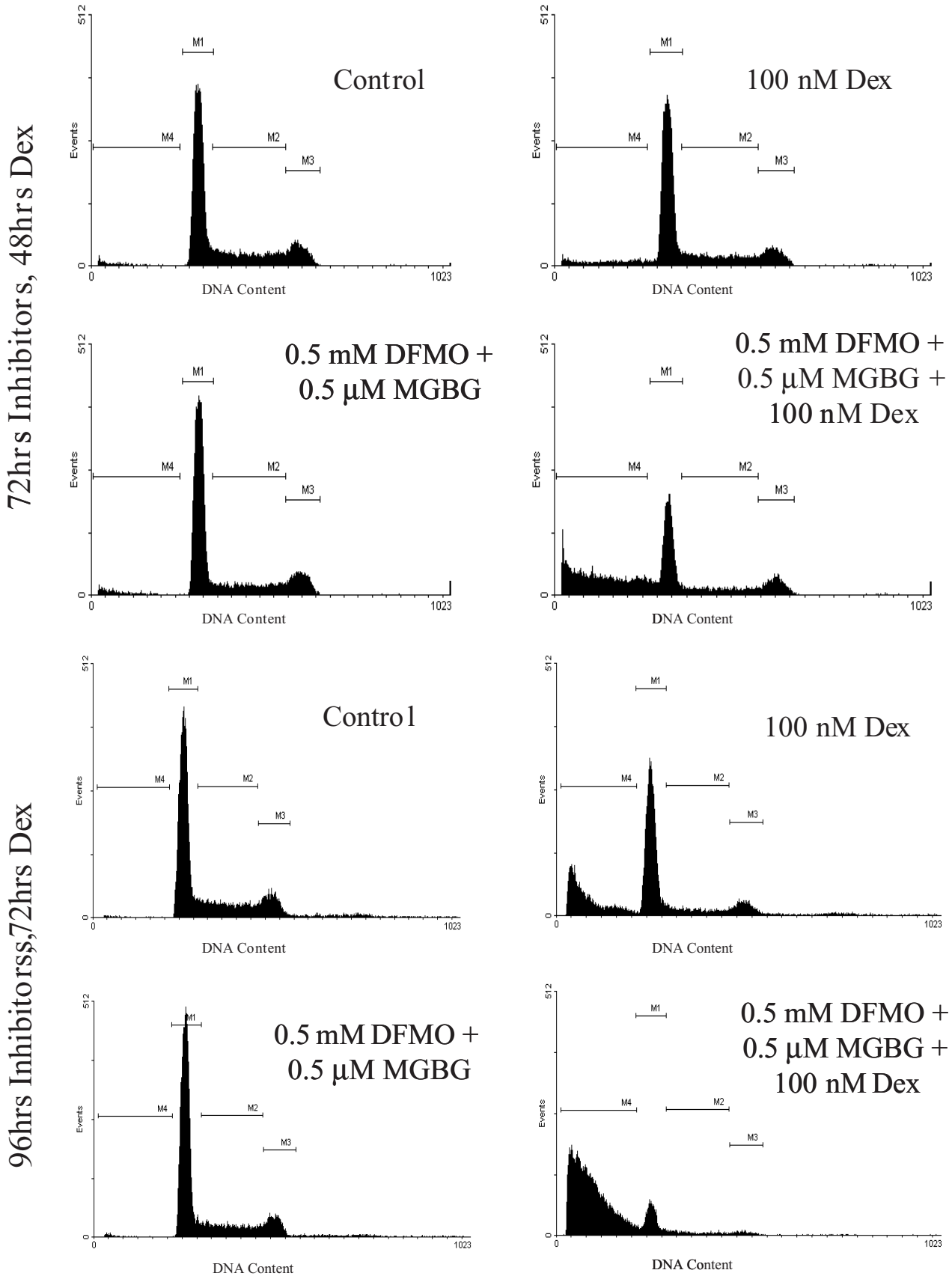


Figure 6. *Constitutive expression of an active form of c-Myc gives little or no protection against Dex-induced apoptosis in polyamine depleted cells. CEM-C7-14 and CEM-MycER-22 cells were treated according to the standard protocol and the number of viable cells was determined by trypan blue dye exclusion at 0, 24, 48, 72, and 96 hours DFMO and MGBG were added at 0 time and 100 nM Dex was added at 24 hours (indicated by the arrows). Error bars represent 1 SD limits; where no bars are seen, the standard deviation fell within the size of the symbol, n=3.*


72hrs Inhibitors, 48hrs Dex
96hrs Inhibitorss,72hrs Dex
Control
100 nM Dex
0.5 mM DFMO +
0.5 µM MGBG
0.5 mM DFMO +
0.5 µM MGBG +
100 nM Dex
Control
100 nM Dex
0.5 mM DFMO +
0.5 µM MGBG
0.5 mM DFMO +
0.5 µM MGBG +
100 nM Dex
M1
M2
M3
M4
Events
DNA Content
0
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DF+MG versus DF+MG+Dex, P=.002) at 72 hours of treatment. As in CEM-C7-14 cells, the addition of Dex to the inhibitors in CEM-MycER-22 cells tended to raise spermidine levels at 72 hours, with no effect over that of inhibitors alone on putrescine and spermine (compare Figure 2, *B* and *D*). At both 48 and 72 hours, the levels of spermidine and spermine were greater in the CEM-MycER-22 cells receiving DFMO plus MGBG and DFMO plus MGBG plus Dex than in the corresponding wild-type CEM-C7-14 cells (Figure 2, *A* versus *C*, *B* versus *D*). As will be seen below, the higher polyamine levels in CEM-Myc-22 cells did not afford strong protection against the three-drug combination.

The Extent and Onset of Apoptotic Cell Death Enhanced by the Combination of Polyamine Pathway Inhibitors and Glucocorticoid

At the concentrations used, the combined effect of DFMO and MGBG was primarily to slow cell growth, lengthening the doubling time from 24 to 48 hours. Figure 3*A*) demonstrates the growth-inhibitory effects on the CEM-C7-14 clone of the two inhibitors together without Dex for 72 hours, 100 nM Dex alone for 48 hours, and that after 72 hours when Dex was added after 24 hours of DFMO plus MGBG. The vehicle-treated control cells continued to grow logarithmically through the course of the experiment. Exposure to Dex alone reproducibly caused cell loss beginning only beyond 24 hours and still incomplete at 48 hours (the time points indicated as 48 and 72 on the abscissa). This is consistent with earlier, longer time course studies showing the full extent of Dex-evoked apoptosis [30,31]. When Dex was added after 1 day of exposure to the polyamine pathway inhibitors, cell loss began earlier than with Dex only and was more extensive during the subsequent 48 hours (compare in Figure 3*A*, $\triangle$, and $\times$ curves; see also Figure 6).

The cell loss seen in Figure 3*A* caused by the three-drug combination appeared to be apoptotic. Glucocorticoids alone are a well-known cause of lymphoid cell apoptosis, but the consequences of adding the polyamine inhibitors and glucocorticoids have had only limited evaluation [23]. Morphologically, the cells appeared to shrink, condense, and lyse (not shown). Phosphatidylserine eversion to the outer surface of the plasma membrane has been shown to precede loss of cell viability [33] and can be assayed by Annexin binding. Early apoptotic cells exclude 7-AAD; therefore, Annexin-positive, 7-AAD−negative cells have undergone phosphatidylserine membrane eversion, but still have an intact cell membrane. By flow cytometry, with Dex alone, 5% of the cells were Annexin-positive, 7-AAD−negative at 59 hours, 35 hours after addition of Dex (Figure 3*B*); but if the cells were first exposed to the polyamine pathway inhibitors for 24 hours, this number increased to 21%.

Both Dex and MGBG have been reported to affect mitochondrial functions adversely [34-36]. We therefore tested for this by use of the dye JC-1, which freely enters cells, where it produces a green fluorescence, unless taken up by active mitochondria, which metabolize it to its red, aggregate form. This conversion requires an intact mitochondrial membrane potential, $\Delta\Psi_m$. Cells that remain green have lost mitochondrial membrane integrity, which causes release of activators of the caspase-3 apoptotic pathway [35]. By this assay, the polyamine pathway inhibitors at these concentrations had little effect (Figure 3*C*). In Dex alone, no change was seen until 64 hours, after which a sharp drop in cells staining red was seen (note interval 64 to 72 hours; Figure 3*C*). When Dex was added 24 hours after the polyamine pathway inhibitors, a two-stage effect on mitochondria was seen. Starting at 48 hours, intact mitochondrial fluorescence was reduced by 10% to 15%, followed by an even deeper decrease between 59 and 64 hours (Figure 3*C*).

For a third test of apoptosis, CEM-C7-14 cells treated according to the standard protocol were evaluated by staining their DNA with PI at the 72-hour time point. Figure 4 and Table 1 show FACScans and percent gated cells from such an experiment. The control panel (Figure 4*A*) illustrates a population of logarithmically growing cells after 72 hours of exposure to vehicle only, with about 98% viability. After 48 hours in 100 nM Dex alone, 24% of the cells had a subdiploid DNA content, typical of apoptosis (Figure 4*B*) and Table 1), and among the remaining cells, the percentage in G_1/G_0 increased to 67%. We have observed [30] and others have confirmed this accumulation of CEM cells in G_1/G_0 due to Dex [18]. After 72 hours, 0.5 mM DFMO, 0.5 μM MGBG, or both combined in no case produced more than 13% apoptotic CEM-C7-14 cells (Figure 4, *C*, *E*, *G*) and Table 1). DFMO for 72 hours, with Dex as well during the final 48 hours, resulted in somewhat greater numbers of subdiploid cells than with Dex alone for 48 hours (30% as compared to 24%; Figure 4, *B* and *D* and Table 1). By 72 hours, however, the three-drug treatment had killed nearly the entire culture, with extensive DNA breakage (Figure 4*H*).

Pretreatment with Polyamine Pathway Inhibitors Followed by Dex Causes Early Induction of Apoptosis

The sequential combination of polyamine pathway inhibitors, followed by addition of Dex, resulted in a noticeable increase in subdiploid cells compared to that seen after 24 hours of Dex alone. At that time, 18% of the cells receiving the combination contained subdiploid amounts of DNA, compared to 2% after Dex alone for a comparable period (data not shown). This acceleration of apoptosis due to the sequential combined treatment is shown by the data in Figures 3, 5 and 6. In Figure 5 and Table 2, the time in Dex

Figure 7. *PI analyses of CEM-MycER-22cells after three-drug treatment. CEM-MycER-22 cells were treated according to the standard protocol, stained with PI, and analyzed by FACScan after the intervals indicated. The data shown are from one of two experiments, which gave very similar results. The percentages of cells in each compartment are presented in Table 3, A and B.*

Table 1. Percent of Cells Gated into Cell Cycle Compartments or Apoptotic from Experiment in Figure 4.

Cell Cycle Percent Gated	Control	0.5 mM DFMO	0.5 μM MGBG	0.5 mM DFMO + 0.5 μM MGBG	100 nM Dex	0.5 mM DFMO + 100 nM Dex	0.5 μM MGBG + 100 nM Dex	0.5 mM DFMO + 0.5 μM MGBG + 100 nM Dex
G_1/G_0	53.4	62.1	53.8	57.2	67.0	63.8	65.3	58.7
S	31.7	25	30.7	23.1	20.4	22.3	21.2	32.9
M	14.8	12.8	15.5	19.5	12.3	13.9	13.5	8.4
Apoptotic	1.7	4.4	2.9	12.8	24.4	30.0	23.4	76.2

required for an equivalent accumulation of PI-stained subdiploid cells is compared after addition of Dex alone (Figure 5, *A* and *B*) or Dex subsequent to DFMO and MGBG. As the data show, pretreatment with DFMO and MGBG followed by 24 hours of added Dex produces the amount of cell death caused after 40 hours of Dex alone (Figure 5, *A* versus *C*); 35 hours of Dex following the polyamine inhibitors result in kill equivalent to 48 hours of Dex alone (Figure 5, *B* versus *D*). The accelerating effect on reduction of numbers of the leukemic cells is shown by the curves with open symbols in Figure 6. The combined, sequential treatment results in significantly reduced numbers of cells 24 hours after Dex is added (triangles), whereas Dex alone begins to show an effect between 48 and 72 hours after addition (squares). Furthermore, the extent of cell loss is greater at each of the time points. In Dex alone, equivalent total cell loss is reached only 1 to 2 days later [30].

Constitutive Expression of c-Myc Does Not Protect Against Dex in Polyamine-Depleted Cells

We have shown that expression of the hybrid protein c-MycER confers significant resistance of CEM cells to Dex-induced apoptosis. The chimeric gene, *Myc-ER*, is expressed constitutively in these cells, and the Myc moiety is functional although the ER is not activated by estrogen [32]. Because the ligand-binding domain of the ER may be activated by nonsteroid effectors, we suspect that some endogenous ligand or interactive pathway chronically activates the chimera. In CEM-MycER-22 cells, the ODC mRNA-lowering effect of Dex alone is blunted but not completely blocked (data not shown). To determine whether polyamine pathway manipulation could sensitize cells, which stably produce an active form of c-Myc, we compared the effects of the standard protocol on CEM-MycER-22 with those on CEM-C7-14 cells (Figure 6). Dex alone caused a six-fold reduction in viable CEM-C7-14 cells 3 days later (open squares). CEM-MycER-22 cells identically treated were reduced only 2.5-fold (closed squares), reproducing the protective effect of constitutive c-Myc expression that we have reported [32]. Pretreatment with DFMO and MGBG for 1 day, followed by 3 days with Dex added, caused extensive additional apoptosis of both CEM clones (open and closed triangles). Clearly, the protective effect of MycER expression was largely overcome, although there were somewhat more viable cells remaining after 96 hours in the CEM-MycER-22 flasks than in the CEM-C7-14 flasks. Figure 7 and Table 3, A and B show data for CEM-MycER-22 cells stained with PI at two time points after various treatments. Again the protection against Dex alone in these cells is shown (compare Figure 4, Panel *B* with upper right panel, Figure 7, and Table 1 with Table 3A). There were clear synergistic effects, however, between the polyamine inhibitors and Dex on the CEM-MycER-22 cells, and these effects overcame the protection to Dex afforded by constitutive c-Myc expression.

In sum, apoptosis was considerably increased with the three-drug treatment as compared to Dex treatment alone in both CEM-C7-14 and CEM-MycER-22 cells (Figures 3*A*, 4, 6 and 7 and Tables 1 and 3, A and B). CEM-C7-14 cells showed more sensitivity to the three-drug treatment than CEM-MycER-22, but synergy was clearly indicated in both cell lines.

Discussion

As hypothesized, glucocorticoids reduce ODC mRNA levels, protein, and enzymatic activity, in a time sequence consistent with dependence on c-Myc. However, under our experimental conditions, this reduction in activity and content of ODC caused only a 50% reduction in putrescine levels (the immediate product of ODC), whereas the downstream polyamines spermidine and spermine increased during the time frame in which Dex proceeded to initiate apoptosis. Thus, we conclude that in this system, a reduction in total polyamine levels is not the key linear consequence of Dex administration that leads to apoptosis.

Table 2. Percent of Cells Gated into Cell Cycle Compartments or Apoptotic from Experiment in Figure 5.

Cell Cycle Percent Gated	100 nM Dex, 40 Hours	100 nM Dex, 48 Hours	Three-Drug Combination, 24 Hours Dex	Three-Drug Combination, 35 Hours Dex
G_1/G_0	58.6	67.0	66.8	63.1
S	23.9	20.4	19.1	20.9
M	16.5	12.3	14.1	15.9
Apoptotic	6.8	24.4	6.5	25.3

Table 3. Percent of Cells Gated into Cell Cycle Compartments or Apoptotic from Experiment in Figure 7.

Cell Cycle Percent Gated	Control	DFMO + MGBG	100 nM Dex	DFMO + MGBG + 100 nM Dex
(A) 72 hours inhibitors, 48 hours Dex				
G_1/G_0	59.9	62.4	59.3	33.4
S	20.9	16.3	16.7	11.5
M	16.5	17.3	15.2	12.4
Apoptotic	2.7	4.0	8.8	42.6
(B) 96 hours inhibitors, 72 hours Dex				
G_1/G_0	62.5	67.9	48.3	11.1
S	20.3	16.9	8.6	4.4
M	16.2	14.1	9.2	2.6
Apoptotic	0.8	1.0	30.6	77.8

As to why the polyamine levels remained elevated when the rate-limiting enzyme had been sharply reduced, one suggestion is that AdoMetDC remains active, feeding into the polyamine pathway beyond ODC, thus contributing to the formation of the downstream polyamines. Thus, until exhausted, the existing putrescine pool could be used to maintain spermidine and spermine. If the cells act to maintain the downstream polyamine levels (by whatever mechanism), it suggests that these must be important and perhaps even protective against the apoptotic process initiated by the glucocorticoid. Consequently, we investigated further the effect of inhibitors of the polyamine pathway alone and in conjunction with Dex.

We first carried out extensive tests on the inhibitors DFMO and MGBG singly, DFMO to inhibit ODC, and MGBG to prevent AdoMetDC production. In experiments not shown here, we found that DFMO slowly decreases the putrescine and spermidine pools, while initially increasing that of spermine, the final product of the pathway. By 48 hours in DFMO alone, putrescine and spermidine were undetectable, whereas spermine had increased somewhat over control levels. After 60 hours or later in DFMO, spermine as well fell to control levels or slightly below. We found that if addition of glucocorticoid was delayed until polyamine pools had been decreased by DFMO, some synergy could be seen in the apoptotic effect. Thus, in our clonal system, we confirm the basic finding of synergism that was described by Choi et al. [23], on uncloned CEM cells, though the kinetics and extent of the effect seem to differ in the two related experimental cellular systems. We found it difficult to obtain consistent results, however, with the DFMO plus Dex combination.

Treatment with MGBG has two effects on polyamines. The inhibitor blocks AdoMetDC and therefore the subsequent amino propyl additions to the pathway. It is also known that MGBG blocks the transport system through which spermine and spermidine enter the cell. In addition, MGBG can cause mitochondrial damage [36]. This effect is closely dose-dependent. Consequently, we conducted extensive preliminary studies to find the minimum effective concentration of MGBG that in combination with DFMO would reduce polyamine levels without causing overt cell death. We found this concentration to be approximately 0.5 μM, a level of the order of magnitude that in mouse leukemic L1210 cells has been shown to cause only slight mitochondrial swelling [36]. In our CEM-C7-14 cells, tests with the dye JC-1 indicated that the combination of 0.5 μM MGBG and 0.5 mM DFMO had no effect on mitochondrial integrity for up to 72 hours (Figure 3*C*) and only a slight effect after 96 hours.

Studies in which the polyamine pathway inhibitors were added simultaneously with Dex showed weak synergism at the times we investigated. The two polyamine pathway inhibitors, singly or combined, caused only a sustained low level of apoptosis. However, addition of the inhibitors long enough to cause significantly lowered polyamines, followed by Dex, resulted in marked synergy. This was seen both as a more rapid onset of apoptosis and as more extensive cell death. We find a general correlation between lowered polyamine levels and enhanced sensitivity to Dex. However, CEM-MycER-22 which we engineered to maintain c-Myc and therefore ODC, showed less reduction of polyamines but only modest — if any — protection against the three-drug combination. Together, our results suggest that a chronic effect of treatment by DFMO and MGBG is to make the cells more sensitive to the glucocorticoid Dex. Lowering total cellular polyamine levels does not seem to be the entire explanation for this synergy. It may be that organelle-specific polyamine reductions or nonpolyamine related effects are relevant. It has been proposed, for example, that the critical apoptotic action of Dex is to cause a loss of mitochondrial membrane integrity [34,35]. Thus, the reduction in cell polyamines, and in particular that of mitochondrial polyamines due to MGBG [37], may make those organelles particularly susceptible to Dex. In CEM-MycER-22 cells, MGBG could have a disproportionate effect on mitochondrial polyamines undetectable in the total polyamine pool. It is also possible that other mechanisms account for the lack of protection in these cells. The mechanism of the Dex effect on mitochondria is unknown, though it presumably requires synthesis of both RNA and protein because blocking these processes protects lymphoid cells from glucocorticoid-induced apoptosis [17,38]. Thus, MGBG, together with DFMO, in a time-dependent manner, renders mitochondria and cells more susceptible to the apoptosis consequent on the altered gene expression induced by Dex.

Many preclinical and clinical cancer trials of DFMO, MGBG, and the combination have been conducted over the

years, with varying degrees of success. DFMO and Dex are considered to be relatively nontoxic [39,40]. MGBG originally showed promise in clinical trials, but was temporarily abandoned due to toxicity [41,42]. After pharmacokinetic studies showed it to have an extraordinary long half-life *in vivo*, new dose schedules allowed MGBG to return to clinical usage [41,42]. In recent phase II trials, the drug showed potential, with little toxicity against AIDS-related lymphoma [43]. DFMO and MGBG, in combination, have been shown to be effective in preclinical and clinical trails, though care must be taken in dosage and schedules if undue toxicity is to be avoided [41,42]. Our three-drug combination *in vitro* suggests that due to the synergy between the polyamine pathway inhibitors and Dex, use of lower, less toxic amounts of MGBG may be possible. MGBG dosage seems to be the main factor in using the polyamine inhibitor drug combination effectively. We show that a subtoxic concentration of MGBG is effective when combined with DFMO plus Dex. This concentration of MGBG does not cause the mitochondrial membrane to depolarize significantly compared to untreated controls (Figure 3*C*). The low level of toxicity due to the polyamine pathway inhibitors alone, seen in CEM-C7-14 cells, is not present in CEM-MycER-22 cells, indicating that at those concentrations, the inhibitors themselves are not an indiscriminate cause of cell death and that the higher polyamine levels in these cells may afford protection (see Figures 4*G* and 7) Rizzo et al. [43] were able to estimate tissue levels of MGBG from one patient with AIDS-related non-Hodgkin's lymphoma who had been treated on a schedule of 600 mg/m^2 MGBG intravenously on days 1 and 8. Two days after the day 8 dose, the patient expired and his tissues were analyzed [43]. On a microgram-of-MGBG-per-gram-of-tissue-wet-weight basis, it can be calculated that the tissue concentrations were of the same order of magnitude as those used herein. It is possible that appropriate protocols of these three drugs may provide a combined chemotherapeutic response of value in certain lymphoid malignancies.

The combined data from our studies suggest that the lethal pathway evoked by glucocorticoids does not employ lowering of polyamines as a necessary and sufficient step. However, polyamines are protective against Dex, and preliminary administration of DFMO and MGBG renders cells more sensitive to the lethal effect of the glucocorticoid. Regardless of precise mechanism, our data suggest the possibility that the three drugs employed here might prove a potent combination in therapeutic circumstances.

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References

[1] Schipper RG, Penning LC, and Verhofstad AJ (2000). Involvement of polyamines in apoptosis. Facts and controversies: effectors or protectors? *Semin Cancer Biol* **10(1)**, 55–68.

[2] Casero RA Jr, and Pegg AE (1993). Spermidine/spermine N^1-acetyltransferase — the turning point in polyamine metabolism. *FASEB J* **7**, 653–61.

[3] Seiler N, Delcros JG, and Moulinoux JP (1996). Polyamine transport in mammalian cells. An update. *Int J Biochem Cell Biol* **28(8)**, 843–61.

[4] Shantz LM, and Pegg AE (1999). Translational regulation of ornithine decarboxylase and other enzymes of the polyamine pathway. *Int J Biochem Cell Biol* **31**, 107–22.

[5] Packham G, and Cleveland JL (1995). The role of ornithine decarboxylase in c-Myc–induced apoptosis. *Curr Top Microbiol Biol Immunol* **194**, 283–90.

[6] Grassilli E, Benatti F, Dansi P, Giammarioli AM, Malorni W, Franceshi C, and Desiderio MA (1998). Inhibition of proteasome function prevents thymocyte apoptosis: involvement of ornithine decarboxylase. *Biochem Biophys Res Commun* **250**, 293–97.

[7] Cohen SS (1998). A Guide to the Polyamines. Oxford Univ. Press, New York, NY.

[8] Jänne J, Alhonen L, and Leinonen P (1991). Polyamines: from molecular biology to clinical applications. *Ann Med* **23**, 241–59.

[9] Sunkara PW, Prakash NJ, Chang CC, and Sjoerdsma A (1983). Cytotoxicity of methylglyoxal bis (guanylhydrazone) in combination with α-difluoromethylornithine against HeLa cells and mouse L1210 leukemia. *J Natl Cancer Inst* **70(3)**, 505–509.

[10] Ask A, Persson L, Oredsson SM, and Heby O (1986). Synergistic antileukemic effect of two polyamine synthesis inhibitors. Host survival and cell cycle kinetic analysis. *Int J Cancer* **37**, 465–70.

[11] Siimes M, Seppänen P, Alhonen-Hongisto L, and Jänne J (1981). Synergistic action of two polyamine antimetabolites leads to a rapid therapeutic response in childhood leukemia. *Int J Cancer* **28**, 567–70.

[12] Seppänen P, Alhonen-Hongisto L, and Jänne J. Combined use of 2-difluoromethylornithine and methylglyoxal bis(guanylhydrazone) in normal and leukemia-bearing mice. *Cancer Lett* **18**, 1–10.

[13] Nakaike S, Kashiwagi K, Terao K, Iio K, and Igarashi K (1988). Combined use of α-difluoromethylornithine and an inhibitor of *S*-adenosylmethionine decarboxylase in mice bearing P388 leukemia or Lewis lung cancer. *Jpn J Cancer Res* **79**, 501–508.

[14] Warrell RP, and Burchenal JH (1983). Methylglyoxal-bis(guanylhydrazone) (methyl-GAG): current status and future prospects. *J Clin Oncol* **1(1)**, 52–65.

[15] Thompson EB (1998). Glucocorticoids and oxysterols in lymphoid apoptosis. *Hormones and Signaling.* Academic Press, San Diego, CA pp. 1–40.

[16] Thompson EB (1999). Mechanisms of T-cell apoptosis induced by glucocorticoids. *Trends Endocrinol Metabolism* **10(9)**, 353–58.

[17] Schwartzman RA, and Cidlowski JA (1994). Glucocorticoid-induced apoptosis of lymphoid cells. *Int Arch Allergy Immunol* **105**, 347–54.

[18] King LL, and Cidlowski JF (1998). Cell cycle regulation and apoptosis. *Annu Rev Physiol* **60**, 601–17.

[19] Thulasi R, Harbour DV, and Thompson EB (1993). Suppression of c-myc is a critical step in glucocorticoid-induced human leukemic cell lysis. *J Biol Chem* **268(24)**, 18306–12.

[20] Zhou F, Medh RD, and Thompson EB (2000). Glucocorticoid mediated transcriptional repression of *c-myc* in apoptotic human leukemic CEM cells. *J Steroid Biochem Mol Biol* **73**, 195–202.

[21] Bello-Fernandez C, Packman G, and Cleveland JL (1993). The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci USA* **90**, 7804–808.

[22] Tabor CW, and Tabor H (1984). Polyamines. *Annu Rev Biochem* **53**, 749–90.

[23] Choi S-H, Kim S-W, Choi D-H, Min B-H, and Chun B-G (2000). Polyamine-depletion induces p27Kip1 and enhances dexamethasone-induced G1 arrest and apoptosis in human T lymphoblastic leukemia cells. *Leuk Res* **24(2)**, 119–27.

[24] Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, and McCarthy RE (1965). Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18**, 522–29.

[25] Norman MR, and Thompson EB (1977). Characterization of a glucocorticoid-sensitive human lymphoid cell line. *Cancer Res* **37**, 3785–91.

[26] Littlewood TD, Hancock DC, Danielian PS, Parker MG, and Evan GI (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23(10)**, 1686–90.

[27] Merali S, and Clarkson AB Jr (1996). Polyamine analysis using *N*-hydroxysuccinimidyl-6-aminoquinoyl carbamate for pre-column derivation. *J Chromatogr. B: Biomed Appl* **675**, 321–26.

[28] Weiss T, Bernhardt G, Buschauer A, Jauch K-W, and Zirngibl H (1997). High-resolution reversed-phase high-performance liquid chromatography analysis of polyamines and their monoacetyl conjugates by fluorescence detection after derivation with *n*-hydroxysuccinimidyl 6-quinolinyl carbamate. *Anal Biochem* **247**, 294–304.

[29] Bradbury BA, Simmons TD, Slater KJ, and Crouch SPM (2000). Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. *J Immunol Methods* **240**, 79–92.

[30] Harmon JM, Norman MR, Fowlkes BJ, and Thompson EB (1979). Dexamethasone induces irreversible G_1 arrest and death of a human lymphoid cell line. *J Cell Physiol* **98(2)**, 267–78.

[31] Johnson BH, Ayala-Torres S, Chan LN, El-Naghy M, and Thompson EB (1997). Glucocorticoid/oxysterol-induced DNA lysis in human leukemic cells. *J Steroid Biochem Mol Biol* **61(1–2)**, 35–45.

[32] Medh RD, Wang A, Zhou F, and Thompson EB (2001). Constitutive expression of ectopic c-Myc delays glucocorticoid-evoked apoptosis of human leukemic CEM-C7 cells. *Oncogene* **20(34)**, 4629–39.

[33] Vermes I, Haanen C, Steffens-Nakken H, and Reutelingsperger C (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* **184**, 39–51.

[34] Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, and Kroemer G (1995). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* **182**, 367–77.

[35] Kroemer G, Dallaporta B, and Resche-Rigon M (1998). The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* **60**, 619–42.

[36] Pathak SN, Porter CW, and Dave C (1977). Morphological evidence for an antimitochondrial action by methylglyoxal-bis(guanylhydrazone). *Cancer Res* **37**, 2246–50.

[37] Byczkowski JZ, and Porter CW (1983). Interactions between bis(guanylhydrazones) and polyamines in isolated mitochondria. *Gen Pharmacol* **14(6)**, 615–21.

[38] Cidlowski JA, King KL, Evans-Storms RB, Montague JW, Bortner CD, and Hughes FM Jr (1996). The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. *Recent Prog Horm Res* **51**, 457–90.

[39] Necheles TF (1979). The Acute Leukemias. *Chemotherapeutic Agents* Stratton Intercontinental Medical Book Corp., New York, NY pp. 126–59.

[40] Meyskens FL Jr, and Gerner EW (1999). Development of difluoromethylornithine (DFMO) as a chemoprevention agent. *Clin Cancer Res* **5(5)**, 945–51.

[41] Von Hoff, DD (1998). There are no bad anticancer agents, only bad clinical trial designs — twenty-first Richard and Hinda Rosenthal Foundation Award Lecture. *Clin Cancer Res* **4**, 1079–86.

[42] Von Hoff, DD (1994). MGBG: teaching an old drug new tricks. *Ann Oncol* **5**, 487–93.

[43] Rizzo J, Levine AM, Weiss GR, Pearce T, Kraynak M, Mueck R, Smith S, Von Hoff DD, and Kuhn JG (1996). Pharmacokinetic profile of mitoguazone (MGBG) in patients with AIDS-related non-Hodgkin's lymphoma. *Invest New Drugs* **14**, 227–34.