Caffeine Prevents Apoptosis and Cell Cycle Effects Induced by Camptothecin or Topotecan in HL-60 Cells

Frank Traganos, Jan Kapuscinski, Jianping Gong, Barbara Ardelt, Robert J. Darzynkiewicz, and Zbigniew Darzynkiewicz

The Cancer Research Institute at New York Medical College, Valhalla, New York 10595

ABSTRACT

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-6,6-dione; CAF) is known to potentiate the cytotoxic effects of DNA damaging agents such as ionizing radiation and alkylating agents. In contrast, however, the cytotoxic and cytostatic activity of aromatic, DNA-intercalating, DNA topoisomerase II inhibitors such as Adriamycin, ellipticine, or mitoxantrone are diminished in the presence of CAF. To resolve whether the protective effect of CAF is associated with a particular mechanism of drug interaction (e.g., intercalation into DNA, inhibition of DNA topoisomerase II), or the aromatic nature of the drug structure, we have presently studied the effects of CAF on the cytostatic and cytotoxic action of camptothecin (CAM) and its less toxic but more water-soluble derivative topotecan (TPT) on HL-60 human myelogenous leukemia cells: both drugs have aromatic structures but are nonintercalating inhibitors of DNA topoisomerase I. By using spectroscopy and titration microcalorimetry, we have also studied the direct interaction between CAF and TPT in solution. Low (20 nM) concentrations of CAM or TPT perturbed progression of HL-60 cells through S-phase, whereas higher concentrations (0.15 μm) of these drugs induced apoptosis; both effects were easily demonstrable after 4 h of treatment. When added simultaneously with CAM or TPT, CAF prevented both effects. The protective effect of CAF was concentration dependent and evident within the concentration range of 1–5 mM; nearly total protection was seen at a CAF concentration of 5 mM. The bathochromic and hypochromic shift in the absorption spectrum of the water soluble compound TPT upon addition of CAF indicated that CAF and TPT interact (stack) in a fashion similar to that previously observed for CAF and DNA intercalators. Microcalorimetric measurements of TPT titration with CAF indicate an exothermic reaction between these compounds (the enthalpy change was ΔH° = -4.2 kcal/mol), which is consistent with a stacking model of CAF-TPT interaction. Thus, the ability of CAF to protect HL-60 cells against the cell kinetic effects of CAM or TPT, as in the case of DNA intercalating topoisomerase II inhibitors, is most likely due to formation of complexes between CAF and these aromatic molecules, which result in reducing the effective concentration of the free form of these drugs available to the cells.

INTRODUCTION

CAF is arguably one of the most widely consumed drugs. Its pharmacological activity varies depending on the dose (concentration) and the type of target cells. In oncology, most studies have focused on the propensity of CAF to potentiate the cytotoxic effects of a variety of DNA damaging agents, such as ionizing radiation, alkylating compounds, cisplatin analogues, etc. (1, 2). The mechanism by which CAF enhances the cytotoxicity of these agents is believed to involve suppression of repair of the potentially lethal DNA lesions induced by such agents (3).

In contrast to this potentiating activity, CAF has been shown to diminish, and under certain conditions to abrogate, the cytotoxic and/or cytostatic effects of several drugs known to interact with DNA by intercalation (4–6). Thus, the effects of doxorubicin, novantrone (mitoxantrone), and ellipticine were markedly reduced when the cells were exposed to these drugs in the presence of CAF (6). Likewise, the toxicity of the classical DNA intercalating fluorochrome, ethidium bromide, was also reduced in the presence of CAF (7).

To date, all agents whose cytotoxic/cytostatic activity has previously been seen to be reduced by CAF share 3 properties: (a) they are flat, aromatic molecules; (b) they interact with DNA by intercalation; and (c) they are DNA topoisomerase II inhibitors. Based on biophysical studies of the interaction between these drugs and CAF in solution, we proposed that the protective effect of CAF may involve sequestration of the drugs via formation of heterologous stacked complexes, which lower the free drug concentration available to the cell. Formation of such complexes is expected to be associated with the aromatic nature of the drug rather than with a particular mechanism of its binding to DNA or the involvement of a particular enzyme (e.g., DNA topoisomerase II). Results of the present study, which demonstrate that CAF reduces the effects of the aromatic, nonintercalating, DNA topoisomerase I inhibitors CAM and TPT, provide additional evidence in support of this mechanism.

MATERIALS AND METHODS

Cell Lines. HL-60 human myelogenous leukemia cells were provided by Dr. Harry A. Crissman of the Los Alamos National Laboratory (Los Alamos, NM). The cells were maintained in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml streptomycin, and 2 μM L-glutamine (GIBCO). The cell have an apparent doubling time of 22 h when growing exponentially over the concentration range of 1 x 10^3 to 1 x 10^6 cells/ml.

Reagents. CAF and CAM [4-ethyl-4-hydroxy-1H-pyrano(3',4':6,7)-indolizino(1,2-b)quinoline-3,14-(4H,12H)dione] were purchased from Sigma Chemical Co. (St. Louis, MO). TPT (no. 104864; 9-dimethylamino-10-hydroxy-campothecin; Smith Kline & French) was kindly provided by Dr. Randall K. Johnson of Smith Kline Beecham Pharmaceuticals (King of Prussia, PA). The stock solution of CAM was prepared in dimethyl sulfoxide at a concentration of 0.5 mM, whereas the other 2 compounds were dissolved in distilled water and diluted in medium or appropriate buffer as described in the text. The chemical structures of both CAM and TPT are shown in Fig. 1.

Drug Treatment of HL-60 Cells. Exponentially growing HL-60 cells were exposed simultaneously to 20 nM CAM and 0.02, 0.5, 1.0, 2.0, or 5.0 mM CAF for 4 h. In addition to control cultures that received no drug, HL-60 cells were incubated (4 h) with 1.0, 2.0, or 5.0 mM CAF alone. The cells from all cultures were then washed in HBSS and fixed in 50% ethanol overnight in the cold (4°C).

In separate experiments, exponentially growing HL-60 cell cultures either received no drug or were exposed to 0.15 μm CAM or TPT. At the time of drug addition, some cultures also received either 0.5, 1.0, 2.0, or 5.0 μm CAF. As above, cultures were washed free of the drug after 4 h of exposure and fixed in 50% ethanol.

A stathmokinetic experiment was performed to determine whether CAF prevented cell transit through S-phase (8). Towards this end, HL-60 cells,

Received 4/22/93; accepted 7/27/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1

This work was supported by USPHS Grant CA 28704, and grants from the Cancer Research Foundation of America and the Carl Inserra Fund. J. G., on leave from the Department of Surgery, Tongji Hospital, Wuhan, China, is supported by the "This Close" Fellowship from the This Close for Cancer Research Foundation. R. J. D., a graduate student of the University of California at Berkeley, worked as a volunteer during summer vacation.

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

3 The abbreviations used are: CAF, caffeine; CAM, camptothecin [4-ethyl-4-hydroxy-1H-pyrano(3',4':6,7)-indolizino(1,2-b)quinoline-3,14-(4H,12H)dione]; TPT, topotecan dihydrochloride (9-dimethylamino-10-hydroxy-campothecin); HBSS, Hank's balanced salt solution; DI, DNA index, where G1 cells have a value of 1.0.
which received either 0 or 5 μM CAF, also received vinblastine sulfate (Sigma) from the ethanol fixative by centrifugation and resuspension in 1.0 ml of 50% ethanol, and the cell cycle distribution analyzed as described. To maximize extraction of small molecular weight DNA from apoptotic cells exposed to 0.15 μM CAM or TPT are expected to contain cells undergoing apoptosis in which the cell’s internucleosomal DNA is degraded by an endogenous nuclease (9). Removal of the degraded DNA lowers the stain-

**Table 1. Effect of CAF on the cell cycle perturbation induced by 20 nM CAM**

<table>
<thead>
<tr>
<th>CAM (μM)</th>
<th>CAF (μM)</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>Relative S peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>36</td>
<td>49</td>
<td>15</td>
<td>1.32</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>28</td>
<td>61(24.5)</td>
<td>11</td>
<td>1.32</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>38</td>
<td>50(2)</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
<td>40</td>
<td>46(0)</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>5.0</td>
<td>38</td>
<td>48(0)</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>27</td>
<td>63(28.6)</td>
<td>10</td>
<td>1.32</td>
</tr>
<tr>
<td>20</td>
<td>0.5</td>
<td>28</td>
<td>63(28.6)</td>
<td>9</td>
<td>1.32</td>
</tr>
<tr>
<td>20</td>
<td>1.0</td>
<td>31</td>
<td>55(12.2)</td>
<td>14</td>
<td>1.40</td>
</tr>
<tr>
<td>20</td>
<td>2.0</td>
<td>35</td>
<td>49(0)</td>
<td>16</td>
<td>1.48</td>
</tr>
<tr>
<td>20</td>
<td>5.0</td>
<td>32</td>
<td>49(0)</td>
<td>19</td>
<td>1.58</td>
</tr>
</tbody>
</table>

* a The cell cycle distribution was determined 4 h after addition of drugs.
* b Relative position of the peak in S-phase (as shown in Fig. 2, thick arrows) expressed as the DNA index of that peak divided by the mean of the G1 population (which has a DI of 1.0).
* c Numbers in parentheses, percentage increase in S-phase cells relative to control, untreated cultures.

which received either 0 or 5 μM CAF, also received vinblastine sulfate (Sigma) at a final concentration of 0.05 μg/ml. Cultures were harvested after 4 h and fixed in 50% ethanol, and the cell cycle distribution analyzed as described below.

**Cell Cycle Analysis.** HL-60 cells from cultures treated with vinblastine sulfate or 20 nM CAM, in the presence or absence of CAF, were rehydrated from the ethanol fixative by centrifugation and resuspension in 1.0 ml of HBSS.

Cells exposed to 0.15 μM CAM or TPT are expected to contain cells undergoing apoptosis in which the cell’s internucleosomal DNA is degraded by an endogenous nuclease (9). Removal of the degraded DNA lowers the stain-

**DNA Gel Electrophoresis.** Untreated and drug-treated HL-60 cells were collected by centrifugation, washed in phosphate buffered saline (without Ca²⁺ or Mg²⁺), and resuspended at a concentration of 5 x 10⁶ cells/0.5 ml of 45 mM Tris-borate buffer, 1 mM EDTA, pH 8.0, containing 0.25% Nonidet P-40 (Sigma) and 0.1% RNase A (Sigma). The mixture was incubated at 37°C for 30 min and then incubated for an additional 30 min at 37°C with 1 mg/ml proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN). Next, a loading buffer (0.1 ml of 0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added and 25 μl of the tube content transferred to the gel. Horizontal 1.5% agarose gel electrophoresis was performed at 2 V/cm for 6 h, and the DNA in gels visualized under UV light after staining with ethidium bromide (5 μg/ml; Polysciences, Inc., Warrington, PA).

**Spectrophotometry.** Light absorption spectra were measured using an IBM 9410 2-beam spectrophotometer interfaced to a Hewlett Packard 9826 computer at 1-nm intervals and stored in digital form. The spectra were corrected for the absorption of the buffer (or CAF solution when it was used) and expressed in the form of molar extinction coefficient (μg⁻¹-cm⁻¹).

**Microcalorimetry.** The measurements were performed using a differential Omega Titration Calorimeter (Microcal, Inc., Northampton, MA). A detailed description of the instrument was presented before (11). Briefly, 2 identical cells (~1.3 ml) were located in an adiabatic shield to which they were thermally shunted by their access tubes. The reference cell was electrically heated at a very slow, constant rate. The difference in temperature between the reference and sample cell was regulated to zero with positive feedback power to the sample cell. A voltage proportional to the sample cell feedback power was sampled by the computer and plotted versus time. When heat was generated (or absorbed) within the sample cell by addition of a titrant, a negative (or positive) compensating deflection appeared in the feedback power, which quickly reestablished zero temperature difference between the 2 cells. The time integral of the power deflection was a measure of the heat generated (or absorbed). The instrument was calibrated by electrically heating the sample with a known quantity of heat. We found the instrument very sensitive and stable with a root-mean-noise level not exceeding 10⁻⁸ cal/s.

Staining of cellular DNA and protein content in individual cells was accomplished as previously described (10) using a solution containing 1.0 μg/ml diamidino-2-phenylindole (kindly provided by Dr. Jan Kapuscinski) and 10 μg/ml sulforhodamine 101 (Eastman Kodak, Rochester, NY), dissolved in 10 mM piperazine-N,N'-bis(2-ethane)sulfonic acid; buffer (Calbiochem, La Jolla, CA) containing 100 mM NaCl, 2 mM MgCl₂, and 0.1% Triton X-100 (Sigma), pH 6.8, at 0-4°C. The blue fluorescence of diamidino-2-phenylindole bound to DNA and red fluorescence of sulforhodamine 101 bound to protein in individual cells was measured with an ICP-22 flow cytometer (Ortho Diagnostics, Westwood, MA) using appropriate dichroic mirror and emission filter combinations (10). The data were collected on a Zeos 386 personal computer using Acqcyte software (Phoenix Flow Systems, San Diego, CA), and the cell cycle distribution of the DNA histograms deconvoluted using Multicycle (Phoenix Flow Systems).

**Fig. 2. DNA frequency histograms of HL-60 cells either untreated (control) or exposed for 4 h to 20 nM CAM and varying concentrations of CAF. Cultures received 20 nM CAM (except control) and either 0 or 0.5, 1, 2, or 5 μM CAF. After 4 h, cultures were harvested, washed free of drugs, and the cells fixed in ethanol. DNA histograms were obtained following cell staining with diamidino-2-phenylindole as described in the text. The cell cycle distributions (G1, S, and G2/M) were analyzed using the Multicycle program. The arrows indicate the mean channel of the S-phase cell accumulation.**
A set of 4 titrations were performed (buffer with buffer, CAF with buffer, buffer with TPT, and CAF with TPT) to correct the CAF-TPT heat of interaction with the heat involved with dilution of the titrant (TPT) and CAF in the sample cell, as well as for the heat resulting from the temperature difference between the titrant and the solution in the cell.

RESULTS

Effect of CAF on CAM-induced Perturbation of S-Phase Progression. Exposure of HL-60 cells to 20 nM CAM resulted in an arrest of cells in S-phase of the cycle. After 4 h incubation, the arrest was characterized by an increased (from 49 to 61%) percentage of cells in S-phase (Table 1). A very characteristic feature of the arrest was an accumulation of cells with DNA content equivalent of that of cells with a DI of approximately 1.3, represented by a peak in S-phase on the DNA frequency distribution histogram (Fig. 2). CAF alone, at concentrations up to 5 mM, had no measurable effect on the cell cycle distribution of HL-60 cells, when analyzed 4 h after addition of the agent (Table 1). Addition of 0.2 or 0.5 mM CAF at the same time as 20 nM CAM neither altered the CAM-induced S-phase accumulation nor affected the position of the S-phase peak, which was still observed at approximately one-third (DI = 1.3) of the distance between G1 and G2 (Figs. 2 and 3B). At a concentration of 1 mM, CAF decreased but did not completely abolish the CAM-induced S-phase block (Table 1).

In addition, the S-phase peak in these cultures shifted slightly to DI = 1.4 (Fig. 2). At a CAF concentration of 2 mM, the CAM-induced S-phase accumulation was nearly abolished (Table 1 and Fig. 3B), though a peak, now in mid-S phase (DI = 1.5), was still apparent (Fig. 2). When the CAF concentration was increased to 5 mM, the cytostatic effects of CAM were no longer apparent (Fig. 3B), and the S-phase peak was dramatically reduced, though a minor elevation could be distinguished at DI = 1.6 (Fig. 2).

CAF Prevents Apoptosis of HL-60 Cells Induced by CAM or TPT. Incubation of HL-60 cells in the presence of 0.15 μM CAM led to a selective apoptosis of S-phase cells, observed as early as 4 h after addition of CAM (Fig. 3), confirming our previous observations (12). A subpopulation of apoptotic cells, characterized by fractional DNA content (13), was present on the DNA frequency histograms to the left of the G1 peak (Fig. 4). The apoptotic mode of cell death in these cultures was confirmed by microscopy: the changes in cell morphology were characterized by typical chromatin condensation and nuclear fragmentation, with preservation of the integrity of plasma membrane (data not shown, but see Ref. 12). The presence of the classical ladder of nucleosomal and oligonucleosomal size particles on DNA gels (Fig. 5) provided additional evidence of apoptosis. In the present experiments, 0.15 μM CAM induced an apoptotic population that comprised approximately 40% of the total number of cells analyzed. The non-apoptotic cell population consisted of predominately G1 and, to a lesser extent, G2M phase cells (Fig. 4). Approximately 3–5% of HL-60 cells normally undergo spontaneous apoptosis in untreated cultures (Table 2). CAF alone, at concentrations up to 5 mM, neither increased the proportion of apoptotic cells nor, at short incubation times, appreciably affected the cell cycle distribution of exponentially growing cells (Table 2).

At low concentrations (0.5 and 1.0 mM), CAF did not prevent apoptosis induced by CAM (Figs. 3A, 4, and 5). Increasing the CAF concentration to 2 mM reduced the apoptotic population to 22% (Figs. 3A and 4); the drop in apoptotic cells was accompanied by an increase (from 6% to 38%) in the percentage of S-phase cells (Table 2) and disappearance of any evidence of endonucleolysis on DNA gels (Fig. 5). At a CAF concentration of 5 mM, the apoptotic response of HL-60 cells to CAM was virtually abolished, regardless of the parameter that was measured (Figs. 3–5, Table 2).

Significant apoptosis in HL-60 cultures, concomitant with a decrease in the proportion of S-phase cells, was also observed in cultures treated with TPT, at similar concentrations as CAM (Fig. 6). The presence of a cell subpopulation with DNA stainability below that of G1 phase cells (Fig. 6) coincided with the appearance of morphologically apoptotic cells observed by fluorescence microscopy (data not shown). However, CAF was even more effective in reversing the apoptosis induced by TPT, compared to equimolar concentrations of CAM. Thus, a significant reduction in the proportion of apoptotic cells was observed in cultures that received TPT and 2 mM CAF (Table 3 and Fig. 3A). In fact, the CAF concentration that reduced apoptosis by 50% was 2.0 mM in CAM-treated cultures and 0.74 mM in TPT-treated cultures (Fig. 3A). In both instances, cultures exposed to 0.15 μM CAM or TPT and 5 mM CAF exhibited a cell cycle distribution similar to unperturbed, exponentially growing control cultures (Figs. 4 and 6).
DNA Content

Fig. 4. DNA frequency histograms of HL-60 cells untreated (control) or exposed to 0.15 μM CAM and varying concentrations of CAF for 4 h. Cultures exposed to CAM alone demonstrated a loss of S-phase cells and the concomitant appearance of apoptotic cells with fractional DNA content (arrows) following staining with diamidino-2-phenylindole. Increasing concentrations of CAF, added at the time of CAM treatment, diminished the appearance of apoptotic cells as well as CAM-induced loss of S-phase cells (see Fig. 3).

Fig. 5. Agarose gel electrophoresis of DNA extracted from control HL-60 cells and cells treated for 4 h with 0.15 μM CAM alone, 2 or 5 mM CAF alone, or combinations of CAM and 0.2-5 mM CAF. Arrow, band in the molecular weight marker lane equivalent to 564 base pairs.

When the above cultures are washed free of drugs and resuspended in fresh, drug-free medium, the cells grew exponentially (data not shown).

The fact that inhibition of CAM or TPT induced apoptosis was not simply the result of inhibition of S-phase progression by CAF could be demonstrated in a stathmokinetic experiment in which cells received either 0 or 5 mM CAF and 0.05 μg/ml vinblastine for 4 h. Cell cycle progression proceeded at 60% of normal (an increase in G2M cells from 13% to 25 versus 33% for control) in the presence of 5 mM CAF; 2 mM CAF had no inhibitory effect on cell cycle progression.

Interactions between CAF and TPT in Solutions. Since both CAM and TPT induced similar degrees of apoptosis in HL-60 cells, and TPT is significantly more water soluble than CAM, the former was used to determine whether CAF formed complexes similar to that observed with DNA intercalating drugs (6). The interactions between CAF and TPT were studied spectrophotometrically and by titration microcalorimetry.

In the first experiment, the light absorption spectrum of free TPT (37 μM in 5 mM N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid buffer and TPT in combination with CAF (10 mM) were deter-
CAF AFFECTS CAM- AND TPT-INDUCED CELL CYCLE CHANGES

Table 2 Effect of CAF on apoptosis induced by 0.15 μM CAM

<table>
<thead>
<tr>
<th>CAM (μM)</th>
<th>CAF (μM)</th>
<th>% apoptosis a</th>
<th>G1</th>
<th>S</th>
<th>G2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>2.0 ± 0.2</td>
<td>37 ± 3</td>
<td>49 ± 3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>2.5 ± 0.5</td>
<td>37 ± 1</td>
<td>44</td>
<td>19 ± 1</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>0.5</td>
<td>44.0 ± 3</td>
<td>84 ± 1</td>
<td>6 ± 3</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>0.15</td>
<td>1.0</td>
<td>43.5 ± 2.5</td>
<td>84 ± 3</td>
<td>8 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>0.15</td>
<td>2.0</td>
<td>22.0 ± 10</td>
<td>53 ± 7</td>
<td>38 ± 4</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>0.15</td>
<td>5.0</td>
<td>2.5 ± 1.5</td>
<td>34 ± 1</td>
<td>52 ± 3</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

a Apoptosis was determined as the percentage of cells with fractional DNA content, located on DNA frequency histograms to the left of the G1 peak (Fig. 4).

The spectra were corrected for absorption of the buffer and, in the case of the TPT-CAF mixture, which is characteristic for aromatic chromophore interaction, such as mitoxantrone, doxorubicin, and ellipticine (4-6, 15). The present data indicate that the perturbation of cell cycle progression

DISCUSSION

As mentioned in the "Introduction," studies from several laboratories demonstrated that CAF is able to reduce the cytostatic and/or cytotoxic effects of DNA intercalating topoisomerase II inhibitors such as mitoxantrone, doxorubicin, and ellipticine (4-6, 15). The present data indicate that the perturbation of cell cycle progression

Fig. 7. Light absorption spectrum of TPT alone (solid line) and after addition of 10 mM CAF (broken line). TPT, at a concentration of 37 μM, was dissolved in a 5 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid buffer containing 0.15 M NaCl, pH 6.8. The spectra were corrected for absorption of the buffer and, in the case of the TPT-CAF mixture for the absorption of CAF (identical amounts of CAF were added to both the sample and reference cuvettes). After measurement and correction for dilution, the spectra were recalculated and expressed as a function of the molar absorption coefficients (ε-1 cm-1).

Table 3 Effect of CAF on apoptosis induced by 0.15 μM TPT

<table>
<thead>
<tr>
<th>TPT (μM)</th>
<th>CAF (μM)</th>
<th>% apoptosis a</th>
<th>G1</th>
<th>S</th>
<th>G2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.5</td>
<td>33</td>
<td>87</td>
<td>4 ± 1</td>
<td>9</td>
</tr>
<tr>
<td>0.15</td>
<td>1.0</td>
<td>14.5</td>
<td>54</td>
<td>39 ± 7</td>
<td>7</td>
</tr>
<tr>
<td>0.15</td>
<td>2.0</td>
<td>7</td>
<td>41</td>
<td>53 ± 6</td>
<td>6</td>
</tr>
<tr>
<td>0.15</td>
<td>5.0</td>
<td>2</td>
<td>36</td>
<td>48 ± 16</td>
<td>16</td>
</tr>
</tbody>
</table>

a Apoptosis was determined as in Table 2 (Fig. 6).

The cell cycle distribution was analyzed 4 h after addition of drugs.

Fig. 8. A, microcalorimetric titration of CAF (1.32 ml at a concentration of 20.6 mM) with TPT (1.75 mM) dissolved in 20 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid, pH 6.8, at 25°C. Ten portions (10 μl each) of the titrant were added at 3-min intervals and the heat exchange (μcal/sec) measured as a function of time (only 6 points are presented for clarity). The curves at each addition represent (from the top) titration of: (a) CAF with buffer; (b) CAF with TPT; (c) buffer with TPT; and (d) buffer with buffer. Curves (a) and (d) represent heats of dilution of CAF and TPT, respectively. Curve (d) was used to correct the results for the heat exchange resulting from differences in temperature between the titrant and the sample cell. Curve (b) is the resultant of all these exchanges combined with the heat of interaction between CAF-TPT. While all titrations were endothermic (heat > 0), it is clear that because the amplitude of curve (b) is much smaller than the sum of curves (a) and (c), the interaction of CAF and TPT is exothermic. B, microcalorimetric titration of CAF with buffer (x), buffer with TPT (o), and CAF with TPT (■) calculated as kcal/mol of titrant injected and plotted versus total titrant concentration after correction for the temperature difference between the titrant and sample cell. The approximate heat of the CAF-TPT interaction was calculated from the above data by subtracting the sum of the first 2 sets of points from the latter (■). The top 3 curves represent the extrapolation of experimental points to 0 titrant concentration. The heat of CAF dilution, while substantial, was constant throughout the titration and, thus, a linear interpolation was made in this case. The extrapolation of the heat of TPT dilution with buffer and with CAF was calculated using the equation Q∞-∞ = φ + A × [TPT] 1/2 (23, 24). Finally, the extrapolated heat of the CAF-TPT interaction was calculated by subtracting the sum of heats of dilutions from the heat of titration of CAF with TPT.
produced by CAF. The protective effect of CAF was concentration dependent: the effect was not apparent at 1 mM and lower CAF concentrations, while maximum protection was observed at 5 mM CAF. Because CAM and TPT are nonintercalating DNA topoisomerase I inhibitors, the present data, therefore, indicate that the protective effect of CAF is exclusive for neither intercalating agents nor for topoisomerase II inhibitors.

CAM and its analogues are known to stabilize the cleavable complex formed between topoisomerase enzymes and DNA and may interfere with the movement of replication forks and/or the transcriptional machinery to cause the unique intercalational DNA cleavage common to the process of apoptosis (16, 17). Since DNA topoisomerase enzymes are phosphorylated in their active state (18, 19), agents that directly or indirectly affect phosphorylation, for instance through protein kinase C or casein kinase II, might alter the ability of CAM (or TPT) to induce accumulation or apoptosis of S-phase HL-60 cells (19, 20). However, previous results have demonstrated that preincubation with CAF immediately before exposure to agents that damage DNA via topoisomerase II had no protective effect, nor did CAF when added 3 or more h after such agents (5). Therefore, a more direct interaction between CAF and these agents appeared more likely (6).

Our previous spectrophotometric studies on the interaction between CAF and DNA intercalators have indicated that the observed protective mechanism of CAF may be a consequence of sequestration of such antitumor drugs in stacking complexes with CAF, thus lowering the effective free drug concentration in the solution and making the drugs less accessible to the cell (6, 14). TPT and CAM are aromatic molecules and are also expected to interact with CAF in a manner similar to the above intercalating drugs. Indeed, the present biophysical studies provide evidence for such interactions. Thus, the spectral shift in the absorption band of TPT in the presence of CAF is very characteristic of interaction between the TPT chromophore and CAF; the shift was observed at >350 nm, i.e., outside of the absorption of CAF. The titration microcalorimetric data provide additional information on the interaction between TPT and CAF; the enthalpy of the reaction (~4.2 kcal/mol) is consistent with a stacking type of interaction (21, 22). The fact that CAM’s solubility could be increased 20-fold in a 0.1 mM solution of CAF over that in water (data not shown) also implies a stacking mode of interaction between CAF and CAM-like structures. However, the mechanisms involved in mixed-aggregation reactions are very complex (14), and a full thermodynamic analysis of CAF-TPT complex formation is outside the scope of this study and will be published separately.

CAF is relatively nontoxic, and even at millimolar concentrations it does not immediately affect cell viability (5). As our data indicate, CAF and its less toxic analogue, pentoxifylline (6), have a propensity to form complexes with other aromatic molecules thus reducing the concentration of the free form of the drug in solution. Many aromatic molecules (e.g., intercalating agents, and/or topoisomerase inhibitors) exert their pharmacological or biological activity at relatively low concentrations, often several orders of magnitude lower than the nontoxic concentrations of CAF or pentoxifylline. These methylxanthines, thus, when included into solutions containing active aromatic compounds, can be used to reduce the effective concentration of the aromatic molecules in free form, and therefore to modulate their activity. Our most recent data indicate that the mutagenic activity of several aromatic compounds is markedly reduced in the presence of CAF. Thus, CAF, and similar nontoxic compounds, can be considered as factors modifying the activity of a variety of active, aromatic molecules and may have practical application in reducing their undesired effects. Among such applications is the possibility of using CAF or similar compounds to neutralize the toxicity of aromatic compounds, e.g., in the instance of overdose.

REFERENCES

Caffeine Prevents Apoptosis and Cell Cycle Effects Induced by Camptothecin or Topotecan in HL-60 Cells

Frank Traganos, Jan Kapuscinski, Jianping Gong, et al.


Updated version  Access the most recent version of this article at: [http://cancerres.aacrjournals.org/content/53/19/4613](http://cancerres.aacrjournals.org/content/53/19/4613)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.