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Effects of Ellipticine on Cell Survival and Cell Cycle Progression in Cultured Mammalian Cells[1]

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ABSTRACT

The effects of ellipticine [5,11-dimethyl-6*H*-pyrido(4,3-*b*)carbazole; NSC 71795] on cell viability, growth, and colony formation were investigated in suspension (Friend leukemia and L1210) and adherent [Chinese hamster ovary (CHO)] tumor cell systems as well as in mitogen-stimulated human peripheral blood lymphocyte cultures. Cell cycle progression and the terminal point of action of the drug were monitored by flow cytometry. Ellipticine was cytostatic for all cell lines tested, blocking cells in G_2 phase following 24 hr constant exposure at concentrations in the range of 1.0 μg/ml. A 10 times higher drug concentration was required to block cells in G_2 if the cells were exposed for only 30 min to the drug followed by 23.5 hr culture in drug-free medium. Formation of CHO cell colonies was inhibited by 50% following exposure to ellipticine for 2 hr at 6.0 μg/ml or for 24 hr at 0.3 μg/ml. Fifty % cell kill in asynchronously growing Friend leukemia and L1210 cells was obtained following exposure to ellipticine for 24 hr at 2.0 μg/ml and 1.15 μg/ml, respectively, whereas human peripheral blood lymphocytes required 66 hr exposure to 1.0 μg/ml to kill 50% of the cells. Phytohemagglutinin-stimulated lymphocytes were remarkably resistant to the cytotoxic effect of ellipticine but did display a dose-dependent inhibition of stimulation and accumulation in G_2 whether the drug was added prior to or during active cell proliferation.

Ellipticine, at cytostatic concentrations, had a marked effect on cellular RNA content. Friend leukemia cells, blocked in G_2 by the drug, doubled their RNA content compared to control cells. L1210 and CHO cells, but not lymphocytes, also increased in RNA content following ellipticine treatment. Drug concentrations which blocked cells in G_2 also led in the case of Friend leukemia and L1210 but not CHO cells to an increase in the proportion of cells with greater than 4C amounts of DNA.

INTRODUCTION

Ellipticine [5,11-dimethyl-6*H*-pyrido(4,3-*b*)carbazole (NSC 71795)] is a plant alkaloid previously shown to be active against the experimental mouse leukemia L1210 *in vivo* (19)[3] and in patients with neoplastic leukemia (24). Biochemical studies have suggested that ellipticine interacts with nucleic acids in cells by intercalation (19, 21, 22). Since many DNA-intercalating agents (*e.g.*, actinomycin D, daunomycin, and Adriamycin) are known anticancer agents, ellipticine might be expected to be useful as an antitumor agent.

Preliminary *in vitro* studies demonstrated that the drug blocked asynchronously growing cells in the premitotic (G_2) phase of the cycle (4). Although there was no evidence for cell cycle phase-specific killing, cells in mitosis and early G_1 phase appeared to be most sensitive to the action of the drug (3, 4). Moderate selectivity in the inhibition of nucleolar 45S RNA synthesis has also been observed (20).

In the present study, the action of ellipticine on the cell cycle kinetics and RNA content of several mammalian cell lines is described. Both cytostatic and cytotoxic drug concentrations were investigated in a variety of cell types under various conditions of ellipticine concentration, time of drug exposure, and state of cell proliferation. Particular emphasis was placed on investigating the effect of the drug on the ability of human peripheral blood lymphocytes, both quiescent and stimulated, to proliferate by mitogenesis. These findings were compared and contrasted with the effect of the drug on a murine leukemic lymphocyte cell line.

MATERIALS AND METHODS

Cells

FL[4] cells (strain 745) were obtained from the Medical Research Institute, Camden, N. J. The cells grown in suspension as described previously (31, 32) were routinely passaged twice weekly by addition of 1×10^6 cells in 1.0 to 9.0 ml of medium twice weekly. The cells were split 1:3 with fresh prewarmed medium on 3 successive days prior to addition of drug in order to ensure asynchronous growth.

The L1210 cells used in this study were kindly provided by Dr. F. Kingsley Sanders of this institute. The cells grown in suspension cultures (32) were routinely passaged by diluting 1:10 in fresh medium every third day. As with FL cells, all studies were done with L1210 cells in logarithmic growth.

CHO cells, originally obtained from Dr. T. T. Puck by Dr. L. Chasin, were maintained as growing monolayer cultures as described previously (16).

Human peripheral blood was obtained by venipuncture from healthy donors. Following isolation of the mononuclear cell fraction on Ficoll:Isopaque (Lymphoprep; Nyegaardo, Oslo, Norway), the cells were rinsed with HBSS, suspended in Eagle's basal medium (Grand Island Biological Co., Grand Island, N. Y.) containing 15% fetal calf serum, and subcultured on plastic dishes to remove most of the monocytes. The non-

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[4] The abbreviations used are: FL, Friend leukemia; CHO, Chinese hamster ovary; HBSS, Hanks' balanced salt solution; PHA, phytohemagglutinin; AO, acridine orange; ICF_{50}, inhibition of colony formation by 50%; LD_{50}, dose at which 50% of the cells are killed.

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adhering cells were then adjusted to a concentration of approximately 5.0×10^5/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-cu cm Falcon tissue culture flasks (Fisher Scientific Co., Pittsburgh, Pa.) at 37° in a humidified atmosphere of 95% air and 5% carbon dioxide. Lymphocytes were stimulated by addition of a 1:100 dilution of reconstituted PHA-M (Grand Island Biological Co.) (9) and cultured as above.

Drugs

Ellipticine was provided by Dr. David Abraham from the Investigational Drug Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment, National Cancer Institute. Drug solutions were prepared for each experiment by dissolving ellipticine at a concentration of 1.0 mg/ml in 0.1 N HCl. Subsequent dilutions were made in HBSS immediately prior to use at 10 times the final concentration desired in the cell culture medium. Vinblastine sulfate was obtained from Sigma Chemical Co., St. Louis, Mo.

Survival Studies

For survival studies on cycling CHO cells, a known number (typically 400 and 2000) of exponentially growing cells were seeded in plastic plates with 35-mm-diameter wells (Costar, Cambridge, Mass.). Following reattachment of the cells (30 min), the drug was added at appropriate concentrations directly to the culture medium. The cells were incubated for 2 or 24 hr in the presence of the drug, then washed twice with HBSS, and refed with fresh medium. Following growth for 7 days, the cultures were washed with HBSS, fixed with Carnoy's fixative, and stained with crystal violet. Cells that were able to form colonies composed of at least 50 cells were scored positive for survival.

The survival of cells in various phases of the cell cycle was studied by collecting CHO cells by mitotic selection, diluting to a known cell concentration and plating as above. Ellipticine was added at appropriate concentrations for a period of 2 hr immediately (mitotic and late telephase-early G_1 cells) or 2 (late G_1), 6 (S), or 9 (late S-G_2) hr after seeding. The degree of synchrony and cell cycle progression following selection has been presented elsewhere (7).

All cell survival studies were carried out in triplicate and repeated at least once. Results are expressed as the percentage of control drug-free cultures, which had a plating efficiency of approximately 80%.

Asynchronously growing cultures of FL and L1210 cells were counted and split into the required number of cultures. Ellipticine was added at appropriate concentrations to all but one control culture for each cell line. Following 24 hr culturing, cell counts were made. Only cells excluding trypan blue were counted, and the percentage of cells relative to the starting concentration is indicated in each case. Only at drug concentrations in excess of 1.0 μg/ml were considerable numbers of trypan blue positive cells obtained. All counts were done in duplicate by hemocytometer on an average of 5×10^2 cells/culture.

Terminal Point of Drug Action

Vinblastine sulfate dissolved in HBSS was added to logarithmically growing FL cells at a concentration of 0.5 μg/ml. A sample of cells was taken immediately and fixed wtih 9 volumes of acetone:70% ethanol (1:1), and then the remainder of the culture was split into 3 parts. After 1 hr, 1.0 and 10 μg ellipticine per ml was added to the remaining 2 aliquots of cells, and samples of all 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 M acetate buffer. Approximately 200 units of RNase (RASE; Worthington Biochemical Corp., Freehold, N. J.) were added to each tube for 1 hr at 37°.

Cell Staining

Simultaneous Staining of DNA and RNA. Cells in suspension (*i.e.*, FL, L1210, or human lymphocyte) taken directly from culture or following trypsinization (CHO) 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 Na_2HPO_4, 0.1 M citric acid buffer (pH 6.0), 1 mM sodium-EDTA, 0.15 M NaCl, and chromatographically purified AO (6 μg/ml; Polysciences, Inc., Warrington, Pa.). This staining reaction and its specificity have been described in previous publications (10, 29, 30). 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 (23), 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) (5).

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 (11-14) 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 (11-14).

Fluorescence Measurements. The green (helical DNA) and red (RNA or denatured DNA) fluorescence and green fluorescence pulse width of individual AO-stained cells were obtained as described previously (25, 26) by use of an FC-200 flow cytometer (Ortho Diagnostic Instruments, Westwood, Mass.) interfaced to a Nova 1220 minicomputer (Data General Corp., Southboro, Mass.).

Interactive computer analysis programs were used to obtain mean values 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

Exponentially Growing CHO Cells. As shown in Chart 1, although a 2-hr incubation with concentrations of up to 1.0 μg ellipticine per ml had little effect on colony formation of exponentially growing CHO cells, ICF_{50} was achieved at a drug concentration of 6.0 μg/ml. When the length of exposure was

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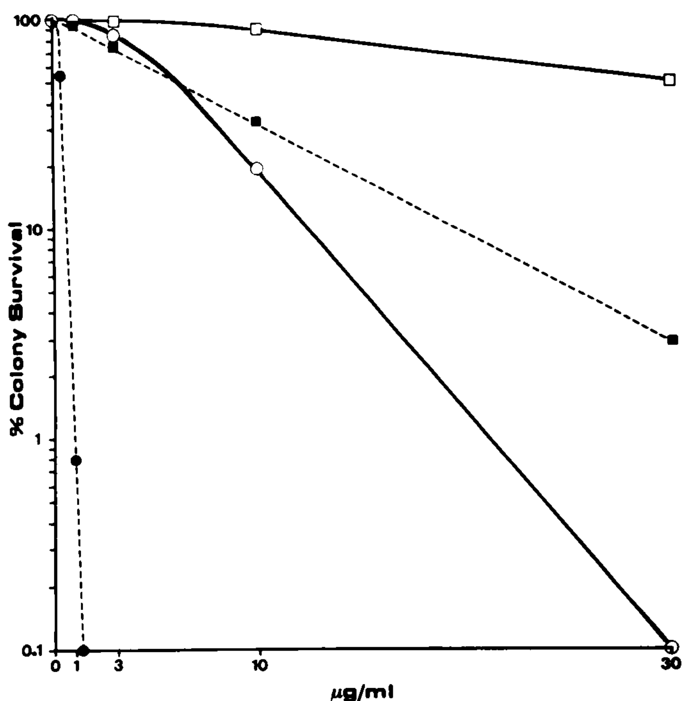

Chart 1. Effect of ellipticine on colony formation of logarithmically growing and stationary CHO cells. Log phase cells were exposed to ellipticine for 2 (○) or 24 (●) hr, washed free of drug, and cultured in drug-free medium. Stationary cultures exposed to ellipticine for 2 (□) or 24 (■) hr were subsequently rinsed, trypsinized, and plated in drug-free medium. Both sets of cultures were plated at 400 and 2000 cells/well. Seven days later, culture plates were rinsed, fixed with Carnoy's fixative, and stained with crystal violet. Each *point* represent the average number of colonies of 50 or more cells from triplicate cultures. The survival rate for untreated control cultures was considered as 100%.

increased to 24 hr, the ICF_{50} fell to 0.3 μg/ml. No colonies were observed following 24 hr incubation with 3.0 μg ellipticine per ml (Chart 1).

Stationary Phase CHO Cells. Stationary CHO cells required significantly higher concentrations of ellipticine to achieve ICF_{50} (Chart 1). Exposure to ellipticine for 2 and 24 hr resulted in an ICF_{50} by 30 and 6 μg/ml, respectively (Table 1).

Synchronized CHO Cells. In order to determine if ellipticine exhibited cell cycle phase-specific cytotoxicity, CHO cells synchronized by mitotic selection were treated with various concentrations of drug at discrete intervals of time following detachment. In each instance, cells were exposed to ellipticine for 2 hr, washed free of drug, and cultured for 7 days. Cells treated immediately after mitotic selection, *i.e.*, cells still in mitosis or early G_1 phase, were slightly more sensitive to ellipticine than CHO cells in the remainder of the cycle (Table 2). The ICF_{50} for these cells was approximately 1.8 μg/ml. Cells in late G_1, early S-mid-S, and late S-early G_2 were slightly more resistant (average ICF_{50}, 2.7 μg/ml) to the drug (Table 2).

Inhibition of Growth of Asynchronous Suspension Cultures

Asynchronous cultures of FL and L1210 cells in logarithmic growth were split, and aliquots were treated with different concentrations of ellipticine. Following 24 hr growth, the number of viable (trypan blue-excluding) cells were counted in each culture. The results are presented in Table 3.

Ellipticine at concentrations below 0.1 μg/ml inhibited the growth of L1210 cells in a dose-dependent manner (Table 3). The drug at 0.1 μg/ml completely inhibited cell growth but was not cytotoxic; more than 90% of the cells were viable at that drug concentration. The LD_{50} for L1210 cells exposed to ellipticine for 24 hr was approximately 1.15 μg/ml (Table 1). At a drug concentration of 5 μg/ml, the majority of cells remaining in culture (75%) failed to exclude trypan blue.

FL cultures exposed to 0.5 μg ellipticine per ml for 24 hr showed no increase in cell number (Table 3). The LD_{50} for FL cells was 2.0 μg/ml; no viable cells could be observed in cultures exposed to 5 μg ellipticine per ml for 24 hr (Tables 1 and 3).

Cell Cycle Progression

The effect of either short-term (30-min pulse) or continuous exposure to ellipticine on the cell cycle distribution of a variety

Table 1

Summary of the cytotoxic and cytostatic action of ellipticine on various cell systems

Cells	Length of exposure to drug (hr)	Cell viability: ICF_{50} (μg/ml)	Cell viability: LD_{50} (μg/ml)	G_2 block[a] (μg/ml)
FL	24		2.0	1.0
L1210	24		1.15	1.0
CHO				
Exponential	24	0.3		1.0
	2	6.0		
Stationary	24	6.0		
	2	30.0		
Lymphocyte				
Nonproliferative	24		>1.0	
	66		1.0	
Proliferative	66		>1.0	0.1

[a] Minimum concentration of ellipticine required to cause a permanent G_2 block. Exposure was for 24 hr for cell lines and 42 hr for stimulated lymphocytes.

Table 2

Effect of a 2-hr pulse of ellipticine on mitotically detached CHO cells

Treatment[a] (hr)	% of colonies formed at the following concentrations of ellipticine[b]: 0.1 μg/ml	1.0 μg/ml	10.0 μg/ml
0-2	97.3 (96.5-100)[c]	65.7 (64.4-67.5)	0.3 (0-0.8)
2-4	99.9 (99.6-100)	85.7 (85.3-86.2)	0.3 (0-1)
4-6	100 (100)	88.8 (84.2-93.8)	0.2 (0-0.5)
9-11	100 (100)	89.4 (89.0-89.9)	0.5 (0-1)

[a] All cells were collected by mitotic detachment and allowed to readhere to the plastic surface for 30 min. Treatment with ellipticine was for 2 hr at the times indicated following detachment and readherence.
[b] Mean of 3 experiments, each done in duplicate, of the percentage of colonies formed in comparison to non-drug-treated cells.
[c] Numbers in parentheses, range of percentage of colony formation for the 3 separate experiments.

Table 3

Effect of 24 hr exposure to ellipticine on cell growth of suspension cultures

The drug was present at the indicated concentrations continuously for 24 hr, at which point duplicate hemocytometer counts of a minimum of 5 × 10^2 cells were made. Only cells excluding trypan blue were included.

Ellipticine (μg/ml)	FL cells: Cell count (× 10^5/ml)	FL cells: %[a]	L1210 cells: Cell count (× 10^5/ml)	L1210 cells: %[a]
0	26.0	509.8	11.0	407.4
0.05	9.5	183.3	4.6	170.3
0.1	7.9	154.9	2.4	88.8
0.5	4.2	82.3	1.8	66.6
1.0	3.5	68.6	1.3	54.1
5.0	0	0	0.2	7.4

[a] Percentage of the starting cell concentration, which for FL cells was 5.1 × 10^5 cells/ml and for L1210 cells was 2.7 × 10^5 cells/ml.

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of cell lines was analyzed by comparing the DNA distribution obtained by flow cytometry of AO-stained cells after various times in culture following drug treatment.

FL Cell Kinetics. FL cells were most sensitive to the action of ellipticine. Chart 2 illustrates the effect of a 30-min exposure to the drug followed by multiple washings and subsequent culturing for 4, 8, and 24 hr.

At a concentration of 0.1 µg ellipticine per ml, FL cells accumulated in S and G_2 + M phases by 4 hr (Chart 2). By 8 hr, cells appear to have passed through mitosis into G_1, since, although the proportion of cells in G_2 + M remained high, the percentage of G_1 cells increased relative to the 4-hr sample (Chart 2*A*). After 24 hr, the distribution still resembled that expected for cells in asynchronous growth, but with fewer cells in S and G_2 + M (Chart 2*A*). Similar results were obtained following a 30-min pulse of ellipticine at a concentration of 1.0 µg/ml. Following a transient block in G_2 + M, there appeared to be a slightly longer delay in the reappearance of cells in G_1 (compare the 8-hr distributions of Chart 2, *A* and *B*), although a normal DNA distribution was observed by 24 hr culture. At 10 µg of the drug per ml, all cells were blocked in late S and G_2 + M by 24 hr; no cells were observed to have passed through mitosis to G_1 (Chart 2*C*).

When FL cells were continuously exposed to the drug, the effect was identical to that observed following a 30-min pulse, but it was obtained with a 10-fold lower drug concentration (Chart 3, *A* to *C*). Thus, 0.01 or 0.1 µg ellipticine per ml present continuously in culture resulted in a transient accumulation of cells in late S and G_2 + M at early times (4 and 8 hr), but by 24 hr the distribution resembled that of an asynchronously growing culture at slightly greater than the original cell density (Chart 3, *A* and *B*). When FL cells were continuously exposed to 1.0 µg ellipticine per ml, a block in cell progression was observed in G_2 + M (Chart 3*C*), which was nearly identical to the effect observed following a 30-min pulse of 10 µg ellipticine per ml described above (Chart 2*C*).

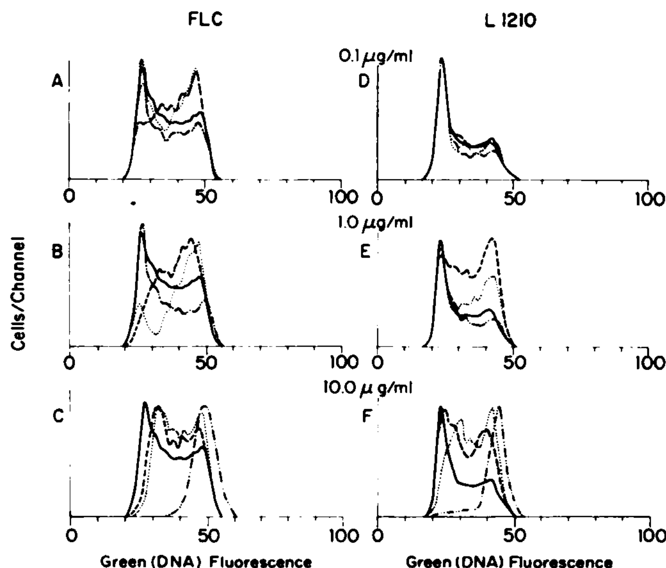

Chart 2. Green (DNA) fluorescence histograms of AO-stained FL and L1210 cells following a 30-min pulse of ellipticine and culturing for various lengths of time. Logarithmically growing FL (*A* to *C*) and L1210 (*D* to *F*) cells were treated with various concentrations of ellipticine, washed free of drug, and cultured in fresh drug-free medium. Samples were taken from untreated 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 5 × 10^3 cells that have been normalized as to height (cells/channel) of the major peak. The *left-most peak* (*e.g.*, as in *A* and *D*) represents G_1 (2C) cells; cells at about twice the green fluorescence of G_1 cells represent G_2 + M (4C) cells; those intermediate, between the 2 phases, constitute DNA-synthesizing S-phase cells.

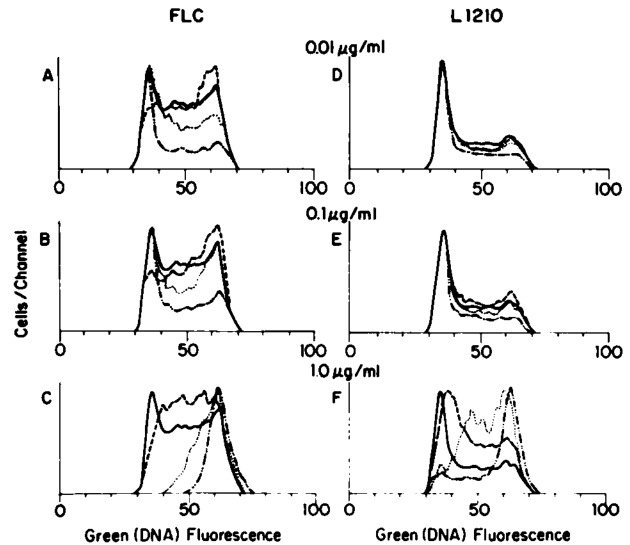

Chart 3. Green (DNA) fluorescence distributions of AO-stained FL and L1210 cells grown continuously in the presence of various concentrations of ellipticine. All conditions are as in Chart 2 except that specimens were taken either prior to addition of the drug (——) or directly from drug containing cultures at 4 (– – – –), 8 (· · · ·) and 24 (– · · –) hr.

L1210 Cell Kinetics. L1210 cells displayed a log lower sensitivity to the drug when compared to FL cells. Thus, a 30-min pulse of 0.1 µg ellipticine per ml had virtually no effect on L1210 cell cycle distribution (Chart 2*D*). When exposed to 1.0 µg/ml for 30 min, followed by culturing in fresh medium, L1210 cells exhibited a transient increase in S and G_2 + M cells at early culture times (4 and 8 hr) as was observed with FL cells at 0.1 µg/ml (Chart 2, *A* and *E*). At the highest concentration tested (10.0 µg/ml), a 30-min exposure resulted in a majority of cells accumulating in G_2 + M, although some cells were observed in G_1 and early S (Chart 2*F*).

Continuous exposure of L1210 cells to 0.01 or 0.1 µg ellipticine per ml had no effect on their cell cycle distribution (Chart 3, *D* and *E*). However, as with FL cells, continuous exposure to 1.0 µg ellipticine per ml resulted in an accumulation of cells in G_2 + M (Chart 3*F*). The block in G_2 + M observed after continuous exposure of L1210 cells to 1.0 µg ellipticine per ml, however, did not result in an accumulation of all cells in G_2 + M as with FL cells (Chart 3, *C* and *F*). Rather, as with a 30-min pulse of 10 µg/ml, a significant proportion of cells remained in G_1 and S phases (Chart 3*F*).

CHO Cell Kinetics. Exposure of CHO cells to ellipticine resulted in a change in the cell cycle distribution similar to that observed with FL cells, although at higher drug concentrations. Thus, as with L1210 cells, continuous exposure to 0.01 or 0.1 µg ellipticine per ml had no effect (not shown), whereas exposure to 1.0 µg ellipticine per ml resulted in a block of cells in G_2 + M, as illustrated in Chart 4. Therefore, CHO cells displayed sensitivity similar to that of L1210 cells. However, like FL cells, but unlike L1210 cells, all CHO cells were blocked in late S and G_2 + M by 1.0 µg ellipticine per ml (Table 1).

Normal Peripheral Blood Lymphocytes. Lymphocytes pu-

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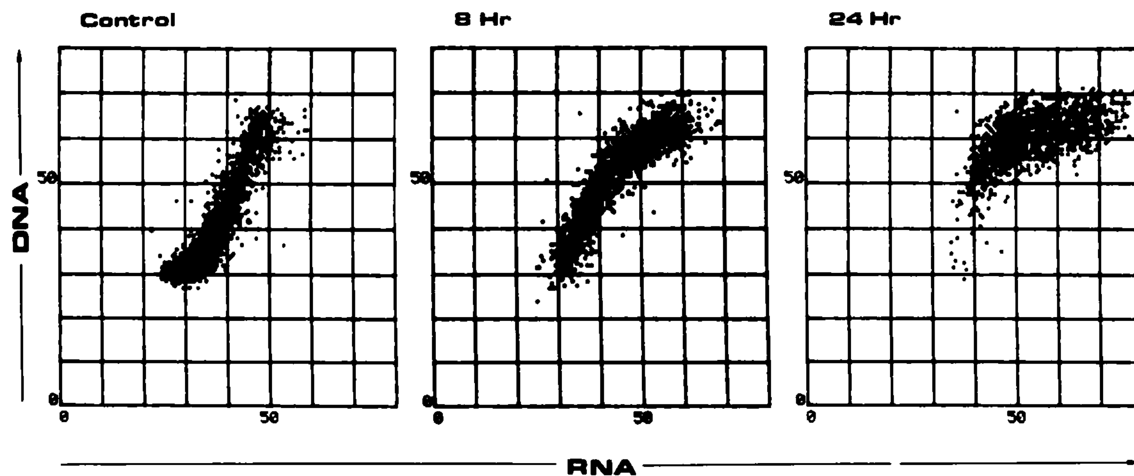

Chart 4. Scatterplots of green (DNA) and red (RNA) fluorescence of CHO cells grown in the absence and presence of 1.0 μg ellipticine per ml. In each scattergram, individual cells are represented by *points* whose distance from the *abscissa* and *ordinate* is a measure of green (DNA) and red (RNA) fluorescence of the cell, respectively. *Left*, asynchronously growing control CHO cells. Following 8 hr culture in the presence of 1.0 μg ellipticine per ml, cells began to accumulate at high DNA levels (G_2 + M) with fewer cells located at lower DNA levels (G_1). By 24 hr, nearly all cells were in late S-G_2 + M. Note the increase in red fluorescence of these cells relative to cells with the same DNA content in control cultures.

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rified from peripheral blood were exposed either briefly (30 min) or continuously to varying concentrations of ellipticine in the absence of mitogens. As illustrated in Chart 5, when exposed briefly to either 0.1 or 1.0 μg or continuously to 0.01 or 0.1 μg ellipticine per ml, the change in viable cell number did not differ from that of the control untreated lymphocytes over the course of 72 hr incubation. However, exposure either for 30 min to 10 μg or continuously to 1.0 μg ellipticine per ml caused a significant decrease in cell viability as assayed by decreased AO green fluorescence (10) (Chart 5). Thus, 45 hr following a 30-min exposure to 10 μg ellipticine per ml, 50% of the lymphocytes were killed (Chart 5). The same proportion of cells was killed after 66 hr of continuous exposure to 1.0 μg ellipticine per ml.

PHA-stimulated Lymphocytes. Continuous exposure of 48- or 72-hr cultures of PHA-stimulated lymphocytes to up to 1.0 μg ellipticine per ml for as long as 42 hr had no effect on cell viability (Table 4). Ellipticine was effective, however, in inhibiting stimulation when added to PHA cultures prior to extensive DNA synthesis (48 hr) or at the point of active proliferation (66 hr). In these situations, ellipticine at concentrations of 0.01 and 0.1 μg/ml lowered the percentage of cells scored as stimulated (Chart 6). When 1.0 μg ellipticine per ml was added to cultures at 48 or 66 hr, there was no further increase in proliferation over the following 42 or 18 hr, respectively, compared to control cultures (Chart 6).

In cultures in which ellipticine was added at 48 hr and monitored over the following 42 hr, the cells could be observed to accumulate in G_2 + M phase in a dose-dependent fashion (Chart 7). Whereas 0.01 μg ellipticine per ml had only a slight effect, treatment with 1.0 μg/ml for 42 hr resulted in a significant proportion (39%) of cells accumulated in G_2 + M phase (Chart 7).

The increase in G_2 + M cells appeared to be dependent upon both the duration of exposure to the drug and the length of time following stimulation that the drug was added. Thus, 1.0 μg ellipticine per ml added at 48 hr resulted, 42 hr later, in an increase in G_2 + M cells from 14 to 39%, whereas if the same dose was added to 66-hr cultures and assayed 24 hr later, the magnitude of the increase in G_2 + M cells was reduced (*i.e.*, an increase from 10 to 24%; Chart 7).

Effect of Ellipticine on RNA Content

As observed in Chart 4, when ellipticine-treated CHO cells were blocked in G_2 + M, their RNase-sensitive red fluorescence increased dramatically. Of the 3 cell lines studied, CHO cells showed the smallest percentage increase in mean RNA content for the G_2 + M population (Table 5). FL cells exposed to 1.0 μg ellipticine per ml for 24 hr accumulated almost entirely in late S and G_2 + M phase (Chart 3) with a doubling of mean RNA content (Table 5). The effect on L1210 cells was intermediate between that of FL and CHO cells (Table 5) at the same drug concentration (1.0 μg/ml).

Increase in Cells with Greater than 4C DNA Content

In addition to increased RNA content, FL and L1210 (but not CHO) cell cultures exposed to concentrations of ellipticine which induced maximal cell accumulation in G_2 + M phase also exhibited a dramatic increase in cells with greater than 4C DNA content (Table 6). Normally, there were 1% or less of cells with greater than a 4C DNA content in these cultures (Table 5). Following 24 hr treatment with 1.0 μg ellipticine per ml, the percentage of these cells increased 6- and 14-fold in FL and L1210 cultures, respectively (Table 3). This phenomenon was not observed in CHO cell cultures (Table 3).

Terminal Point of Action of Ellipticine

In order to determine the point in the cell cycle at which ellipticine exerted its effect, the drug was added to an FL cell culture to which 0.5 μg vinblastine sulfate per ml had been included for the previous 1 hr. By calculating the time between addition of ellipticine and cessation of accumulation of mitotic cells in culture, the precise point in the cell cycle in which cells were blocked could be calculated. Ellipticine, at a concentration of 1.0 μg/ml, resulted in a plateau of mitotic cell accumulation 40 min after addition of the drug (Chart 8). This would

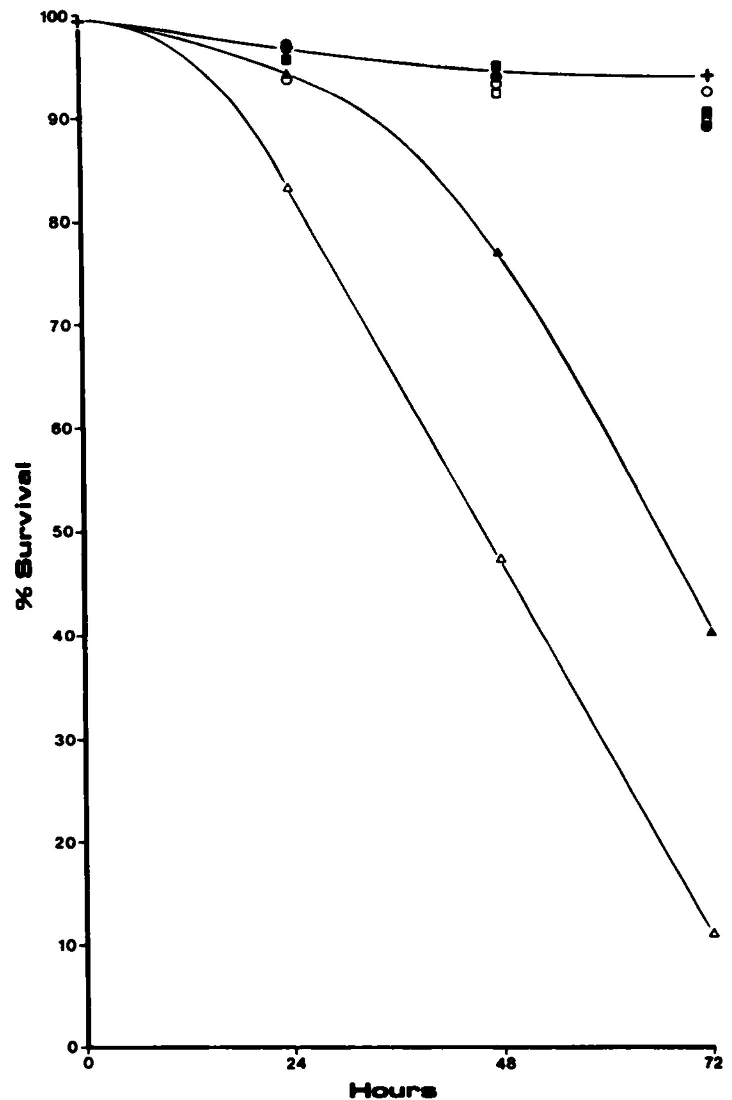

Chart 5. The effect of treatment of human peripheral blood lymphocytes with ellipticine on cell viability. Human peripheral blood lymphocytes were prepared as described in "Materials and Methods" and exposed either for 30 min (○, □, △) or continuously (●, ▼, ■) to various concentrations of ellipticine. Dead or dying cells were determined by their low green (DNA) fluorescence (*i.e.*, green fluorescence less than G_0 nonstimulated lymphocytes). All cultures were prepared in duplicate and exposed either for 30 min to 0.1 (○), 1.0 (□), or 10 (△) µg or continuously to 0.01 (●), 0.1 (■), or 1.0 (▲) µg ellipticine per ml. A minimum of 5 × 10^3 cells were examined at each time point. +, untreated lymphocyte control cultures.

suggest that for FL cells the terminal point of action of ellipticine was in G_2, 40 min prior to mitosis.

Since this method of analysis also permits visualization of the distribution of cells in the remaining phases of the cycle (13), it could be simultaneously demonstrated that ellipticine does not affect the transit of cells through any other portion of the cycle. The fluorescence distributions in Chart 9, *A* and *C*, demonstrate that cells leave the G_1 and S compartments with the same kinetics in vinblastine-treated culture with or without ellipticine. The only difference in the distributions was the obvious lack of accumulation of mitotic cells in ellipticine-treated cultures at longer incubation times (Chart 9*C*).

As cells accumulate in G_2 and mitosis, their relative sensitivity to acid-induced DNA denaturation *in situ* increased (11–14). This was reflected as an increased α_t value for mitotic cells (Chart 9*B*), where α_t represents the ratio of denatured to total (native plus denatured) DNA (8). In addition, G_2 cells in vinblastine-treated cultures had a higher mean α_t than did the interphase population as a whole (Chart 9*B*). However, the G_2 cells accumulated as a result of ellipticine treatment had a lower mean α_t than interphase cells (Chart 9*D*), indicating a decreased sensitivity to denaturation by acids and, thus, an altered chromatin structure.

When cultures were exposed to 10 µg ellipticine per ml, there was no observable increase in mitotic cells at any time point; rather, there was a decrease in the percentage of mitotic cells at 4 hr and after (Chart 8). Thus, 10 µg ellipticine per ml represents a cytotoxic concentration for FL cells when present for extended lengths of time, resulting in an immediate cessation in cell progression and, eventually, cell death.

Table 4

Effect of ellipticine on survival of PHA-stimulated lymphocytes

Culture (hr)	Treatment (hr)[a]	% of viable cells at the following concentrations of ellipticine			
		0 µg/ml	0.01 µg/ml	0.1 µg/ml	1.0 µg/ml
48	0	91.4			
66	18	91.8	87.4	91.4	84.8
90	42	86.4	87.4	90.8	80.9
90	18		88.5	86.3	88.3

[a] Length of time ellipticine was present in the culture prior to analysis.

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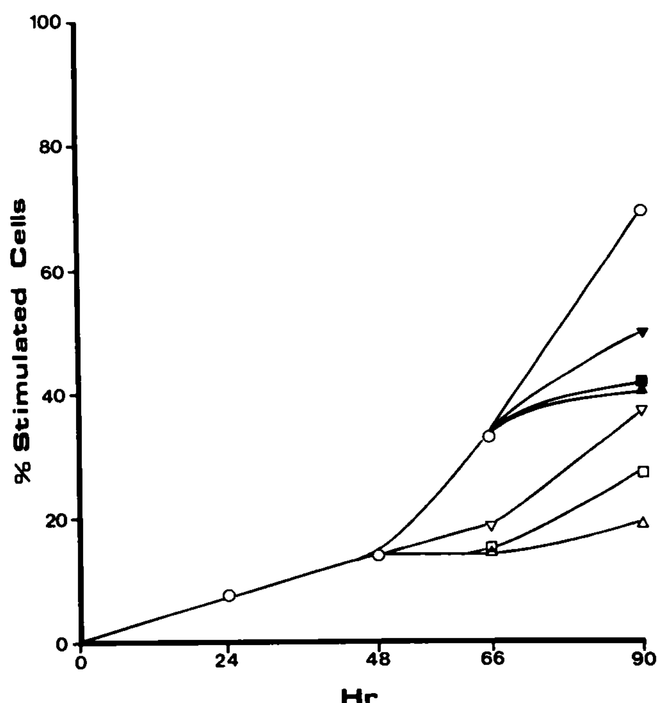

Chart 6. Effect of ellipticine on PHA stimulation of human peripheral blood lymphocytes. PHA-stimulated cultures were prepared as in "Materials and Methods." The cultures when stained with AO as described (30) could be divided into nonstimulated (G_0) cells and stimulated lymphocytes based on their increase in red (RNA) fluorescence (10). PHA-stimulated cultures were split at 48 and 66 hr, and 0.01 (▽, ▼), 0.1 (□, ■), or 1.0 (△, ▲) µg ellipticine per ml were added. A minimum of 5 × 10^{3} cells were analyzed in control (○) and drug-treated cultures for the percentage of stimulated cells.

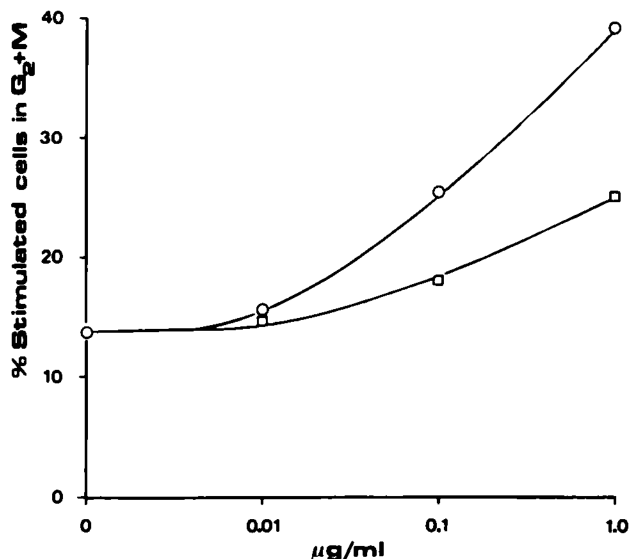

Chart 7. The percentage of increase of G_2 cells in ellipticine-treated PHA-stimulated lymphocyte cultures. The percentage of cells with 4C levels of DNA (G_2 + M) was determined by use of an interactive computer program. Ellipticine was added either prior to (48 hr, ○) or during (72 hr, □) active cellular proliferation in these cultures. All cultures were sampled following a total of 90 hr incubation.

DISCUSSION

The cytostatic and cytotoxic effects of ellipticine *in vitro* depend on a variety of factors. As discussed below, not only did the various cell types respond differently to the drug, but, in addition, the duration of drug exposure, the proliferative or quiescent state of the cells during drug treatment, and, to a certain extent, the phase of the cell cycle all were important. Some, if not all, of these factors may be relevant in assessing the utility of a prospective antitumor agent.

Cell Type Differences. The 3 permanent cell lines studied varied little in the range of ellipticine required to produce a clear cytostatic effect under equivalent conditions of cell concentration and length of exposure. Thus, continuous exposure of FL and L1210 cells (Chart 3) and CHO cells (Chart 4) to 1.0 µg ellipticine per ml resulted in an accumulation of a majority of cells in G_2 by 24 hr (Table 1). Lower concentrations of the drug (0.01 and 0.1 µg/ml) produced transient perturbations of FL (Chart 3, *A* and *B*) but not of L1210 (Chart 3, *D* and *E*) or CHO cells (not shown). Interestingly, relatively low concentra-

Table 5

Effect of ellipticine on cellular RNA content in various cell lines

Cell line	Mean G_2 RNA content: Control	Mean G_2 RNA content: Ellipticine (1.0 µg/ml)	RNA_D/RNA_C [a]
FL	27.3	54.5	1.99
L1210	29.2	45.4	1.55
CHO	46.8	57.7	1.23

[a] Ratio of mean G_2 RNA content of drug-treated *versus* control cultures.

Table 6

Fraction of cells with greater than 4C DNA content

Cell line	% of cells with >4C DNA content: Control	% of cells with >4C DNA content: Ellipticine [a]	D/C [b]
FL	1.0	6.3	6.30
L1210	0.9	12.5	13.88
CHO	2.4	2.8	1.17

[a] Conditions of treatment were 1.0 µg ellipticine per ml present continuously in culture for 24 hr.
[b] Ratio of cells with greater than 4C DNA content in drug-treated *versus* control cultures.

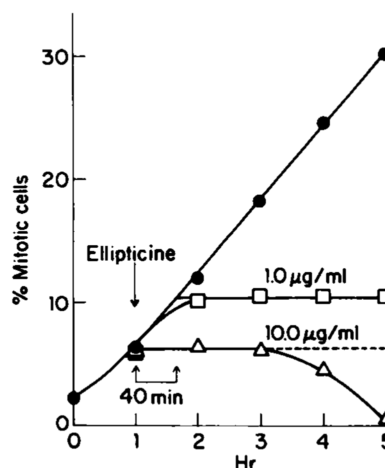

Chart 8. Terminal point of action of ellipticine in FL cells. Logarithmically growing FL cells were treated with 0.5 µg vinblastine sulfate per ml and split into 3 cultures. After 1 hr of incubation, 1.0 and 10 µg ellipticine per ml were added to 2 of the cultures. Ten-ml samples of cells were removed at appropriate times and fixed as described in "Materials and Methods." Following RNase treatment, cells were stained with AO after acid denaturation in a pH 1.5 buffer (see "Materials and Methods"). The percentage of mitotic cells was calculated automatically using an interactive computer program which allows appropriate thresholds to be set, thereby separating interphase from mitotic cells.

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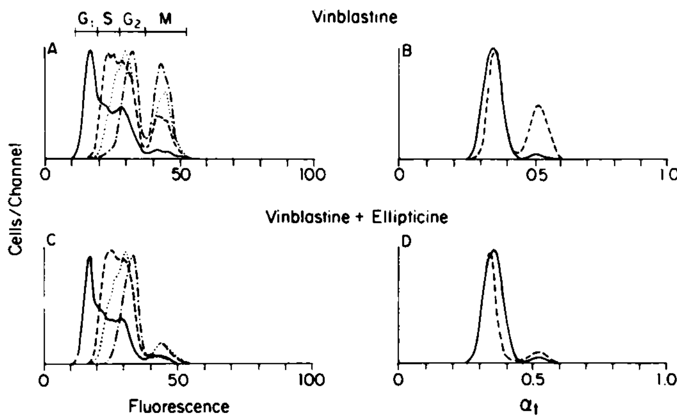

Chart 9. The fluorescence and α_t distribution of interphase and mitotic FL cells, grown for various lengths of time in the presence of vinblastine and the presence or absence of 1.0 μg ellipticine per ml and denatured in a pH 1.5 buffer. Cell treatment and staining was as in "Materials and Methods." *A*, fluorescence distribution of cells grown in the presence of 0.5 μg vinblastine per ml for 0 (——), 3 (----), 5 (····), and 7 (-·-) hr. *C*, fluorescence distribution of identical samples to which 1.0 μg ellipticine per ml was added at 1 hr for the same time points as in *A*. The relative fluorescence intensities allow for the discrimination of cells in G_1, S, G_2, and mitosis. *B* and *D*, the distribution of interphase and metaphase cells along the α_t axis. *B*, cells treated with vinblastine only for 0 (——) or 5 (----) hr. *D*, cells treated with vinblastine plus ellipticine as in *C* for either 0 (——) or 5 (----) hr. Note the modest increase of cells in metaphase following treatment with vinblastine plus ellipticine (*D*) compared to the large increase in mitotic cells observed in cultures treated with vinblastine alone (*C*).

tions of ellipticine (0.1 μg/ml) produced a G_2 block in stimulated lymphocyte cultures (Chart 7), although the length of exposure of the cells in these cultures was considerably longer (42 hr) compared to the experiments on the permanent cell lines.

The cytotoxic effects of ellipticine were monitored in several ways. CHO cells which grow attached to the culture vessel and form discrete colonies when plated at low cell densities were used to examine the effect of ellipticine on clonability. This is a particularly sensitive assay of drug cytotoxicity, since it reflects both the effect of the drug on the proliferative potential of the cells and the immediate cell kill. Continuous exposure of asynchronously growing CHO cells for 24 hr to various concentrations of ellipticine resulted in an exponential inhibition of colony formation (Chart 1). The dose required for ICF_{50} under these culture conditions was 0.3 μg/ml (Table 1).

Short-term cell killing of suspension cultures was monitored by scoring cells able to exclude trypan blue. Thus, cell viability was based on the preservation of the integrity of the cell membrane; the disruption of cellular integrity is generally considered to be a less sensitive assay of cytotoxicity than loss of proliferative potential.

FL and L1210 cells were roughly similar in their response to 24 hr continuous drug exposure (Table 3). The LD_{50}'s for these cell lines were 2.0 and 1.15 μg/ml for FL and L1210 cells, respectively (Table 1). As a comparison, following 24 hr continuous exposure to 1.0 μg ellipticine per ml, only 5% of the unstimulated lymphocytes failed to exclude trypan blue (Chart 5).

Exposure of lymphocytes previously stimulated with PHA to varying concentrations of ellipticine had little effect on cell viability (Table 4). Thus, while the viability of non-drug-treated PHA cultures was typically in the range of 90%, 18 hr exposure of these cells to ellipticine concentrations as high as 1.0 μg/ml had no additional effect on viability (Table 4).

It would appear, therefore, that while human peripheral blood lymphocytes are more sensitive to the cytostatic action of ellipticine, they are considerably more resistant to cell killing than either of the leukemic cell lines. This was true whether the drug was added in the absence of or subsequent to stimulation by PHA (Table 1).

Duration of Exposure. If the time of exposure of cells to ellipticine was reduced from 24 to 2 hr, then a 10 times higher drug concentration was required to produce a comparable (*i.e.*, permanent) G_2 block when monitored at an equivalent point (24 hr) in time (Chart 2).

Inhibition of colony formation of asynchronously growing CHO cells as determined by the dose required for ICF_{50} was also dependent upon the length of drug exposure. The ICF_{50} for asynchronously growing CHO cells exposed to ellipticine for 2 hr was 20 times higher than if cells were cultured for 24 hr in the presence of the drug (Table 1). In addition to a difference in the ICF_{50}, short-term exposure to the drug (2 hr) resulted in a shoulder at low concentrations (1.0 μg/ml and below) which preceded the exponential portion of the curve (Chart 1). The presence of a shoulder region may be due to the fact that cells accumulate damage which ultimately leads to a lethal effect and that survivors have sublethal damage which is repaired (15).

A log lower efficiency in cell killing of unstimulated human lymphocytes was also observed when comparing continuous *versus* short-term (30 min) exposure to ellipticine. In the latter case, a significant decrease in cell survival was observed only after exposure to the relatively high concentration of 10 μg ellipticine per ml (Chart 5).

Thus, if the length of drug exposure was reduced from a period in excess of the cell generation time to one covering a relatively small portion of the cell cycle, a 10 to 20 times higher drug concentration was required to produce a comparable cytostatic or cytotoxic effect.

Stationary or Quiescent *versus* Cycling Cells. CHO cells grown to a high cell density (stationary cultures), in contrast to exponentially growing CHO cells, required a significantly higher drug concentration to achieve ICF_{50} (Table 1). This was the case whether the cells were exposed to the drug for 2 or 24 hr (Chart 1; Table 1). Likewise, as noted above, unstimulated human lymphocytes (quiescent cells) were more resistant to the cytotoxic action of ellipticine than were the leukemic cell lines (Tables 1, 3, and 4).

The basis for increased resistance of quiescent or stationary cells is not well understood. One may speculate that quiescent or stationary cells have either altered membrane permeability (decreased penetration of the drug) or different chromatin structure (more condensed) which may limit the binding of the drug and thus decrease its cytotoxicity.

Cell Cycle Specificity. The sensitivity of synchronized CHO cells to ellipticine varied somewhat, depending upon the phase of the cell cycle (Table 2). It should be pointed out, however, that the increased sensitivity of mitotic-early G_1 cells as presently noted was not nearly as dramatic as was observed by others for DON cells in which mitotic and early G_1 cells were up to 250 times more sensitive to ellipticine than G_2 cells (3, 4). These quantitative differences between CHO and DON cells may be due to a variety of factors, including the difference in the methods of obtaining synchronized cells (*i.e.*, DON cells were synchronized by brief Colcemid arrest) and the differences in the length of the G_1 phase of DON *versus* CHO cells

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[mean of 6.5 hr for CHO (7) *versus* 1 to 2 hr for DON (3)]. The basis for the differential sensitivity of G_1 cells is not known; perhaps, as in the case of stationary *versus* cycling cells, the sensitivity may be modulated by changes in chromatin structure.

Increase of Cells with Greater than 4C DNA Content (Terminal Point of Action). Interestingly, while suboptimal concentrations of ellipticine resulted in a transient accumulation of cells in G_2 + M, perhaps as a result of increased cell transit time through G_2 phase, higher concentrations of the drug which were effective in blocking cells in G_2 (1.0 μg/ml) also resulted in a significant increase in cells with greater than a 4C amount of green (DNA) fluorescence in 2 of the 3 cell cultures studied (Table 6). The exact mechanism responsible for this observation is not known. The terminal point of action of ellipticine, 40 min prior to mitosis (Chart 8), coincides with the point of action of several inhibitors of protein synthesis, such as puromycin and cycloheximide (28). Thus, the drug may inhibit cell division-dependent RNA or protein synthesis (3) or results (as has been described for DON cells) in chromosome damage (3), thereby interfering with spindle fiber attachment or movement. In either instance, the result would be a block in late G_2 phase or mitosis. However, with FL and L1210 cultures, some cells may either proceed directly into the next round of DNA synthesis without passing through mitosis or undergo endomitosis. The latter phenomenon was observed in FL cells treated with anthracenedione (17), a DNA-intercalating agent. Regardless of the mechanism responsible for the appearance of cells with greater than a 4C content of DNA, survival of these cells in culture is limited; cell death occurs within 72 hr of removal of ellipticine.[5]

RNA Content. An additional observation relating to the mechanism of action of the drug was the increase in RNA content of cells blocked in G_2 by ellipticine (Table 5). While some tightly bound DNA-intercalating agents such as actinomycin D and daunomycin have been observed to affect RNA chain elongation, easily dissociable intercalators such as proflavin, ethidium, and ellipticine did not affect RNA chain elongation, although they did exhibit a moderate selectively in inhibiting nucleolar 45S RNA synthesis (20). Intercalators of this type, such as ethidium, are also capable of intercalation into double-stranded helical regions of RNA (18), interfering with the processing of preribosomal RNA (27) and the integrity and function of RNA in protein synthesis (2).

Since the major RNA class stained by AO in this system is rRNA (9), the observation of a net increase in RNA content could be due either to a slower breakdown of rRNA (increased half-life) or, in the absence of cell division, continued accumulation of rRNA even at a reduced rate of synthesis, or both. This phenomenon has been observed previously in cells blocked in G_1 either by growth at nonpermissive temperatures (1) or by hydroxyurea or 5-fluorodeoxyuridine (6, 7) and blocked in G_2 by the action of other agents such as anthracenedione (NSC 287513) (16) and dihydroxyanthraquinone (NSC 279836) (32).

Chromatin Changes. G_2 cells blocked by the drug had decreased susceptibility of DNA *in situ* to acid denaturation (decreased mean α_t; Chart 9) as compared with G_1 or S cells in the absence of the drug. Lower α_t values generally indicate decreased chromatin condensation, since mitotic cells (11), differentiated erythroblasts (31), and G_0 lymphocytes (13, 14), all characterized by highly condensed chromatin, are more sensitive to acid denaturation (increased α_t) than their undifferentiated or actively cycling counterparts. Intercalating agents, among them ellipticine and several of its analogs, increase the melting temperature of DNA in solution (3). Whether this is directly responsible for the observed increase in the stability of ellipticine-treated cells to acid denaturation is not clear. However, since the effect of the drug on cellular proliferation and transcription probably arises as a result of its interaction with DNA *in situ*, it is not surprising that this interaction was also manifested as a change in the acid-induced denaturability of treated cells, *i.e.*, in an alteration in chromatin structure.

[5] F. Traganos, D. P. Evenson, L. Staiano-Coico, Z. Darzynkiewicz, and M. R. Melamed, manuscript in preparation.

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