Action of Dihydroxyanthraquinone on Cell Cycle Progression and Survival of a Variety of Cultured Mammalian Cells

Frank Traganos,2 Donald P. Evenson, Lisa Staiano-Coico, Zbigniew Darzynkiewicz, and Myron R. Melamed

In this manuscript, the authors investigate the effects of dihydroxyanthraquinone on cell cycle kinetics of various cultured mammalian cells. The drug, dihydroxyanthraquinone (DHAQ), is shown to alter cell cycle kinetics in a dose-dependent manner. Exposure to DHAQ results in the accumulation of cells in G2 phase, with optimal drug concentrations not only blocking cells in G2 but in the case of Friend leukemia, producing a 40 and 70% increase in RNA content. The analog is approximately 200 times more potent in vitro than anthrancenedione. The results obtained with DHAQ were compared to those obtained previously with a nonhydroxylated analog, anthracenedione (NCS 279836), and were found to be more potent. DHAQ is also more potent in the above in vivo test systems than anthrancenedione (NCS 279836), a nonhydroxylated analog, indicating its potential as a chemotherapeutic agent.

INTRODUCTION

DHAQ is one of several polycyclic aromatic compounds selected as a potentially new chemotherapeutic agent based on the possibility of its binding to nucleic acids by intercalation. Information provided by the Drug Evaluation Branch of the National Cancer Institute (Bethesda, Md.) reveals that DHAQ is active in a number of in vivo tumor systems, including i.p. B16 melanoma, i.p. Colon 26, i.p. L1210 leukemia, and i.p. P388 leukemia. In addition, DHAQ, in contrast to Adriamycin, produces a significant increase in life span when tested against i.v. transplanted L1210 leukemia and moderate activity against P388/Adria. The agent is effective in the above systems when administered i.p., i.v., and s.c., but not p.o.

MATERIALS AND METHODS

Cells

FL cells, strain 745, were obtained from the Medical Research Institute, Camden, N. J. The cells were maintained in suspension culture in Dulbecco’s modified Eagle’s medium containing 2.5 × 10⁻² M 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid buffer (Grand Island Biological Co., Grand Island, N. Y.) and 1000 mg glucose per ml, and supplemented with 15% fetal calf serum (Grand Island Biological), 100 units penicillin per ml, and 100 mg streptomycin per liter (Grand Island Biological). In addition, 4 × 10⁻⁵ M L-glutamine (Microbiological Associates, Bethesda, Md.) was freshly added to each 500 ml of stock culture medium. Stock cultures of FL cells were maintained at 37° in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were routinely passaged by addition of 1 × 10⁶ cells into 1.0 ml to 9.0 ml of the above medium twice weekly. However, in order to insure logarithmic growth prior to addition of drugs, the cells were split 1:3 with fresh medium on 3 consecutive days.

The L1210 cells used in this study were kindly provided by Dr. F. Kingsley Sanders of this Institute. The cells, grown in suspension cultures containing Roswell Park Memorial Institute Medium 1640 (Grand Island Biological) supplemented with L-glutamine, penicillin, streptomycin, and 15% fetal calf serum at the same concentrations as for FL cells, were routinely pas-

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3 The abbreviations and trivial names used are: DHAQ, dihydroxyanthraquinone; AO, acridine orange; lCF@50, 50% inhibition of colony formation.
saged by diluting cells 1:10 in fresh medium every second day. As with FL cells, all studies were done with L1210 cells in logarithmic growth.

Chinese hamster cells (line CHO originally obtained from Dr. T. T. Puck by Dr. L. Chasin) were maintained as growing monolayer cultures in F12 medium (Grand Island Biological) as previously described (10). The cultures were routinely passed following trypsinization by splitting at a ratio of 1:20 twice weekly.

Human peripheral blood was obtained by venipuncture from healthy donors. Following isolation of the mononuclear cell fraction on Ficoll:Isopaque (Lymphoprep; Nyegaard, Oslo, Norway), the cells were rinsed with HBSS, suspended in Eagle’s basal medium (Grand Island Biological) containing 15% fetal calf serum, and subcultured on plastic dishes to remove most of the monocytes. The nonadhering cells were then adjusted to a concentration of approximately 5.0 x 10⁶/ml in Eagle’s basal medium containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, antibiotics, and 15% fetal calf serum. Unstimulated cultures were incubated in 25-ml Falcon tissue culture flasks (Fisher Scientific, Fairlawn, N. J.) at 37° in a humidified atmosphere of 95% air and 5% carbon dioxide. Lymphocytes were stimulated either before or after the addition of the drug. Lymphocytes, obtained as described above, were plated at a higher original cell concentration which had been incubated with various concentrations of DHAQ under identical conditions. FL and L1210 cell cultures were seeded at 2.5 to 3.0 x 10⁵ cells/ml, whereas lymphocytes were tested at a higher original cell concentration (1 x 10⁶ cells/ml) since no cell growth was expected. At various concentrations. The drug was either washed out and the cells resuspended in drug-free medium after 30 min culture or the cultures were incubated for the full 24 hr in the presence of the drug. Lymphocytes, obtained as described above, were incubated with DHAQ under identical conditions. FL and L1210 cell cultures were seeded at 2.5 to 3.0 x 10⁵ cells/ml, whereas lymphocytes were tested at a higher original cell concentration (1 x 10⁶ cells/ml) since no cell growth was expected. The number of viable cells, as defined by their ability to exclude trypan blue, was determined by hemocytometer count for each culture, and the relative increase or decrease in viable cells/ml was plotted as the percentage of the original cell concentration. The viability of untreated FL and L1210 cell cultures was always greater than 95%, while 98% of the untreated lymphocytes excluded trypan blue.

Urdeine Incorporation

Incorporation of [5-³H]uridine (New England Nuclear, Boston, Mass.) into FL cells was determined by addition of the precursor (0.2 μCi/ml; 27.6 Ci/mmol) to cell suspensions which had been incubated with various concentrations of DHAQ for 2 hr. After 30 min incubation, the cells were collected on Whatman GF/D glass fiber filters (Fisher Scientific) and washed 3 times with 5.0 ml of ice cold 5% trichloroacetic acid (Sigma Chemical Co., St. Louis, Mo.) and twice with 5.0 ml of 95% ethanol. After drying overnight at room temperature, the filters were placed into vials containing 10 ml of Aquasol-2 (New England Nuclear) and assayed for amount of acid-insoluble radioactivity in a Packard Tri-Carb liquid scintillation counter.

Terminal Point of Drug Action

Vinblastine sulfate (Sigma) dissolved in HBSS was added to logarithmically growing FL cells at a concentration of 0.5 μg/
A sample of cells was taken immediately and fixed with 9 volumes of a 1:1 mixture of acetone:70% ethanol, and then the remainder of the culture was split in half. After 1 hr, 0.05 μg DHAQ per ml was added to one-half of the culture, and samples of both cultures were taken at appropriate intervals afterwards and fixed as above. Following fixation overnight at 0–4°, each sample was pelleted by centrifugation and resuspended in 1.0 ml of a buffer containing 30% ethanol in 0.05 mM acetate buffer, pH 6.0. Approximately 200 units of RNase (RASE; Worthington Biochemical Corp., Freehold, N. J.) was added to each tube for 1 hr at 37°.

Cell Staining

Simultaneous Staining of DNA and RNA. Cells in suspension (i.e., FL, L1210, and human lymphocytes), taken directly from culture or following trypsinization (CHO cells), were made permeable by the addition of 0.2 ml of cell suspension to 0.4 ml of a solution containing 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X-100 (Sigma). The cells were stained with AO 30 sec later by adding 1.2 ml of a solution containing 0.2 M Na2HPO4: 0.1 M citric acid buffer (pH 6.0). 1 mM sodium EDTA, 0.15 M NaCl, and 6 μg per ml AO (chromatographically purified; Polysciences, Inc., Warrington, Pa.). This staining reaction and its specificity have been described in previous publications (5, 25, 26). Briefly, under these staining conditions, AO intercalates into double helical nucleic acids (predominantly DNA in this case), fluorescing green (530 nm) in blue light, as does the dye monomer (13), while it ‘‘stacks’’ in polymeric form on single-stranded nucleic acids (in this case RNA) with a metachromatic shift in maximum emission to red (640 nm) (1).

Differential Staining of Mitotic Cells. The AO staining reaction used to identify mitotic cells by flow cytometry also has been discussed in great detail in previous publications (6–9) and was applied here to determine the terminal point of drug action. Fixed RNase-treated cells (0.1-ml cell suspension) were added to 0.1 M HCl/KCl buffer (0.4 ml) at pH 1.5 for 30 sec. The cells were then stained by addition of 2 ml of AO solution (8 μg/ml) in 0.2 M phosphate:0.1 M citrate buffer at pH 2.6 (6–9).

Fluorescence Measurements

The 2-color fluorescence is emitted from AO-stained cells as they traverse the elliptically focused beam of a 488-nm argon ion laser in the flow cytometer (FC 200; Ortho Diagnostic Instruments, Westwood, Mass.). The red fluorescence (measured in a band from 600 to 650 nm) and green fluorescence (measured in a band from 515 to 575 nm) emissions were separated by optical filters and measured by separate photomultipliers, and their integrated values were recorded and stored in a Nova 1220 minicomputer (Data General Corporation, Southboro, Mass.) that was interfaced to the instrument (22). Single cells were distinguished from cell doublets, and their nuclear diameters were determined by the green fluorescence pulsewidth of the stained nuclei, i.e., the time taken for the cell nucleus to pass through the argon ion laser beam (21). The integrated fluorescence intensity at both wavelengths and the pulsewidth values for a minimum of 5 × 10⁵ cells were recorded for each sample; cell doublets and higher aggregates were excluded from analysis.

Interactive computer analysis programs were used to obtain mean value and normalized histograms of fluorescence for populations and subpopulations as illustrated in the charts. The computer-drawn displays were obtained with a Tektronix 4010-1 graphics display terminal (Tektronix Inc., Beaverton, Oreg.).

RESULTS

Inhibition of Colony Formation

The ability of DHAQ to inhibit colony formation was determined over a wide concentration range. As shown in Chart 2 and Table 1, ICF₅₀ was achieved at a drug concentration of 3.2 ng/ml following 24 hr exposure to the drug and 6.5 ng/ml if the drug was removed after only 30 min incubation. In either instance, no colonies were observed at drug concentrations in excess of 50 ng/ml. Stationary phase CHO cells required a significantly higher concentration of DHAQ to inhibit colony formation by 50%. For the same exposure times, 30 min and 24 hr, the ICF₅₀ for stationary CHO cells was 0.23 and 0.024 μg/ml, respectively (Chart 2 and Table 1).

In order to determine if DHAQ exhibited cell cycle phase-specific inhibition of colony formation, CHO cells were treated with various concentrations of drug at discrete intervals of time following mitotic selection (Chart 3). In each instance, cells were treated with DHAQ for 2 hr at the appropriate concentration, washed free of drug, and cultured for 7 days. Cells treated immediately after mitotic selection and during reattachment (i.e., mitotic and early G₁ cells) appeared to be the most resistant, with an apparent ICF₅₀ of 5.5 ng/ml (Chart 3). CHO
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Table 1
Effect of DHAQ on cell survival and colony formation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>State*</th>
<th>Length of drug exposureb (hr)</th>
<th>ICF50 (µg/ml)</th>
<th>LD50c (µg/ml)</th>
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<tr>
<td>CHO</td>
<td>Exponential</td>
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<td>0.003</td>
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<td></td>
<td></td>
<td>24</td>
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<td></td>
<td>Stationary</td>
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<td>0.23</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-early G1</td>
<td>2</td>
<td>0.0055</td>
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</tr>
<tr>
<td></td>
<td>Late G1</td>
<td>2</td>
<td>0.0012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>2</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Late S-G2</td>
<td>2</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>FL</td>
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<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210</td>
<td>Exponential</td>
<td>0.5</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Unstimulated</td>
<td>0.5</td>
<td>&gt;5.0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>24</td>
<td>&gt;5.0</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates whether cells were in stationary or exponential growth or, in the case of CHO cells examined after mitotic selection, the phase of the cell cycle.

b The majority of the cells were in during drug treatment. Lymphocytes were not mitogen stimulated.

c FL and L1210 cells and lymphocytes were counted after 24 hr exposure or 30 min exposure followed by 23.5 hr growth in drug-free medium. All CHO cell cultures, regardless of duration of drug exposure, were examined for colony formation after 7 days.

c LD50, dose lethal to 50% of cells.

cells in S and early G2 phase (i.e., 6 and 9 hr following mitotic detachment) were less resistant, having an IFC50 of approximately 2 ng/ml. Cells in late G1 were slightly more sensitive to the action of the drug; 1.2 ng of DHAQ per ml were required to inhibit colony formation by 50% (Chart 3).

Growth and Viability of Cells in Suspension Culture following Treatment with DHAQ

The effects of 30 min and 24 hr incubation of FL and L1210 cells and human lymphocytes with various concentrations of DHAQ are illustrated in Chart 4. DHAQ had 2 effects on FL and L1210 cells. At concentrations at or below 0.01 µg/ml, FL and L1210 cells incubated for either 30 min or 24 hr were inhibited, to various degrees, from proliferating as extensively as untreated cultures but did not contain increased numbers of trypan blue positive cells (not shown). Above 0.01 µg DHAQ per ml, fewer than the original starting concentration of viable cells were present in culture after incubation for either 30 min or 24 hr with the drug (Chart 4). FL cells were generally more sensitive to DHAQ than were L1210 cells (Table 1), whereas incubation of lymphocytes for 24 hr in the presence of 5.0 µg DHAQ per ml did not reduce the viable cell number to 50% of control values (Chart 4).

Cell Cycle Progression

The effect of short-term (30-min pulse) and continuous exposure to DHAQ on the cell cycle progression of a variety of cell lines was evaluated by comparing the green fluorescence histograms (representing relative DNA content per cell) produced by AO staining and flow cytometric analysis of 3 to 5 × 10^3 cells.

FL Cell Kinetics. FL cells were most sensitive to the action of DHAQ. Chart 5, A to C, illustrates the effect of a 30-min pulse of various concentrations of the drug followed by multiple washings and subsequent culturing for 4, 8, and 24 hr. As is obvious from the green (DNA) fluorescence histogram in Chart 5A, 1 ng of DHAQ per ml had little or no effect on the distribution of FL cells throughout the cell cycle at any point following the 30-min pulse. However, 10 ng/ml was sufficient to cause an accumulation of cells in S and G2 + M at the expense of G1 cells as early as 4 hr following removal of the drug (Chart 5B). By 8 hr, nearly all cells had accumulated in late S and G2 + M, and by 24 hr the majority of cells contained 4C levels of green (DNA) fluorescence, with the remainder of the cells appearing to contain greater than 4C amounts of DNA (Chart 5B). At 0.1 µg DHAQ per ml, cells accumulated in G2 + M but also appeared to be blocked in G1 and S at 4 hr (Chart 5C). At longer culture times, a greater proportion of cells accumulated in G2 + M, and by 24 hr there appeared to be a significant number of cells with greater than 4C levels of green (DNA) fluorescence values (Chart 5).

When FL cells were continuously exposed to drug in culture, the effect on the cell cycle distribution was almost identical to that following a 30-min pulse of DHAQ, but was obtained at shorter culture times with a 10 times lower concentration of drug (Chart 6, A to C). Thus, at 1 ng/ml continuous exposure, cells began piling up in G2 + M by 4 hr in culture (Chart 6A). Following 24 hr exposure, most cells accumulated in G2 + M, although some G1- and S-phase cells remained and some cells with greater than 4C levels of green (DNA) fluorescence ap-
Chart 4. Viability of L1210 and FL cells and human lymphocytes following a 30-min pulse or 24 hr continuous exposure to DHAQ. The drug was added to L1210 and FL cells growing exponentially at an original concentration of 2.5 to 3.0 x 10^6 cells/ml and to freshly separated human lymphocytes seeded at a concentration of 1 x 10^6 cells/ml. DHAQ was added at appropriate concentrations and washed out following 30 min exposure followed by resuspension of cells in drug-free medium (□, ○, Δ), or left for the duration (24 hr) of the experiment (■, ◯, A). Aliquots of cells were then mixed with 0.4% trypan blue in a cell:stain ratio of 4:1. Cell counts were done by hemocytometer, and the relative increase or decrease in viable cells relative to the original cell concentration was plotted. The experiments were repeated with essentially identical results.

Chart 5. Green (DNA) fluorescence histograms of AO-stained FL (A to C) and L1210 (D to F) cells following a 30-min pulse of DHAQ. Logarithmically growing FL and L1210 cells were treated with various concentrations of DHAQ, washed free of drug, and cultured in fresh drug-free medium. Samples (0.2 ml) were taken from control cultures (— — — — ) and from cultures following 4 (— — — — ), 8 (—— — ), and 24 (— — — ) hr growth and stained with AO as described in Materials and Methods." Each histogram represents the green (DNA) fluorescence distribution of 6 x 10^5 cells which have been normalized as to height (cells/channel) of the major peak for ease of comparison. The left-most peak (e.g., as in A and D) represents G1 (2C) cells. The cells at about twice the green fluorescence of G1 cells represent G2 + M (4C) cells; those intermediate, between the 2 phases, constitute DNA-synthesizing S-phase cells. Cells with green (DNA) fluorescence above the G2 + M value are considered to be cells with greater than a 4C DNA content.

Chart 6. Green (DNA) fluorescence distribution of AO-stained FL (A to C) and L1210 (D to F) cells grown continuously in the presence of various concentrations of DHAQ. All conditions are as in Chart 5 except that specimens were taken either prior to addition of the drug (——) or directly from drug containing cultures at 4 (— — — — ), 8 (—— — ), and 24 (— — — ) hr.

L1210 Cell Kinetics. L1210 cells displayed a logarithmically lower sensitivity to the drug when compared to FL cells. Thus, a 30-min pulse of 1 ng DHAQ per ml had no effect on L1210 cells, as with FL cells, yet a 10 times higher dose, which had a dramatic effect on FL cells, appeared to result in a minor, transient accumulation of L1210 cells in G2 + M (Chart 5). Since the proportion of cells in G2 + M decreased after 24 hr at this drug concentration (although it remained above control levels), it would appear that 10 ng DHAQ per ml resulted in a slight increase in cell transit time through G2 (Chart 5D). Following a 30-min pulse of 0.1 μg/ml, the distribution of L1210 all cells contained either 4C (G2 + M) or greater than 4C DNA fluorescence values (Chart 6, B and C).

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cells appeared to parallel the results obtained with FL cells at 10 ng DHAQ per ml (Chart 5F); namely, by 24 hr nearly all L1210 cells had accumulated either in G2 + M or at higher ploidy levels (Chart 5F). The effect of a 30-min pulse of 0.1 μg DHAQ per ml on L1210 cells did differ slightly from the effects of a 10-ng/ml pulse of DHAQ on FL cells to the extent that by 4 hr following the pulse relatively fewer L1210 cells had accumulated in late S and G2 + M and by 24 hr a smaller percentage of L1210 cells had appeared with greater than 4C levels of green (DNA) fluorescence than was the case for FL cells at the lower drug concentration (Chart 5, B and F).

This difference in sensitivity between L1210 and FL cells was also manifested following continuous exposure of the cells to DHAQ. Thus, at the lowest drug concentration (1 ng/ml), little more than a slight, transient accumulation of L1210 cells in G2 + M could be observed, although this concentration of drug had a dramatic effect on FL cells (Chart 6, A and D). The results of continuous exposure of L1210 cells to 0.1 μg of drug per ml were similar to those observed for both FL and L1210 cells at 10 ng DHAQ per ml (Chart 6, E and F).

**CHO Cell Kinetics.** The effect of DHAQ on CHO cells was qualitatively different than that observed for cells grown in suspension culture, i.e., FL and L1210 cells. Thus, a 30-min pulse at 10 ng DHAQ per ml resulted in a transient accumulation of CHO cells in S and G2 + M phase of the cycle (Chart 7A). At 4 hr following removal of the drug, a greater proportion of cells appeared in S phase (Chart 7A). By 8 hr, cells appeared to accumulate in G2 + M, and by 24 hr a more or less normal distribution was observed (Chart 7A).

Exposure of CHO cells for 30 min to 0.1 μg DHAQ per ml followed by culturing in the absence of the drug resulted in the accumulation of nearly all cells in G2 + M by 24 hr (Chart 7B). At a drug concentration of 1.0 μg/ml, the effect was such that by 8 hr many cells were still in early S phase and by 24 hr cells appeared to be accumulated in late S phase and early G2 (Chart 7C).

Continuous exposure of CHO cells to as little as 1 ng DHAQ per ml resulted in preferential accumulation of cells in G1 and G2 + M at the expense of S-phase cells (Chart 7D). At 10 times higher concentration of DHAQ (10 ng/ml), the accumulation of cells in G2 + M became apparent, with a small percentage of cells either blocked in G1 or capable of traversing G2 + M initially but accumulating G1 in the subsequent cell cycle (Chart 7E). Finally, continuous exposure of CHO cells to 0.1 μg DHAQ per ml again resulted in an accumulation of cells in late S and G2 + M phases of the cycle by 24 hr (Chart 7F).

Table 2 summarizes the results of the various treatments on the 3 mammalian cell lines tested.

**Human Peripheral Blood Lymphocytes**

Human peripheral blood lymphocytes were treated for 2 hr with various concentrations of DHAQ, washed, and cultured either in the absence or presence of optimal concentrations of DHAQ. Analysis of the effect of the drug on both cell viability and the percentage of cells responding to the mitogen was based on observations described previously in detail (5); namely, stimulated lymphocytes may be identified following staining with AO as described above by their characteristic increase in red (RNase-sensitive) fluorescence. This population may be further subdivided into G1, S, and G2 + M cells based on their green (DNA) fluorescence. Dead or dying cells also have a characteristic pattern which may be readily quantitated (5). Thus, human peripheral blood lymphocytes stimulated by PHA can be subdivided into nonresponsive G0 lymphocytes, stimulated lymphocytes in G1, S, or in G2 + M phase of the cell cycle, and dead or dying but intact lymphocytes (5).

As can be observed in Chart 8A, in the absence of PHA there was a dose-dependent increase in cell death with increasing time in culture. Thus, while the percentage of dead and dying cells in unstimulated cultures not treated with DHAQ increased from 1 to 18% by 66 hr, slightly less than 50% of the lymphocytes remained viable after an equivalent time in culture in the presence of 0.01 μg of DHAQ per ml. The proportion of viable cells in unstimulated cultures was reduced to approximately 10% after treatment with either 0.1 or 1.0 μg of DHAQ per ml for 66 hr (Chart 8A).

If drug treatment was followed by culturing the lymphocytes in the presence of PHA, a greater percentage of cells remained viable when compared to nonstimulated cultures at all drug concentrations (Chart 8B). Thus, at concentrations of 0.01, 0.1, and 1.0 μg DHAQ per ml, 66, 41, and 41% of the cells remained viable, respectively, after 66 hr in culture (Chart 8B).

While the addition of PHA to the culture affected cell viability in the presence of the drug at longer culture times, fewer cells responded to the mitogen as the dose of drug was increased (Chart 8B). In control cultures, 32% of the lymphocytes responded to PHA by 66 hr in culture (38% of viable cells). Only 10, 8, and 7% of the cells responded to PHA following treat-

### Table 2

**Minimum DHAQ concentration at which a G2 block was observed**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>30 min exposure</th>
<th>24 hr exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>L1210</td>
<td>0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>FL</td>
<td>0.01</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Cells were exposed to the drug for 30 min, washed, and cultured in drug-free medium. Based on 24-hr cultures in each case.
Effects of DHAQ on Cell Kinetics

Effect of DHAQ on PHA-stimulated Lymphocytes

The effect of DHAQ on lymphocytes previously stimulated with PHA depended upon when the drug was added. Addition of drug following 48 hr of culture in the presence of PHA, i.e., at a point where few cells are actively cycling, resulted in a nearly linear inhibition of stimulation with increasing concentration of DHAQ (Chart 9). However, if the drug was added at 66 hr and the percentage of stimulated cells was compared with control cultures 6 hr later, there was no decrease observed in the percentage of stimulated cells or perturbation in the cell cycle distribution of cells up to a drug concentration of 1.0 μg DHAQ per ml.

Effect of DHAQ on RNA

Coincident with the accumulation of cells in the G2 + M phase of the cycle following treatment with DHAQ was an increase in the red fluorescence intensity. As described previously and confirmed by us in this series of experiments, the red fluorescence of cells stained with AO under the present conditions is almost entirely sensitive to RNase (4, 5, 26). Thus, with the cell lines used in the present study, at least 70% and in most cases greater than 80% of the red fluorescence was abolished following brief treatment with RNase (10).

Chart 10 illustrates both the extensive increase in red fluorescence of AO stained cells treated with 10 ng DHAQ per ml as compared to control cells and the extent to which that red fluorescence was sensitive to RNase treatment.

To determine whether drug treatment affected the rate of RNA synthesis, FL cells were treated for 2 hr with various concentrations of DHAQ. Coincident with the accumulation of cells in the G2 + M phase was an increase in the red fluorescence intensity. As described previously and confirmed by us in this series of experiments, the red fluorescence of cells stained with AO under the present conditions is almost entirely sensitive to RNase (4, 5, 26). Thus, with the cell lines used in the present study, at least 70% and in most cases greater than 80% of the red fluorescence was abolished following brief treatment with RNase (10).

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concentrations of DHAQ, ranging from 1.0 ng to 1.0 μg per ml. At that point, 0.2 μCi of [5-3H]uridine per ml were added to each culture for 30 min, and then the cells were collected on glass fiber filters and the amount of acid-insoluble radioactivity was assayed by scintillation counting. As can be seen in Chart 11, at low concentrations of DHAQ (1 ng/ml) there was a slight increase in incorporation of [5-3H]uridine. However, as the concentration of drug was increased, there was a linear decrease in incorporation within the range of 1 ng to 1.0 μg DHAQ per ml.

Since the drug blocks cells in the G2 + M phase of the cell cycle, the red fluorescence of these cells selected by appropriate thresholding along the green (DNA) fluorescence histogram was determined both under conditions of continuous exposure and following a 30-min pulse of various concentrations of the drug. When plotted as a function of time following exposure to DHAQ, 1 ng/ml (30-min pulse) had little or no effect on RNA accumulation in FL cells (Chart 12). However, the same concentration of DHAQ (1 ng/ml), when present continuously, resulted in both an accumulation of cells in G2 + M by 24 hr (Chart 6A) and a 70% increase in mean red fluorescence of the G2 + M cells (Chart 12). DHAQ, at a concentration of 10 ng/ml, whether administered as a 30-min pulse or present continuously during culturing, resulted in an almost identical time course of accumulation of FL cells in G2 + M (Charts 5B and 6B) and an increase in the red (RNA) fluorescence of these cells by 140% (Chart 12; Table 3). However, at higher drug concentrations (0.1 μg/ml) either set of culture conditions (30-min pulse or continuous presence) resulted in only a modest (approximately 20%) increase in mean red fluorescence of the G2 + M cells (Chart 12).

The change in red fluorescence of the G2 + M population of L1210 cells followed a somewhat different pattern, although it was also correlated with the extent of cells blocked in G2 + M; namely, neither a 30-min pulse of 1 or 10 ng (Chart 5, D and E) nor continuous exposure to 1 ng (Chart 6E) of DHAQ per ml had much effect on the DNA distribution of L1210 cells or on the mean red fluorescence of the G2 + M population (Chart 13). However, continuous exposure to 10 ng/ml and a 30-min pulse of 0.1 μg/ml had significant and nearly identical effects on the DNA distribution of L1210 cells (Charts 5F and 6E) and also resulted in a maximum (70%) increase in mean red fluorescence of G2 + M cells after 24 hr under these culture conditions (Chart 13; Table 3). As was observed with FL cells, continuous exposure to 0.1 μg DHAQ per ml resulted in only a modest (12%) increase in mean red fluorescence of G2 + M cells by 24 hr (Chart 13).

The RNA content of CHO cells was unchanged following 24

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**Chart 11.** Incorporation of radioactive uridine into FL cells following a 2-hr treatment with DHAQ. Logarithmically growing cells were incubated for 2 hr with various concentrations of DHAQ. The cells were then incubated for 30 min with 0.2 μCi [5-3H]uridine per ml, collected onto glass filters, and counted as in "Materials and Methods." Each point represents the average of duplicate cultures. Background counts (i.e., the amount of radioactivity retained by filters to which an equal amount of [5-3H]uridine was added in the absence of cells) was subtracted from each point.

**Chart 12.** Relative red (RNase sensitive) fluorescence of G2 + M cells from FL cell cultures grown continuously in or following a 30-min pulse of various concentrations of DHAQ. Cells in G2 + M were selected by appropriate computer thresholding along the green (DNA) fluorescence histograms. Cells were treated as in Charts 5 and 6 with either 0.001 (□), 0.01 (●), or 0.1 (△) μg DHAQ per ml for 30 min and grown in fresh medium or were incubated continuously with 0.001 (●), 0.01 (■), and 0.1 (△) μg DHAQ per ml. Each point represents from 1 to 3 × 10^3 cells; bars, 3 S.E.
hr exposure to 10 ng DHAQ per ml, i.e., culture conditions which resulted in maximal increases in FL and L1210 cell RNA content (Table 3).

**Terminal Point of Drug Action**

In order to determine more precisely at what phase in the cell cycle cells were blocked in the presence of DHAQ, cultures of FL cells were exposed first to vinblastine, followed 1 hr later by addition of drug. Techniques have been developed by which fixed, RNase-treated cells can be stained with AO and the percentage of mitotic cells can be scored rapidly by automated cytometry (6–9). Thus, samples from control-vinblastine- and drug-vinblastine-treated cultures were obtained at 30-min or 1-hr intervals, and the percentage of mitotic cells present was scored automatically. If the drug results in a complete block of cells at some point prior to mitosis, then the length of time between the addition of drug and the plateau in the accumulation of mitotic cells in drug-treated cultures will represent the time interval between the terminal point of action of the drug and mitosis (the point of action of vinblastine). A concentration of 50 ng DHAQ per ml was chosen, since it would be sufficient to ensure a complete rather than a transient block of FL cells yet not so high as to result in significant cell death. At 50 ng DHAQ per ml, the plateau in the accumulation of drug-treated cells was achieved by 15 min, i.e., cells were blocked in G2 phase rather than mitosis and the block preceded the point of action of vinblastine by 15 min (Chart 14). This experiment was repeated with a second set of FL cell cultures with precisely the same results.

**DISCUSSION**

Continuous exposure of FL, L1210, and CHO cell cultures to sublethal doses of DHAQ (i.e., in the range of 1 to 10 ng/ml) resulted in an accumulation of cells in G2 by 24 hr (Charts 5 to 7 and 14; Table 2). Under these culture conditions, FL cells were clearly 10 times more sensitive to the cytostatic action of DHAQ than were the other 2 cell lines. No cycle-specific block was observed following treatment of PHA-stimulated lymphocytes with DHAQ (Chart 8).

Limited exposure of cells to the drug (i.e., a 30-min pulse of DHAQ followed by resuspension of cells in drug-free medium) resulted in a G2 block in FL, L1210, and CHO cells by 24 hr, but at a 10 times higher drug concentration (Charts 5 to 7; Table 2). Again, FL cells were more sensitive to the cytostatic effect of DHAQ when compared to L1210 and CHO cells.

The effect of DHAQ on cell growth, viability, and proliferative potential was determined by 2 different techniques. One approach based on cell counts and trypan blue dye exclusion measures the immediate effect of the drug on cell growth and viability and was applied to the suspension cultures, FL and L1210 cells, as well as to unstimulated lymphocytes. A second technique, inhibition of colony formation, measures not only the immediate effects of the drug on cell survival but, in addition, the effect of the drug on the proliferative potential of the cells, since at least 4 consecutive doublings are required to form a macroscopically visible (50-cell) colony. The latter technique was applied to CHO cells, since they grow attached to a substrate and generally manifest a high cloning efficiency.

Twenty-four hr exposure to drug concentrations below 0.01 μg/ml affected proliferation but not viability of FL and L1210 cells and human lymphocytes. However, exposure to concentrations in excess of 0.01 μg/ml for 24 hr resulted not only in...
complete inhibition of proliferation of FL and L1210 cells but also in a decrease in cell viability (Chart 4). Again, FL cells were more sensitive than L1210 cells (dose lethal to 50% of the cells was 0.3 μg/ml and 0.8 μg/ml, respectively).

Higher concentrations of DHAQ were required for 50% cell kill if the drug was removed after 30 min and the cells were grown in drug-free medium for the remaining 23.5 hr as compared with continuous exposure to the drug (Chart 4; Table 1). Lymphocytes treated for either 30 min or 24 hr were remarkably resistant to the cytotoxic effect of the drug. Even after 24 hr exposure to 5.0 μg DHAQ per ml, a majority of lymphocytes in culture excluded trypan blue (Chart 4).

The differential inhibition of colony formation of exponentially growing versus stationary CHO cells by DHAQ paralleled the results of the effect of the drug, described above, on cycling (FL and L1210) cells versus quiescent (G0) lymphocytes; namely, stationary phase CHO cells, if exposed to DHAQ for 24 hr, were nearly 8 times more sensitive to the drug than their exponentially growing counterparts (Chart 2; Table 1). There was nearly a 40-fold difference between the ICFso of stationary and exponentially growing CHO cell cultures when the cells were exposed to DHAQ for only 30 min (Table 1).

Since CHO cells are easily synchronized by mitotic selection, the ability of cells exposed to the drug at various phases of the cell cycle to form colonies could be tested. Following a 24-hr pulse of DHAQ, the ICFso of mitotic and early G1 cells was 3 to 4 times lower than for cells in the remainder of the cycle, especially late G1 cells (Chart 3; Table 1).

Two separate series of experiments were performed on human lymphocytes to assay the effect of the drug on various parameters of lymphocyte growth and stimulation. In one set of experiments, lymphocytes were pretreated with DHAQ for 2 hr and then grown in the absence or presence of PHA. Thus, the cytotoxic effect of DHAQ on stimulated (cycling) and nonstimulated (quiescent) lymphocytes could be compared. In addition, the ability of the drug to suppress stimulation by PHA could be investigated. In the second series of experiments, PHA-stimulated lymphocytes were treated with DHAQ at 42 and 66 hr of stimulation to investigate the effect of the drug on the early and late proliferative phases of stimulation, respectively (15).

Lymphocyte stimulation was studied by flow cytometry. As described previously in detail (5), AO-stained lymphocytes could be classified into subpopulations which include unstimulated (G0) lymphocytes and, upon mitogen stimulation, G1, S, and G2 + M stimulated lymphocytes based upon their increase in RNA or DNA content. Intact, dead, or dying cells could be determined by their decreased green fluorescence, indicating nuclear disintegration (5). Since the cell cycle stage of a large number (5 x 10⁶) of individual cells can be characterized by this technique, the analysis of PHA stimulation by flow cytometry is much more precise and informative than results obtained either by determining the incorporation of radioactive thymidine or by morphological identification of blast transformation.

Significant cell death occurred after 42 and 66 hr of culture of nonstimulated lymphocytes treated with 0.1 and 1.0 μg DHAQ per ml for 2 hr (Chart 8A). PHA-stimulated cultures incubated for 42 hr following a 2-hr pulse of 0.1 or 1.0 μg DHAQ per ml contained comparable numbers of dead cells (Chart 8). However, by 66 hr, considerably fewer dead cells appeared in PHA-stimulated than in nonstimulated cultures (approximately 60% versus 90%). These results were unlikely to be due to a compensatory increase in viable cells as a result of PHA-induced cell proliferation, since proliferation was substantially inhibited by DHAQ (Chart 8B). Therefore, continuing proliferation among stimulated lymphocytes appears to rescue some lymphocytes that would otherwise die in nonstimulated cultures.

If lymphocytes were first stimulated with PHA and then incubated with the drug starting at 42 or 66 hr of culture (i.e., early in or midway through the proliferative phase of stimulation), cell proliferation was inhibited in a dose-dependent fashion at the earlier time point but was completely unaffected by as much as 1.0 μg DHAQ per ml at the later time point (Chart 9). Thus, if the drug was added prior to maximal proliferation of stimulated lymphocytes, stimulation and/or cell proliferation was inhibited. If, however, lymphocytes were already actively cycling, a short-term exposure (6 hr) to various concentrations of DHAQ had little effect.

As a result of the ability to simultaneously stain both DNA and RNA in individual cells, it was observed that the RNA content among G2 cells affected by the drug increased. This observation was consistent with the earlier results obtained with the analog, anthracenedione (10). The increase in RNA content of G2 cells was observed for FL and L1210 but not CHO cells (Charts 10, 12, and 13; Table 3). The greatest increase in RNA content occurred at a DHAQ concentration of 0.01 μg/ml for both FL and L1210 cells (Charts 12 and 13). That drug concentration was the minimum dose required to produce a consistent G2 block (Chart 6) but was the maximum dose which inhibited cell proliferation but did not affect cell viability (Chart 4) in both cell lines.

To determine if RNA synthesis increased as a result of treatment with DHAQ, drug-treated cells were pulsed with radioactive uridine following a 2-hr incubation with the drug (Chart 11). High concentrations of DHAQ (0.1 and 1.0 μg/ml) suppressed RNA synthesis. However, following exposure to 0.001 or 0.01 μg DHAQ per ml for 2 hr, incorporation of radioactive precursor was either unaffected or slightly enhanced. It is at these lower concentrations, albeit for longer exposure times, that the maximal increases in RNA content among G2 cells in FL and L1210 cell cultures were observed (Charts 12 and 13; Table 3). It is interesting to note that similar increases in RNA content were observed when stimulated lymphocytes or CHO cells were prevented from cycling by agents such as hydroxyurea and 5-fluorodeoxyuridine (2, 3).

DHAQ, at concentrations and exposure times which were effective in blocking cells in G2 and which resulted in increased RNA content of the drug-treated population, also resulted in an increase in cells with greater than a 4C level of DNA (Charts 5 and 6). Thus, while normally 1.5% or less of the FL or L1210 cells had greater than a 4C DNA content (10), the proportion of cells in this locus increased substantially in FL cells following a 30-min pulse of 10 ng or 0.1 μg per ml or 24 hr exposure of 1.0 ng to 0.1 μg DHAQ per ml and in L1210 cells following a 30 min exposure to 0.1 μg/ml or 24 hr continuous exposure to 10 ng or 0.1 μg DHAQ per ml (Charts 5 and 6). This phenomenon was not observed with CHO cells (Chart 7).

The exact mechanism responsible for these observations is not known. Endoreduplication of eukaryotic chromosomes can occur spontaneously (14, 20) or can be induced by a variety...
of agents (11, 12, 6–19, 23). Anthracenedione, an analog of DHAQ, was observed to induce endoreduplication in FL cells (10). This observation of an increased percentage of cells with a greater than 4C level of DNA may be correlated with the fact that, at the lowest concentrations of DHAQ sufficient to prevent passage of cells through mitosis to G1, no increase in mitotic cells could be observed. Rather, the terminal point of action of DHAQ was calculated to be 15 min prior to mitosis for FL cells (Chart 14), which coincides with the point of action of several inhibitors of protein synthesis (24). It is also possible that DHAQ may induce disruption of the mitotic process by some other mechanism. Nevertheless, most cells would be blocked in G2 as a consequence of drug treatment, but in cell lines such as FL and L1210 a number of cells may proceed directly into the next round of DNA synthesis without passing through mitosis.

Preliminary evidence suggests that DHAQ binds to nucleic acids by intercalation. Thus, DNA and RNA are both implicated as cellular targets for DHAQ. Additional information on the mechanism of action of the drug is required, however, before a thorough understanding of the low toxicity of the drug for normal cells but its high antitumor activity can be understood. Nevertheless, the combination of in vivo data and the present in vitro results suggest that DHAQ may be a promising antitumor agent, particularly since there appears to be a clear difference in sensitivity to the drug between leukemic cell lines and normal human lymphocytes.

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