ABSTRACT

Exposure of L1210 cells to DNA-intercalating antitumor drugs Novantrone (mitoxantrone; 20 ng/ml), doxorubicin (0.5 µg/ml), ellipticine (5 µg/ml), or the doxorubicin analogue AD198 (0.4 µg/ml), for 1 h, results in inhibition of cell proliferation, arrest of cells in the G2 phase of the cell cycle, and an increase in the number of cells entering higher DNA ploidy. These effects are significantly reduced when 5 mM concentrations of caffeine or pentoxifylline are present either simultaneously with, or, in some cases, when added for 1 h immediately following pulse exposure to the drug. Both caffeine and pentoxifylline alone (5 mM) have little effect on cell growth or cell cycle progression. The possible mechanism of cell protection against intercalating drugs provided by caffeine was studied spectrophotometrically by measuring the interaction between Novantrone and the caffeine chromophore and in a model system using permeabilized L1210 cells and measuring the effect of caffeine in reducing binding of the intercalating dye acridine orange to cellular DNA and RNA. The data indicate that the observed protection of cells against intercalating drugs by caffeine or pentoxifylline is most likely a consequence of the direct interaction between the methylxanthines and the planar aromatic molecules of the intercalating drugs: formation of caffeine-drug complexes in solution effectively lowers the concentration of the free drug and thereby reduces its pharmacological activity. The principle of selective entrapment of the intercalator by compounds like caffeine may be considered in designing strategies to modulate the activity of intercalating drugs in vivo, e.g., in lowering drug toxicity when inadvertently applied at too high doses.

INTRODUCTION

CAF, a methylxanthine, has a multiplicity of effects on cells in culture. At high concentrations (>10 mM), CAF prolongs the duration of the G2 phase (1, 2), inhibits enzymes required for DNA synthesis (3, 4), and causes increased chromatid condensation (5, 6). When combined with a wide variety of DNA-damaging agents (e.g., ionizing radiation, alkylating agents, cisplatinum analogues, hydroxyurea, etc.), CAF enhances cell killing, presumably by shortening the time of cell cycle arrest normally caused by such agents, thereby limiting the repair of potentially lethal DNA damage (7). However, earlier reports of Ross et al. (8), Ganapathi et al. (9), and Iliakis et al. (10) indicated that CAF can diminish the cytotoxic and/or cytostatic effects of the intercalating agent, DOX. CAF was also capable of reducing the toxicity of the classical DNA intercalator, ethidium bromide, possibly by reducing the intracellular concentration of the drug (11).

We recently reported that CAF can reverse the cytotoxic and cell kinetic effects of another intercalating drug, NOV, on Chinese hamster ovary cells, mouse lymphocytic leukemia L1210, and human promyelocytic HL-60 cells (12). One-h exposure of cells to CAF alone, up to a concentration of 5 mM, had no significant effect on subsequent cell growth or cell cycle progression. This concentration of CAF, however, given together with a pulse of NOV, dramatically reduced both the cytotoxic and cytostatic effects of the latter in all the cell lines tested (12). These observations raised the question as to whether the ability of CAF to reverse the cytokinetic effects of two intercalating antitumor agents may be a more general phenomenon common to a wider range of intercalating agents. This prompted us to test the effects of CAF on additional intercalating drugs as well as to try to elucidate the mechanism by which CAF is so effective in reducing the pharmacological effects of these drugs.

In the present study, we systematically tested, under comparable conditions, the efficacy of CAF and TRN in reducing the cytostatic effects of the intercalating drugs NOV, ELP, DOX, and the highly lipophilic N-alkyl derivative of DOX, AD198 (13), on L1210 cells in culture. The mechanism of this phenomenon was studied in two ways. In competition experiments, the binding of the fluorescent, intercalating probe AO to nucleic acids in intact, permeabilized L1210 cells was assayed in the presence and absence of CAF by flow cytometry. AO structurally resembles many intercalating antitumor agents and its spectral characteristics are well known (Ref. 14 and references therein). In addition, the direct interactions between AO and CAF and NOV and CAF were determined in solution studies by spectrophotometry. The data suggest that at least some of the observed effects of CAF in modulating the activity of intercalating drugs may be due to direct interaction between these agents and CAF. These interactions may reduce the effective concentration of the free monomeric form of the drug in solution, which is then reflected as diminished pharmacological activity.

MATERIALS AND METHODS

Cell Lines

L1210, a murine leukemic cell line used throughout this study, was grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (GIBCO, Grand Island, NY) as described previously (15). Within the cell concentration range of 1–6 × 10^6/ml, growth was exponential with an apparent doubling time of approximately 11 h.

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2 To whom requests for reprints should be addressed, at The Cancer Research Institute, 100 Grasslands Road, Elmsford, NY 10523.

3 The abbreviations used are: CAF, caffeine; TRN, trental, pentoxifylline; NOV, Novantrone, mitoxantrone; DOX, doxorubicin, 14-hydroxydaunomycin hydrochloride; ELP, ellipitcin; AD198, N-benzyladoumonycin-14-valerate; AO, acridine orange; DAPI, diamidino-2-phenylindole; HBSS, Hank's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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CAFFEINE REVERSES THE EFFECTS OF DNA INTERCALATORS

Drugs

CAF (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) and TRN [3,7-dihydro-3,7-dimethyl-1H-purine-2,6-dione] were obtained from Sigma Chemical Co. (St. Louis, MO). Both agents were dissolved in RPMI 1640 medium to form a 0.05 mM stock solution; heating at 70°C for 30 min was necessary to completely dissolve the CAF. NOV (1,4-dihydroxy-5,8-bis[2(2-hydroxy)aminoethylaminio]-9,10-anthracenedione) and ELP [5,11-dimethyl-6H-pyrido(4,3-b)carbazole] were provided by the Investigational Drug Branch of the National Cancer Institute and were dissolved at a concentration of 1.0 mg/ml in 0.1 N HCl followed by further dilutions in HBSS (GIBCO) or medium (15, 16). DOX hydrochloride (14-hydroxydaunomycin) from Sigma was dissolved as a 1.0-mg/ml stock solution in 5 mM Hepes buffer containing 0.15 N NaCl at pH 7.0. Further dilutions of DOX were in RPMI 1640 medium. AD198 (N-benzyladriamycin-14-valerate), an N-alkyl derivative of DOX synthesized by Dr. Mervyn Israel of the University of Tennessee College of Medicine and provided by Dr. Milan Potmesil of New York University School of Medicine, was dissolved in HBSS to form a stock solution of 0.1 mg/ml; AD198 is lipophilic and requires time to dissolve.

Drug Treatment

Single Drug Treatment. Suspension cultures of L1210 cells (10 ml) in 25-cm² tissue culture flasks were treated for 1 h with 5 mM CAF, 5 mM TRN, or the appropriate concentration of drug (i.e., 20 ng/ml NOV; 0.5 μg/ml DOX; 5 μg/ml ELP; 0.4 μg/ml AD198), centrifuged for 5 min at 900 rpm, resuspended in 10 ml fresh, prewarmed complete medium, and grown for an additional 24 h in RPMI 1640 medium. Untreated, control cultures were centrifuged and resuspended in fresh medium as above. Cell counts were performed at 1 h posttreatment with 5 mM CAP (or TRN), followed by counting trypan blue-excluding cells on a hemocytometer. In some instances, cell proliferation was expressed as the ratio of the difference in cell count between 1 and 24 h of the drug-treated cultures compared to control, untreated cultures.

Multiple Drug Treatment. In addition to the cultures treated with a single intercalating agent, CAF or TRN, parallel cultures were treated for 1 h with the appropriate concentration of a particular drug plus 5 mM CAF (or TRN) or first exposed for 1 h to a pulse of drug, followed by a 1 h exposure to 5 mM CAF (or TRN), which was then removed and fresh drug-free medium added. L1210 cells were also exposed continuously to 5 mM CAF (24 h). In addition, cultures treated with 5.0 μg/ml ELP alone or in combination with 5 mM CAF were exposed, following the removal of ELP or ELP + CAF, to medium containing 5 mM CAF for the remaining 24 h of culture. In each instance, cell aliquots (0.2 ml) were taken for cell counts after the final resuspension in drug-free medium and again at 24 h after the addition of the first agent. Each set of experiments was repeated at least 3 times with essentially the same results; the data presented in histogram form are generally of one representative set of experiments.

Cell Cycle Analysis

Aliquots containing several (2–5) ml of cell suspension were harvested at the appropriate time and centrifuged at 900 rpm for 5 min, and the cells were resuspended in 0.5 ml of HBSS. A 0.2-ml aliquot of the concentrated cell suspension was then diluted with 2.0 ml of ice-cold staining solution consisting of 2 μg/ml DAPI (Polysciences Inc., Warrington, PA)-0.1% (v/v) Triton X-100 (Sigma)-0.1 μM pipaziner-N,N′-bis(2-ethane) sulfonic acid buffer, pH 6.4 (Calbiochem, La Jolla, CA)-2 mM MgCl₂ (17). DAPI fluorescence of individual cells was measured with an Ortho Diagnostics ICP-22 flow cytometer (Becton Dickinson Immunocytometry Systems, Mountainview, CA) using a UGI UV excitation filter and an emission filter transmitting between 450 and 510 nm. The fluorescence signals from a minimum of 1 × 10⁶ cells were recorded as a 1024-channel frequency distribution on an IBM XT personal computer, translated into a 256-channel ASCII file, and analyzed on an IBM AT computer using the Multicycle program (Phoenix Flow Systems, San Diego, CA). The coefficient of variation of the mean value of the G₁ peak of the DNA distribution of untreated L1210 cell cultures was typically 2.0%.

Effect of CAF on AO Interaction with Nucleic Acids

Flow Cytometry. Cells from an exponentially growing L1210 culture were suspended in whole medium at a concentration of 1–5 × 10⁶ cells/ml. Small aliquots (0.2 ml) were removed and treated for 30 s with HBSS (control) or CAF at a final concentration varying from 0.1 to 10.0 μM. Each aliquot, in turn, was stained with the metachromatic dye AO using a modified one-step procedure (18). Thus, 0.6 ml of a solution containing 5 μg/ml AO (Polysciences), 0.07% (v/v) Triton X-100, 10⁻² M EDTA, and 0.15 N NaCl was added to 0.2 ml of cells in medium. Under such staining conditions, the cells become permeable as a result of the detergent treatment. The AO binds stoichiometrically to double-stranded DNA by intercalation and fluoresces green, while RNA, rendered single stranded by the procedure, binds the dye by a complex reaction leading to condensation of the dye-nucleic acid complex and emits red luminescence (phosphorescence) following excitation with blue light. The term luminescence, which covers both simple fluorescence and phosphorescence, is used throughout the text to describe the emission of AO bound to single-stranded nucleic acids (14).

The mean green and red luminescence of the G₁ population of the L1210 cells was measured immediately following addition of the stain with an Ortho Diagnostics FC 200 flow cytometer (Becton Dickinson); green fluorescence was measured in a band from 515–570 nm and red luminescence at wavelengths >610 nm. One of the control samples, which did not receive CAF, was treated for 15 min with 40 μg/ml RNase (RASE; Worthington Biochemical Corp., Freehold, NJ) at room temperature; the red luminescence remaining following RNase treatment was considered to be due to emission of AO bound to DNA. The luminescence values of 1–3 × 10⁶ cells were collected in list mode on a Compaq 386 personal computer using the Acqcyte software (Phoenix Flow Systems).

Spectrophotometry. Aliquots (2 ml) containing 5–20 μM drugs dissolved in buffer (see figure legends for details) were placed in a quartz cuvette inserted into the thermostatic holder of an IBM 910 UV-visible light-scanning spectrophotometer. The absorption spectra of free dye and the control (buffer only) were measured at 1-nm intervals and stored on a Hewlett Packard 9826 computer. Samples were then treated with small volumes (10–50 μl) of a stock solution of CAF (0.1 M), incubated for 10 min, and scanned again.

The recorded spectra were processed numerically by computer. This process included the subtraction of the spectra of the blank and recalibration of data to allow them to be expressed as molar extinction coefficients. The initial concentrations of drugs were obtained from the absorption of the samples before addition of CAF measured at the isobestic points [λ = 470 nm, E₀ = 4.33 × 10⁶ M⁻¹ cm⁻¹ for AO (19); λ = 682 nm, E₀ = 8.36 × 10⁶ M⁻¹ cm⁻¹ for NOV (20)], where E₀ is the molar absorption coefficient at wavelength λ. The concentration of the drug at the titration points was calculated based on the initial concentration and the diluting factors. The linear extrapolation to [drug]/[CAF] = 0 was used for spectra calculation of the drug-CAF complexes, and the Marquardt-Levenberg algorithm-based program (Sigmaplot; Jandel Scientific, Corte Madera, CA) was used to analyze the multicomponent spectra quantitatively.

RESULTS

Cell Growth and Cell Cycle Distribution

Novantrone. As previously demonstrated (12), a 1-h pulse of 5 mM CAF has a minor effect on the growth of L1210 cells. In the present study, a 1-h pulse of 5 mM CAF inhibited cell growth by <5% when monitored at 24 h, while the cell cycle distribution remained virtually unchanged (not shown).

NOV alone (20 ng/ml, 1 h) decreased the cell growth rate by
52% (Table 1), increased the proportion of cells in the G1M phase, and caused the appearance of cells cycling at a higher (>G2M) DNA ploidy level (Table 1; Fig. 1). Addition of 5 mM CAF during the 1 h of NOV treatment partially reversed the growth rate inhibition caused by NOV (from 48 to 83% of control growth; Table 1) and returned the cell cycle distribution to a near normal pattern (Fig. 1).

Interestingly, this activity was not confined to CAF since TRN, a less toxic derivative of CAF, had an almost identical ability to reverse the effects of NOV on L1210 growth and cell cycle kinetics. Thus, while an equivalent concentration of TRN (5 mM) alone had little effect on cell growth, it almost completely reversed the growth inhibitory effects of NOV in this experiment, from 39% of control growth for the drug alone to 87% with the simultaneous addition of TRN (Table 2). The cell cycle distribution of cultures treated for 1 h with NOV + TRN resembled that of control cultures receiving neither agent (Table 2).

Post-treatment in which the 1-h pulse of CAF followed 1-h exposure to NOV failed to reverse the NOV-induced growth inhibition (49 versus 48% of control cell growth; Table 1) but did result in a shift of the cell cycle distribution manifested as an S rather than a G2 phase accumulation (Fig. 1).

Doxorubicin. A 1-h pulse of 0.5 μg/ml DOX followed by growth in fresh medium caused a 74% inhibition in cell growth (Table 1). However, as shown in Fig. 2, simultaneous addition of 5 mM CAF increased proliferation 360% over that observed with DOX alone, within the DOX concentration range of 0.1 to 1.0 μg/ml.

In parallel to overcoming the effect of DOX on the cell growth rate (Table 1), a simultaneous pulse of CAF in the presence of DOX returned the cell cycle distribution to near normal (Table 1; Fig. 1). Postaddition of CAF to DOX-treated cells had a very modest effect on cell growth relative to cultures treated with DOX alone (37 versus 26% of control, respectively; Table 1). The cell cycle distribution of L1210 cells treated first with DOX followed by CAF appeared to fall intermediate between cultures treated with DOX alone and those treated simultaneously with DOX + CAF (Fig. 1). Thus, few higher ploidy cells were observed and more cells reentered the G1 phase than in DOX-treated cultures (Fig. 1). As with NOV, prior treatment with CAF had no protective effect on cultures subsequently exposed to a 1-h pulse of DOX.

Ellipticine. ELP, at a concentration of 5 μg/ml for 1 h, reduced cell growth to 20% of control values (Table 1). A 1-h

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Table 1 Modulation of the effects of different intercalating drugs on the cell cycle and cell growth of L1210 by CAF

<table>
<thead>
<tr>
<th>Culturedrug</th>
<th>CAF conditions</th>
<th>Cell cycle distribution*</th>
<th>Cell growth (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G1</td>
<td>S</td>
</tr>
<tr>
<td>CON</td>
<td></td>
<td>32 ± 1</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>NOV</td>
<td></td>
<td>14 ± 1</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>NOV + CAF</td>
<td></td>
<td>27 ± 2</td>
<td>61 ± 3</td>
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<tr>
<td>NOV - CAF</td>
<td></td>
<td>18 ± 1</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>DOX</td>
<td></td>
<td>34 ± 2</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>DOX + CAF</td>
<td></td>
<td>4 ± 1</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>DOX - CAF</td>
<td></td>
<td>30 ± 2</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>ELA</td>
<td></td>
<td>13 ± 2</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>ELP</td>
<td></td>
<td>3 ± 1</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>ELP + CAF</td>
<td></td>
<td>27 ± 2</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>ELP - CAF</td>
<td></td>
<td>39 ± 2</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>AD198</td>
<td></td>
<td>22 ± 4</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>AD198 + CAF</td>
<td></td>
<td>25 ± 4</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>AD198 - CAF</td>
<td></td>
<td>31 ± 1</td>
<td>53 ± 3</td>
</tr>
</tbody>
</table>

* The cell cycle distributions represent the means ±SD of at least three separate experiments. All distributions were determined 24 h after the start of the experiment.

Drug treatment consisted of a 1-h pulse of 20 ng/ml (45 nM) NOV, 0.5 μg/ml (0.9 μM) DOX, 5 μg/ml (20 μM) ELP, or 0.4 μg/ml (0.56 μM) AD198.

Cells with DNA content greater than twice G1 phase cells, —, no change in higher ploidy level cells relative to control cultures (e.g., 1-2%).

---

Fig. 1. DNA frequency distributions of L1210 control (CON) cells and cells treated for 1 h with NOV or DOX alone, in combinations with 5 mM CAF, or followed by a 1-h pulse of 5 mM CAF. DNA distributions were obtained following cell staining with DAPI as described in the text. Calculations of cell cycle distribution into G1, S, G2M, and >G2M compartments were obtained using the Multicycle program on an IBM AT personal computer; S phase has been shaded for clarity. L1210 cultures received no drug or 20 ng/ml NOV or 0.5 μg/ml DOX for 1 h, followed by 23 h growth in drug-free medium. Other cultures received a 1-h pulse of NOV or DOX as above plus 5 mM CAF or a 1-h pulse of NOV or DOX, followed by a 1-h pulse of 5 mM CAF. After removal of the drugs, the cells were cultured in drug-free medium. All DNA distributions were obtained 24 h after the start of the experiment.

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pulse of ELP caused a dramatic G2 phase accumulation and, as with NOV, an increase in cells cycling at higher ploidy (Table 1; Fig. 3). Thus, under these culture conditions, virtually no L1210 cells divided, leading to almost total loss of G1 phase cells (Fig. 3).

Simultaneous cell treatment with 5 mM CAF + ELP partially reversed both growth inhibition (Table 1) and the cell cycle effects of ELP. Although considerable numbers of cells continued to accumulate in G2M (20 versus 10% in control), cells continued to divide with virtually no increase in the number of higher DNA ploidy cells (Fig. 3). If, however, the 1-h CAF pulse followed ELP treatment, the cell cycle distribution returned to normal (Table 1; Fig. 3) and cell growth increased further, although not to normal limits (Table 1). Pretreatment with a 1-h pulse of CAF had no protective effect on subsequent exposure to ELP.

ELP-treated cultures were also exposed to CAF for extended periods. Thus, in cultures exposed to ELP for 1 h, washed free

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Cell cycle distribution</th>
<th>Cell growth (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOV* MEX*</td>
<td>G1 S G2M &gt;G2M</td>
<td></td>
</tr>
<tr>
<td>- -</td>
<td>36 ± 2 55 ± 1 9 ± 1 —</td>
<td>100</td>
</tr>
<tr>
<td>+ -</td>
<td>17 ± 3 43 ± 2 29 ± 4 11 ± 1 —</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>- CAF</td>
<td>38 ± 1 53 ± 1 9 ± 1 —</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>- TRN</td>
<td>37 ± 1 56 ± 1 7 ± 1 —</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>+ CAF</td>
<td>37 ± 2 54 ± 1 9 ± 2 —</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>+ TRN</td>
<td>34 ± 1 56 ± 3 10 ± 2 —</td>
<td>87 ± 4</td>
</tr>
</tbody>
</table>

* NOV treatment (+) consisted of a 1-h pulse of 20 ng/ml followed by resuspension in drug-free medium. Cell cycle distribution and cell growth were determined 24 h after the start of the experiment as described in "Materials and Methods." —, none.

Methylxanthines (MEX) CAF and TRN were added at a concentration of 5 mM for 1 h in the absence or presence of NOV. The cells were then washed free of drugs, resuspended in drug-free medium, and returned to culture for an additional 23 h.

Cells with DNA content greater than twice G1 phase cells. —, no change in higher ploidy cells relative to control cultures (e.g., 1-2%).

Mean ± SD of at least 3 separate experiments.

Fig. 2. Proliferation of L1210 cells treated with different concentrations of DOX in the absence and presence of 5 mM CAF. Cultures were treated for 1 h with varying concentrations of DOX or DOX plus 5 mM CAF, followed by 23 h growth in drug-free medium. Cell counts of trypan blue-excluding cells were made at 1 and 24 h. Cell proliferation was calculated as the difference in cell number between 24 and 1 h in the drug-treated cultures divided by the difference in control cultures at the same time points; thus, a value of 1.0 indicates no difference in the proliferation rate compared to control, while 0 indicates no cell growth in drug-treated cultures. A 1-h pulse of CAF alone, decreases cell growth by 5-10%.

of drug and cultured for 23 h in medium containing 5 mM CAF, there was a decrease in cell proliferation compared to ELP treatment alone (19 versus 27% of control, respectively), while the cell cycle distribution remained markedly perturbed (Table 3). If CAF was present during ELP treatment and added with the medium following washing, growth remained depressed (30.0% of control) and the cell cycle distribution continued to contain considerable numbers of cells cycling at a higher DNA ploidy level (Table 3).

N-Benzyladriamycin-14-valerate. AD198, an N-alkyl derivative of DOX, when applied to L1210 cells as a 1-h pulse at a
Table 3  Long-term exposure of ELP-treated LI210 cells to CAF

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>ELP°</th>
<th>CAP*</th>
<th>Cell cycle distribution</th>
<th>Cell growth (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G₁</td>
<td>S</td>
</tr>
<tr>
<td>−−</td>
<td>31 ± 1</td>
<td>58 ± 2</td>
<td>11 ± 1</td>
<td>−</td>
</tr>
<tr>
<td>1 h</td>
<td>33 ± 1</td>
<td>57 ± 3</td>
<td>10 ± 2</td>
<td>−</td>
</tr>
<tr>
<td>24 h</td>
<td>39 ± 1</td>
<td>48 ± 3</td>
<td>13 ± 2</td>
<td>−</td>
</tr>
<tr>
<td>−−</td>
<td>2 ± 1</td>
<td>31 ± 3</td>
<td>55 ± 5</td>
<td>12 ± 3</td>
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<td>+24 h</td>
<td>18 ± 1</td>
<td>32 ± 1</td>
<td>36 ± 2</td>
<td>14 ± 3</td>
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<tr>
<td>+24 h</td>
<td>18 ± 2</td>
<td>51 ± 3</td>
<td>16 ± 2</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

° ELP treatment (+) consisted of a 1-h exposure to 5 µg/ml, followed by culture in drug-free medium. Cell cycle distribution and cell growth were determined 24 h after start of experiment as described in "Materials and Methods."

* CAP treatment was for 1 or 24 h in the absence of drug (−, 1 h; or −, 24 h, respectively) or added to ELP-treated cells and for the remaining 23 h after removal of ELP (+, 24 h) or only for 23 h after removal of ELP (+, 23 h).

Means ± SD of at least 3 separate experiments.

Concentration of 0.4 µg/ml, inhibited cell growth by 41% (Table 1). This same treatment led to a characteristic G₂ cell accumulation with a concomitant decrease in G₁ phase cells at 24 h (Table 1; Fig. 3), although maximum cell kinetic effects were generally observed at 48 h (13).

Simultaneous exposure of cells to AD198 and 5 mM CAF did little to change either the cell cycle distribution (Fig. 3) or the inhibition of cell growth observed following exposure to AD198 alone (Table 1). However, if the 1-h pulse of CAF followed the 1-h exposure to AD198, the cell cycle distribution returned to near normal (Table 1; Fig. 3) and the inhibition of cell growth was partially overcome, i.e., the growth rate increased from 59 to 72% of control cell growth (Table 1).

Fig. 4. Intensity of green (DNA) or red (RNA) luminescence of LI210 cells stained with AO in the presence of varying concentrations of CAF. Exponentially growing LI210 cells were treated with 0–10 mM CAF, followed by staining with AO, in the presence of CAF (see "Materials and Methods"). The green and red luminescence, representing AO binding to cellular DNA and RNA for 10³ G₁ phase cells, was obtained by flow cytometry and expressed in arbitrary units. Solid symbols, luminescence associated with control cells following 15 min incubation with 40 µg/ml RNase; the red luminescence remaining following RNase treatment is believed to be the result of dye binding to DNA.

A modified one-step AO-staining procedure was used, and untreated LI210 cells were exposed to CAF at various concentrations within seconds of staining and measurement by flow cytometry. Under such conditions, CAF, at 0.1 mM or less, had no effect on staining of either DNA or RNA by AO (Fig. 4). However, as the concentration of CAF was increased, the red luminescence of AO-RNA complexes decreased dramatically along a straight line on a semilog plot of CAF concentration versus luminescence (Fig. 4). At the same time, CAF, up to a concentration of 5 mM, had little or no effect on AO green fluorescence, indicative of dye binding to double-stranded DNA; at 10 mM CAF, both the green fluorescence and red luminescence of the LI210 cells fell below the levels observed in cells in which all RNA had been digested with RNase (Fig. 4).

Fig. 5. A, spectrophotometric titration of AO (initial concentration, 4.5 µM in 0.01 M NaCl-5 mM Hepes, pH 6.8) with CAF. ——, absorption spectrum of AO alone; ——, spectra of AO in the presence of CAF at 0.5, 1.5, 2.5, 3.5, 4.5, and 5.3 mM; ——, extrapolated spectrum of AO-CAF complex formed at high CAF/dye ratio. Only a part of the recorded spectra are shown. B, comparison of the calculated absorption spectra. AO monomer ( ) and AO dimer ( ), data from Ref. (14) and AO-CAF complex at high CAF/dye ratio (— — —); see A). C analysis (deconvolution) of the spectrum of the mixture of AO (19.4 nM) and CAF (1.5 mM) in a 0.1 M phosphate buffer containing NaCl (total [Na⁺], 0.42 M), pH 5.8 ( ) into three component spectra: AO monomer (——), AO dimer (——), and AO-CAF complex at high CAF/dye ratio (— — —; see also B). These component spectra are scaled in proportion to their molar fraction in the mixture (0.459, 0.211 and 0.330, respectively). Top of C, residue of the analysis (the difference between the measured and the weighted sum of the component spectra).
CAF complex at high CAF/drug ratio ( ). The spectral data for the NOV component spectra: NOV monomer ( ), NOV dimer ( ), and NOV-CAF complex ( ) in 0.15 M NaCl-5 min Hepes, pH 6.8 ( ) into three expressed as a weighted sum of the component spectra. This component spectrum, in all probability, reflects the presence of extrapolation (Fig. 5).

The monomer concentration of the drugs, as a function of CAF concentration, was examined next. The result of titrations of AO and NOV with CAF are presented in Fig. 7. From these data, it can be seen that the monomer fraction of the drugs (AO or NOV) decreased from approximately 60% in the absence of CAF to <5% when the CAF concentration reached the 8-10 mM range.

**CAF-Drug Interaction in Solution**

Aqueous solutions of CAF showed little light absorption above 300 nm in contrast to AO which has maximum absorption at 450–500 nm. This difference in absorption bands makes it possible to study the interaction between CAF and AO spectrophotometrically. As is evident from Fig. 5A, the spectrum of AO is red shifted (9 nm) in the presence of CAF, indicating changes in the electronic structure of the AO chromophore resulting from the formation of complexes between these two compounds. The observed phenomenon is reversible (after dilution; data not shown) which indicates an equilibrium state between the free components and the complex and that the interaction is of the nonbonding (noncovalent) type. The spectrum of the complex, therefore, can only be calculated by extrapolation (Fig. 5).

No isosbestic points were observed, which indicates that more than two components were present in the mixture. The multi-component spectrum, in all probability, reflects the presence of the AO monomer in equilibrium with the AO dimer and AO-CAF complex. The spectra of the AO monomer and dimer are known and can be calculated as previously described (14) (Fig. S4). Because the major portion of total dye molecules are represented by these three components (i.e., AO monomer, dimer, and AO-CAF complex), the resulting spectrum can be expressed as a weighted sum of the component spectra. This treatment is presented in Fig. 5C. The weighted coefficients represent, in this case, molar fractions of the AO monomer, AO dimer, and the AO-CAF complex.

A similar procedure was applied to the NOV-CAF mixture yielding the molar fraction of NOV monomer, NOV dimer, and NOV-CAF complexes in the mixture (Fig. 6).

The present data confirm a previous study (12) in which CAF was shown to be capable of reversing the cell kinetic effects of NOV and extend that observation to DOX, ELP, and AD198. However, it is important to note that the ability of CAF to affect intercalator-induced perturbation of cell growth and cell cycle kinetics depends on both the timing and duration of CAF exposure, since CAF alone affects cell growth: 24-h continuous exposure of L1210 cells to 5 mM CAF, which as a 1-h pulse has little or no effect on cell growth, inhibited growth by nearly 50% and caused a significant G1 phase accumulation (Table 3).

In the original study, Ross et al. (8) assayed survival of L1210 cells after exposure for 1 h to various intercalators, followed by growth in agar for 14–17 days in the absence or presence of CAF. Under these conditions, DOX cytotoxicity was diminished by CAF, while enhanced cell killing was observed when ELP-actinomycin D-, and hycanthone-treated cells were exposed to CAF for 14–17 days (8). Interestingly, if the CAF treatment was limited to <3 h, no enhancement of ELP-induced cytotoxicity was observed (8). In the present study, continuous exposure to CAF following a 1-h pulse of ELP had either no effect on or increased ELP-induced inhibition of cell growth (Table 3), while 1-h CAF treatment simultaneous with or immediately following pulse exposure to ELP resulted in a significant reversal of ELP-induced growth inhibition (Table 1) and returned the cell cycle distribution of L1210 cells to near normal (Fig. 3). This same sensitivity to the duration of CAF exposure was also demonstrated for NOV-treated cells. Whereas, in one set of experiments, a 1-h exposure to CAF in the presence of a 1-h pulse of NOV increased cell growth from 48 to 83% of control (Table 1), we previously reported (12) that extending CAF treatment for an additional 23 h reduced growth from 52% following treatment with NOV alone to 28% following a 1-h pulse of NOV and continuous exposure to CAF.

The second part of this study was aimed toward revealing the mechanisms by which CAF reduces the pharmacological activity of intercalating drugs. From the physiochemical point of view, the mixture of CAF, intercalators, and nucleic acids represent a complex, multiple equilibria system in which several types of interactions exist which are relevant to the observed...
phenomenon. (a) Monomeric forms of both CAF and intercalating drugs interact with single- and double-stranded nucleic acids (14, 20–23). (b) CAF, in solution, undergoes isodesmic self-association, i.e., it has the ability to form indefinite-sized aggregates (21). (c) Aromatic cations such as AO and NOV form aggregates in aqueous solutions, although, at micromolar concentrations of these cations, the size of the aggregates are probably limited to dimers (14, 24). (d) CAF and intercalators can form mixed aggregates. This is evident from spectral studies presented in Figs. 5 and 6. Additional support of this conclusion comes from the observations of Stockert (25) who noted shifts in the AO absorption spectrum and changes in the fluorescence intensity of AO-CAF solutions. Likewise, studies with the intercalating, fluorescent dye ethidium bromide, whose intracellular concentration was reportedly reduced in the presence of CAF, can be explained by formation of CAF-intercalator complexes (11). While the spectra of CAF-drug complexes presented in Figs. 5 and 6 most likely represent drug monomer complexed with an unknown number of CAF molecules (the structure favored by high CAF/drug ratios), there is no reason to believe that drug dimerization cannot also be associated with CAF. The method of monomer calculation used in this study, therefore, only gives approximate results.

The interactions described in c and d above can be quantitated spectrophotometrically by measurement of the concentration of the free intercalator monomer in equilibrium with all types of complexes as described above. A mathematical model which can be applied to the complex mixed-aggregate situation has been recently developed in our laboratory and will be published elsewhere. The approximate calculation presented here indicates that CAF significantly reduces the free drug (monomer) concentration in solution. These data strongly suggest that, because of the formation of CAF-intercalator complexes, the suppression of the effects of intercalating drugs on cell growth may be a direct consequence of the reduction in availability of the monomeric forms of these drugs for interaction with nucleic acids in live cells.

The experiments in which CAF was used to modulate stainability of DNA and RNA with AO in permeabilized cells provides additional support for the mechanism described above. AO is considered to be a model intercalator. Its binding to single- and double-stranded nucleic acids is well characterized and the fluorescence properties of the dye allow study by both flow cytometric and spectrophotometric techniques (26).

AO has two distinct modes of interaction with nucleic acids. This fluorochrome intercalates between the adjacent base pairs of double helical nucleic acids and, when bound in this form, fluoresces green, with maximum emission at 530 nm (26). Interaction of AO with single-stranded nucleic acids involves formation of a complex which undergoes condensation and subsequent precipitation (26, 27). In the condensed or precipitated form, AO exhibits red luminescence, with maximum emission at 640 nm. These different modes of binding provide for differential AO staining of DNA and RNA; AO complexes with DNA fluoresce in the green, while red luminescence results from AO-DNA complexes (28). The differential staining of DNA and RNA in the cell requires use of AO at a specific concentration, which ensures an excess of AO per binding site, and still leaves a relatively high concentration of free AO (about 20 μM, when used in approximately 0.4 M Na+) in solution. When the concentration of free AO decreases below that value, the ability of this dye to condense RNA is lost, but DNA stainability remains virtually unchanged (29). As presently observed, the addition of CAF to permeabilized cells first reduced the stainability of RNA in these cells (red fluorescence), and only at very high concentrations, did CAF affect the AO stainability of DNA (green fluorescence) (Fig. 4). Since CAF was added immediately before staining of permeabilized cells with AO, the observed changes in AO binding cannot be due to differences in RNA content. These results, therefore, suggest that either (a) CAF interacted directly with AO, causing the formation of CAF-AO complexes, thereby reducing the concentration of free AO in solution or (b) in addition to binding directly to AO, CAF also bound to nucleic acids (preferentially RNA), competing with and, thus, reducing the available binding sites for the dye. In light of the evidence that CAF has a relatively low affinity for nucleic acids (21–23), the contribution of the second process would appear less important.

The spectrophotometric studies (Figs. 5 and 6) provided evidence that the reduced stainability of RNA with AO was, most likely, a consequence of the reduction in the concentration of monomeric AO, due to CAF-AO complex formation. The light absorption spectrum of the mixture of AO and CAF can be decomposed into components representing the dye monomer, dye dimer, and dye-CAF complex (Fig. 5B). Using these data, we found that the monomer (free) AO concentration was lowered considerably from about 60% in the absence of CAF to <10% in 5 mM (the concentration used in this study) or <5% in 8–10 mM CAF (Fig. 7). Such a dramatic decrease in AO monomer concentration is expected to directly and primarily effect AO binding to single-stranded RNA as illustrated in Fig. 4 but have little effect on binding to DNA (28, 29). Indeed, when AO was used alone but at a 6-fold lower concentration (starting from 20 μM), the decrease in cell stainability (RNA luminescence) was similar to that observed with 5 mM CAF (data not shown).

It is not obvious, at present, why CAF is most effective in inhibiting the action of NOV and DOX when added at the same time as the intercalators, while in the case of ELP, inhibition is higher when CAF is added after the 1-h ELP pulse and with AD198 CAF has no effect when added simultaneously with, but a modest effect when added subsequent to, the 1-h AD198 pulse (Table 1). CAF does have solubilizing activity however, such that it may displace intercalators already bound to nucleic acids (30). It should also be noted that ELP itself is not active but must be converted to 9-hydroxy-ELP within the cell (31). This conversion may occur somewhat later, following the initial 1-h pulse treatment, i.e., at a time when CAF is most active in reducing the effects of ELP.

Finally, it is clear that the ability of CAF to modulate the effects of intercalating agents is neither unique, since TRN has similar effects, nor is it confined to a single cell type; as was previously reported (12), CAF reverses the cell growth and cell cycle kinetics of NOV in human promyelocytic leukemia HL-60 and Chinese hamster ovary cells. As shown in Table 2, TRN, at the same concentration and under the same treatment conditions as CAF, produced nearly identical results in inhibiting the effects of NOV. Thus, the replacement of the N1 methyl group of CAF with a bulkier acetyltetramethylene substituent [CH₃CO(CH₂)₄-] does not appear to alter the ability of these methylxanthines to reverse the inhibitory effects of intercalators nor enhance the lethality of DNA damaging agents.

The concentrations of CAF presently seen to reduce the effects of intercalators are relatively high, clearly above the pharmacological doses of this alkaloid (4). There are, however, analogues of CAF such as TRN which, at equimolar concentra-
tions, are as effective but are less toxic. The principle of selective entrapment of intercalators by such compounds may, therefore, be considered in the clinic in designing strategies for modulating the activity of these drugs. One of the obvious applications of such a strategy may be mopping up intercalators which have been misapplied or applied at too high a dose.

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Caffeine Modulates the Effects of DNA-intercalating Drugs in Vitro: A Flow Cytometric and Spectrophotometric Analysis of Caffeine Interaction with Novantrone, Doxorubicin, Ellipticine, and the Doxorubicin Analogue AD198

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