Modulation of Adriamycin and N-Trifluoroacetyladriamycin-14-valerate Induced Effects on Cell Cycle Traverse and Cytotoxicity in P388 Mouse Leukemia Cells by Caffeine and the Calmodulin Inhibitor Trifluoperazine

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ABSTRACT

1,3,7-Trimethylxanthine "caffeine" (CAF) is reported to induce a differential effect on the cytotoxicity of the DNA intercalators actinomycin-D (ADR) and adriamycin (ADR). In the present study the effect of caffeine and/or trifluoperazine in modulating cell cycle traverse, drug accumulation, and cytotoxicity of anthracyclines was evaluated. The survival in soft agar of P388 mouse leukemia cells treated with ADR (0.05-0.25 μg/ml) alone for 1 h was 1.2- to 3-fold lower when the cells were incubated for 24 h in drug-free medium versus medium supplemented with 2 mM CAF. In contrast, for P388 cells treated with ADR in the presence of 2 mM CAF for 1 h and subsequently incubated for 24 h in the absence or presence of 2 mM CAF, cell kill based on colony formation in soft agar was 2- to 20-fold lower than in ADR-treated cells never exposed to 2 mM CAF. In cells treated continuously for 24 h with ADR (0.01-0.05 μg/ml) or the DNA nonbinding ADR analogue N-trifluoroacetyladriamycin-14-valerate (AD32) (0.05 and 0.1 μg/ml) the survival in soft agar was 3- to 20-fold higher in the presence versus the absence of 2 mM CAF. The decreased cytotoxicity in cells treated with ADR or AD32 in the presence of CAF was accompanied by a significant reduction in the accumulation of cells in G2, however, in cells treated with ADR or AD32 in the presence of 2 mM CAF plus 5 μM trifluoperazine the decreased G2 accumulation was not accompanied by a reduction in anthracycline cytotoxicity. The modulation by CAF of ADR and AD32 cytotoxicity did not correlate with decreased cellular ADR and AD32 accumulation. Results from this study indicate that CAF markedly reduces the cytotoxicity of ADR or AD32 and trifluoperazine circumvents the effects of CAF.

INTRODUCTION

The anthracycline antitumor antibiotics represent a group of drugs extensively studied and are widely recognized for their experimental and clinical antitumor activity against a spectrum of tumors (1). ADR is one of the most potent anthracyclines and is clinically important in the treatment of a variety of human malignancies (2, 3). Cellular drug accumulation and cytotoxicity of ADR is concentration, time, and temperature dependent (3-6). The binding of ADR to nucleic acids and the subsequent disruption of DNA and RNA synthesis is widely accepted as a major mechanism of action (6). Although the binding to DNA by some anthracyclines probably accounts for the cytotoxicity, the potent antitumor effects of semisynthetic anthracyclines which bind weakly or not at all to DNA suggest a complexity in the biological effects of anthracyclines. AD32 is a semisynthetic analogue of ADR (7). AD32 is rapidly accumulated by cells (8), does not bind to DNA (9, 10) and in experimental tumor systems is reported to have greater antitu-
mortality similar to P388 cells maintained in DBA/2 mice by weekly transplantation. Trifluoperazine was a generous gift from Dr. Carl Kaiser, Smith Kline and French Laboratories, Philadelphia, PA. Caffeine was obtained from Aldrich Chemical Co., Milwaukee, WI. AD32 was a generous gift from Dr. Mervyn Israel, Department of Pharmacology, University of Tennessee Center for Health Sciences, Memphis, TN.

Anthracycline Cytotoxicity in Vitro. Drug treatment was carried out using P388 cells in suspension culture, and cytotoxicity was determined using a soft-agar colony-forming assay. Stock solutions of ADR, CAF, and TFP were prepared in sterile glass distilled water and a stock solution of AD32 was prepared in DMSO. RPMI 1640 supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 10% FBS was used to prepare working dilutions of the various drugs. In AD32-treated cells, the final concentration of DMSO was 0.5%. No cytotoxicity due to DMSO alone at 0.5% was observed.

Drug exposure for 1 h involved treatment of log-phase cultures of P388 cells in RPMI 1640 supplemented with 10% FBS, with ADR (0.05, 0.1, 0.25, and 0.5 μg/ml) in the absence or presence of 2 mM CAF. Following treatment the cells were centrifuged (80 x g) and washed twice with drug-free RPMI 1640 supplemented with 10% FBS. After the final washing, the cells treated with ADR alone and ADR plus 2 mM CAF were resuspended in RPMI 1640 supplemented with 10% FBS and 10 μM 2-mercaptoethanol in the absence or presence of 2 mM CAF, and incubated for an additional 24 h at 37°C. Cell survival and cell cycle phase distribution analysis in control and treated cells were then determined by soft-agar colony assay and flow cytometry, respectively.

Drug exposure studies for 24 h were carried out with log-phase cultures of P388 cells in RPMI 1640 supplemented with 10% FBS and 10 μM 2-mercaptoethanol and treated as follows: anthracycline alone; anthracycline plus 2 mM CAF; anthracycline plus 5 μM TFP; and anthracycline plus 2 mM CAF and 5 μM TFP. Controls included same medium alone and media supplemented with 2 mM CAF and/or 5 μM TFP. The anthracylines and concentrations studied were ADR (0.01, 0.025, and 0.05 μg/ml) and AD32 (0.05 and 0.1 μg/ml). Other concentrations of AD32 were not studied since preliminary studies demonstrated that cell survival at 0.025 and 0.2 μg/ml was 100 and <99%, respectively, with or without 2 mM CAF and/or 5 μM TFP. Soft-agar colony assay and flow cytometry were used to determine cell survival and cell cycle phase distribution, respectively, in the control and treated P388 cells.

Soft-Agar Colony-forming Assay. Cell counts in control and treated P388 cells following continuous drug exposure for 24 h or at the end of the 24-h incubation period following 1-h drug treatment were determined by trypan blue dye exclusion using a hemacytometer. Samples were centrifuged (80 x g) and washed twice with drug-free RPMI 1640 supplemented with 10% FBS and 1.5 x 10⁵ trypan blue dye-excluding cells/Petri dish, plated in triplicate using 35- x 10-mm Petri dishes. The plating medium was RPMI 1640 supplemented with 20% FBS, 10 μM 2-mercaptoethanol, conditioned medium from the culture supernatant of P388 cells, and 0.3% agar. The plated cells were incubated at 37°C for 120 h in a humidified 5% CO₂ plus 95% air atmosphere and colonies (>50 cells) in untreated control and treated plates counted in an Omnicom Feature Analysis System II (Bausch & Lomb, Rochester, NY). In control untreated P388 cells, the plating efficiency under these conditions was approximately 30%.

Cell Cycle Phase Distribution Analysis by Flow Cytometry. The P388 cells following treatment for 1 and 24 h with anthracycline in the absence and presence of CAF and/or TFP were analyzed for cell cycle phase distribution in a Becton-Dickinson FACS IV multiparameter flow cytometer using propidium iodide-stained nuclei. Control and treated cells were washed twice with ice-cold 0.85% sodium chloride solution and the cell pellet was resuspended in hypotonic propidium iodide staining solution (23). The nuclei were maintained in propidium iodide staining solution for 24 h at 4°C, centrifuged (300 x g), and fixed in 70% ethanol. The fixed nuclei were centrifuged (300 x g) and resuspended in hypotonic propidium iodide staining solution prior to analysis for cell cycle traverse perturbations. At least 10,000 nuclei were analyzed in each sample, and the fraction of cells in G₁, S phase, and G₂ + M of the cell cycle was determined as reported previously (24).

Anthracycline Accumulation in Vitro. P388 cells (1 x 10⁶ cells/ml) in RPMI 1640 supplemented with 10% FBS were treated in vitro with ADR or AD32 at concentrations of 0.05, 0.1, and 0.5 μg/ml in the absence and presence of 2 mM CAF at 37°C. Additional studies on ADR accumulation in P388 cells treated for up to 8 h at 37°C with ADR (0.01, 0.025, 0.05, and 0.1 μg/ml) in the absence or presence of 2 mM CAF and/or 5 μM TFP were also done. Duplicate 1-ml aliquots of cells retrieved at the end of 1, 2, 4, 6, and 8 h of incubation were centrifuged (100 x g) and washed twice with 7 ml of ice-cold 0.85% sodium chloride solution. Following the final wash, the cell pellet was resuspended in 50% ethanol-0.3 M hydrochloric acid, mixed thoroughly in a vortex mixer, centrifuged at 700 x g, and ADR or AD32 content in the supernatant determined fluorometrically (18, 25) in an Amino-Bowman spectrofluorometer (American Instrument Co., Silver Spring, MD). Excitation and emission wavelengths of 470 and 585 nm, respectively, were used for fluorometric analysis of ADR and AD32. Standard curves prepared with ADR or AD32 were used for computation of anthracycline content which was expressed as ng/10⁶ cells. Thin layer chromatographic analysis of P388 cell extracts following treatment with ADR or AD32 with CAF or TFP revealed little, if any, breakdown of parent drug, suggesting that the fluorometric analysis represents uncharged cellular anthracycline levels.

RESULTS AND DISCUSSION

Cytotoxicity and Cell Cycle Traverse in P388 Cells Treated with Adriamycin and Caffeine for 1 h. The survival in soft agar of P388 cells treated with or without CAF during and/or after ADR for 1 h is shown in Fig. 1. Treatment with 2 mM CAF alone for 1 h and/or subsequently for 24 h was noncytotoxic. In P388 cells treated with ADR alone for 1 h, the cytotoxicity was dose dependent, and at ADR concentrations >0.1 μg/ml, the survival was <10% of the untreated control. The magnitude of reduction in survival of P388 cells treated with ADR for 1 h and subsequently incubated for 24 h in media supplemented with 2 mM CAF was ADR dose dependent. Although little
reduction in cell kill due to CAF was observed at ADR, 0.05 and 0.5 μg/ml, the survival at ADR, 0.1 and 0.25 μg/ml was 4- and 2-fold higher, respectively. In contrast to these results, cell kill in P388 cells treated for 1 h with ADR in the presence of 2 mM CAF was reduced 2-fold at ADR concentrations of 0.05 and 0.5 μg/ml, and at ADR, 0.1 and 0.25 μg/ml, the survival was 8- and 25-fold higher, respectively. Compared to treatment with ADR in the presence of CAF for 1 h, the further reduction in cytotoxicity observed when cells were treated with CAF during and after ADR exposure was minimal at ADR, 0.05 and 0.1 μg/ml and 2.5- and 4-fold at ADR, 0.25 and 0.5 μg/ml, respectively.

Fig. 2 shows the cell cycle phase distribution of P388 cells treated for 1 h with or without CAF during and/or after ADR exposure. Similar to controls, no significant cell cycle perturbations were observed in caffeine-treated cells. ADR dose-dependent accumulation of cells in the G2 + M fraction and a corresponding decrease in the G1 fraction were apparent, and the subsequent incubation of similarly treated cells in media with 2 mM CAF showed a near-complete reversal of this block. In cells treated for 1 h with ADR in the presence of CAF, accumulation of cells in the G2 + M fraction was comparable to that with ADR alone, and a significant reduction of this block occurred only in cells subsequently incubated for 24 h in the presence of 2 mM CAF.

Effect of Caffeine on the Cytotoxicity and Cell Cycle Traverse of P388 Cells Treated with Adriamycin for 24 h. The effect of continuous treatment with ADR in the absence and presence of 2 mM CAF for 24 h on cytotoxicity in P388 cells is presented in Fig. 3. Cell kill with ADR (0.01 μg/ml) was 60% and at concentrations of ADR between 0.025 and 0.1 μg/ml the survival was <1%. Similar to results with the 1-h treatment the cytotoxicity of ADR was reduced in the presence of CAF. At ADR (0.01 μg/ml) there was a 2-fold reduction in cytotoxicity due to CAF, and at 0.025 and 0.05 μg/ml, the enhancement in survival of CAF-treated cells was 23- and 3-fold, respectively. However, little reduction in cytotoxicity due to CAF was observed at ADR, 0.1 μg/ml.

The distribution of cells in G1, S phase, and G2 + M of the cell cycle following treatment with ADR in the absence and presence of CAF for 24 h is shown in Fig. 4. The cell cycle phase distribution following treatment with 2 mM CAF alone...
with ADR and AD32 alone, a dose-dependent cytotoxicity and accumulation of cells in G2 + M was evident. Similar to results with ADR, reduced accumulation of cells in G2 + M and cytotoxicity was observed following treatment with AD32 in the presence of CAF. In agreement with our earlier data (18) the effects of TFP on enhancing cytotoxicity of AD32 were less marked than with ADR. In contrast to the reduction in the cell kill due to CAF and enhancement in cytotoxicity with TFP in cells treated with ADR or AD32, the survival of P388 cells treated with ADR or AD32 in the absence and presence of 2 mM CAF plus 5 μM TFP were comparable. Furthermore, in the presence of CAF plus TFP, the marked reduction in accumulation of cells in G2 + M due to CAF was not accompanied by a reduction in cytotoxicity as observed earlier with CAF alone.

Effect of Caffeine and Trifluoperazine on the Cellular Accumulation of Adriamycin and N-Trifluoroacetyladiamycin-14-valerate. The cellular accumulation of ADR and AD32 in the absence and presence of 2 mM CAF in P388 cells is shown in Fig. 5. The uptake of ADR was concentration and time dependent, in contrast to AD32, wherein differences in drug levels were only concentration but not time dependent. Cellular accumulation of ADR and AD32 in the absence and presence of CAF following treatment with ADR (0.05 μg/ml) and AD32 (0.05 and 0.1 μg/ml) AD32 was comparable. At ADR (0.1 μg/ml) a reduction in cellular ADR levels due to CAF was more apparent at 2 and 4 h than at 1 h. Maximal reduction (ADR, 40%; AD32, 20%) in anthracycline accumulation in the presence of CAF was apparent only in cells exposed to ADR or AD32, 0.5 μg/ml.

The effects of caffeine and/or TFP on cellular accumulation of ADR in cells treated with ADR (0.025, 0.05, and 0.1 μg/ml) for up to 8 h is shown in Fig. 6. In cells treated with ADR (0.01 μg/ml), the accumulation of ADR was comparable in the absence and presence of CAF. Data are the mean from triplicate experiments. $K_{\text{a}}$, SE.

| Table 1 Effect of CAF and TFP on the cell cycle traverse and survival in soft agar of P388 mouse leukemia cells treated with ADR and AD32 for 24 h | Cell cycle phasedistribution (%) of control | Survival (%) of control |
| Treatment | G1 | S | G2 + M | Media control | 34 | 55 | 11 |
| 2 mM CAF | 45 | 44 | 11 | 100 ± 4
d | 5 μM TFP | 36 | 53 | 11 | 100 ± 3.9 |
| 2 mM CAF + 5 μM TFP | 46 | 44 | 10 | 100 ± 4.3 |
| ADR (0.01 μg/ml) + 2 mM CAF | 26 | 50 | 24 | 41 ± 2 |
| ADR (0.01 μg/ml) + 5 μM TFP | 43 | 44 | 13 | 91.7 ± 2.2c |
| ADR (0.01 μg/ml) + 5 μM TFP | 24 | 37 | 39 | 1.9 ± 0.1c |
| ADR (0.01 μg/ml) + 2 mM CAF + 5 μM TFP | 48 | 35 | 18 | 31 ± 4c |
| ADR (0.025 μg/ml) | 20 | 30 | 50 | 1.1 ± 0.4 |
| ADR (0.025 μg/ml) + 2 mM CAF | 36 | 43 | 21 | 23.1 ± 4.2f |
| ADR (0.025 μg/ml) + 5 μM TFP | 10 | 30 | 60 | 0.1 ± 0.03f |
| ADR (0.025 μg/ml) + 2 mM CAF + 5 μM TFP | 38 | 37 | 25 | 0.7 ± 0.3f |
| ADR (0.05 μg/ml) | 9 | 25 | 66 | 1.1 ± 0.3 |
| ADR (0.05 μg/ml) + 2 mM CAF | 36 | 37 | 27 | 3.3 ± 0.7f |
| ADR (0.05 μg/ml) + 5 μM TFP | 5 | 18 | 77 | 0.7 ± 0.2f |
| ADR (0.05 μg/ml) + 2 mM CAF + 5 μM TFP | 32 | 24 | 44 | 0.6 ± 0.1f |
| AD32 (0.05 μg/ml) | 33 | 49 | 18 | 72.7 ± 7.9 |
| AD32 (0.05 μg/ml) + 2 mM CAF | 50 | 37 | 13 | 89.5 ± 3.2 |
| AD32 (0.05 μg/ml) + 5 μM TFP | 31 | 45 | 24 | 44.5 ± 10.1f |
| AD32 (0.05 μg/ml) + 2 mM CAF + 5 μM TFP | 46 | 43 | 11 | 67.8 ± 3.7f |
| AD32 (0.1 μg/ml) | 31 | 40 | 29 | 11.6 ± 2.3 |
| AD32 (0.1 μg/ml) + 2 mM CAF | 57 | 34 | 9 | 39.3 ± 2.1c |
| AD32 (0.1 μg/ml) + 5 μM TFP | 21 | 41 | 38 | 1.2 ± 0.3c |
| AD32 (0.1 μg/ml) + 2 mM CAF + 5 μM TFP | 56 | 32 | 12 | 13.8 ± 1.7c |

* Data on cell cycle phase distribution are from a representative experiment; SDs were <15% of the means in replicate experiments.

† Mean ± SE of triplicate experiments. Survival is based on colony counts.

‡ Significantly different from ADR or AD32 alone, at $P < 0.01$.

§ Significantly different from ADR + CAF or AD32 + CAF, at $P < 0.01$.

¶ Significantly different from ADR + TFP or AD32 + TFP, at $P < 0.01$.

** Data on cell cycle phase distribution are from a representative experiment; SDs were <15% of the means in replicate experiments.

†† Mean ± SE of triplicate experiments. Survival is based on colony counts.
ence or presence of CAF, TFP, and CAF plus TFP (results not shown). At concentrations of ADR (0.025, 0.05, and 0.1 μg/ml), a reduction (25–30%) and increase (20%) in ADR levels were observed in the presence of CAF and TFP, respectively. However, in cells treated with similar concentrations of ADR in the presence of CAF plus TFP, ADR levels were still approximately 20% lower than in cells treated with ADR alone.

Results on ADR accumulation (Fig. 5) and cytotoxicity (Fig. 1) in cells treated with ADR (0.5 μg/ml) in the presence of CAF suggest a correlation between reduced ADR levels and cell kill. However, following treatment with ADR (0.05 and 0.1 μg/ml) wherein CAF had minimal effects on ADR accumulation at 1 h (Fig. 5), a 2- to 7-fold reduction of ADR cytotoxicity in the presence of CAF was observed. The effects of CAF and/or TFP on cytotoxicity (Table I) and cellular levels of ADR (Fig. 6) further suggest that the magnitude of reduction (~30%) in ADR accumulation by CAF does not account for the 2- to 20-fold increase in survival, since restoration of ADR cytotoxicity with TFP is not accompanied by similar alterations in cellular ADR levels.

Steady state accumulation within an hour and the absence of noteworthy reduction due to CAF of cellular AD32 levels also demonstrate that reduction in cytotoxicity by CAF is dependent on mechanism(s) other than reduction of anthracycline levels. Overall, the effects of CAF on reducing ADR and AD32 cytotoxicity were less apparent at higher concentrations of anthracycline wherein marked reductions in drug accumulation are observed.

CAF is reported to synergistically enhance the cytotoxicity of DNA intercalators (17), nitrosoareas (26), bleomycin (27), neocarzinostatin (28), nitrogen mustard (29), and thiota (30). Our results demonstrate that the modulation of ADR cytotoxicity is dependent on the presence of CAF during and/or after ADR treatment as well as the concentration of ADR. Reduced cytotoxicity with CAF is not restricted to DNA binders like ADR, since similar results were also observed with the DNA nonbinding ADR analogue AD32. The observation that TFP reverses CAF-induced alterations in the cytotoxicity of ADR- and AD32-treated cells is noteworthy and suggests the role for calcium homeostasis. The antagonistic effect of TFP could be related to either its inhibitory effect on calcium-regulated events which are activated due to the release of calcium by CAF from stores in the endoplasmic reticulum (31) or to effects on calcium mobilization.

In summary, this study demonstrates that CAF markedly reduces the cytotoxicity of ADR and AD32 by mechanisms which are not directly related to alterations in cellular anthracycline levels. The antagonistic effects of TFP and CAF suggest the role for a common target which determines the cytotoxic response to anthracyclines. The identification of the molecular basis for the effects of CAF and TFP in anthracycline-treated cells should provide information on the mechanisms of action and determinants of a cytotoxic effect.

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