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Interaction of Caffeine with Anthracycline Antibiotics: Effect of Caffeine on Anthracycline Cytotoxicity

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Abstract

We studied the interaction of caffeine (CAF) with DNA-intercalating antitumor drugs, anthracycline antibiotics, *in vitro* and *in vivo*. The results of treatment with anthracycline alone were compared to those observed after treatment with CAF and intercalating drugs. The present study demonstrates that CAF markedly reduces the cytotoxicity of doxorubicin (DOX) and DOX analogues by a mechanism which is directly related to alterations in cellular anthracycline levels *in vitro*. The results of these studies also showed that the activity of DOX *in vivo* can be modified by CAF. These 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 drug, which is then reflected as diminished pharmacological activity.

Introduction

Doxorubicin (DOX) is one of the most useful chemotherapeutic agents currently available. Pirarubicin (THP) and epirubicin (EPI) are DOX analogues which have shown equivalent anitumor effect and less cardiac injury than DOX[1-5]. These antibiotics were considered to be cytotoxic by intercalating into cellular DNA[6-8]. The binding of the drug to nucleic acid and the subsequent disruption of DNA synthesis is widely accepted as a major mechanism of the pharmacological action of anthracycline[9,10].

In oncology, most studies have focused on the propensity of ceffeine (CAF) to potentiate the cytotoxic effects of a variety of DNA damaging agents such as a ionizing radiation, alkylating compounds, cisplatinum analogues, etc.[11-18]. The mechanism by which CAF enhanced the cytotoxicity of these agents is believed to involve suppression of repair of the potentially lethal DNA lesions induced by such agents[19,20]. These facts of the potentiating effect by CAF suggest that the compound has useful implications in clinical application for

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cancer chemotherapy. 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 intercalation[21]. Thus, the effects of doxorubicin (DOX), *n*-trifluoroacetyldoxorubicin, novantrone, and ellipticine were markedly reduced when the cells were exposed to these drugs in the presence of CAF[22-24]. Likewise, the toxicity of the classical DNA intercalating fluorochrome, ethidium bromide, was also reduced in the presence of CAF[25]. The results of the present study, which demonstrate that CAF reduces the effects of DNA topoisomerase II inhibitor anthracyclines, provide evidence in support of the mechanism.

Materials and Methods

Drugs Doxorubicin was kindly supplied by Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. THP was kindly provided by Meiji Seika Co., Ltd., Tokyo, Japan. CAF was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Rosewell Park Memorial Institute (RPMI) medium 1640 and its supplements were obtained from Nissui Seiyaku Co., Ltd., Tokyo, Japan. Fetal bovine serum (FBS) was purchased from GIBCO (Life Technologies, Inc., U.S.A.). Penicillin G potassium and streptomycin sulfate were purchased from Meiji Seika Co., Ltd., Tokyo, Japan. All other chemicals were of analytical reagent grade. Each drug was dissolved in fresh medium and filtered through a 0.20 μm syringe filter (Corning, Co., NY, U.S.A.).

Cell lines and cell culture Murine P388 leukemia (P388) cells were kindly supplied by Japanese Cancer Research Resource Bank, Tokyo, Japan. P 388 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 10% FBS, 5×10^{-5}M 2-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml). They were grown in an atmosphere of 5% CO_2 and 95% air at 37°C in a humidified incubator. The cell line was maintained by twice a week passage into fresh medium.

Animals Females DBA/2 and DBA/2×C57BL/6 (BDF_1) mice were obtained from Japan SLC Inc., Hamamatsu, Japan. Through the experimental period, they were bred at the Laboratory Animal Center of our college and kept in cages in a room maintained at 23± 1°C and 50±5% relative humidity. The animals were fed a commercial pellet diet (CE-2, Clea Japan, Inc., Tokyo, Japan). The P 388 cells line was passed weekly through the DBA/2 mice.

Cytotoxic activity studies The effect of a drug on cellular proliferation was determined by seeding cells in culture at a concentration of 1×10^5 cells/ml. P388 cells were exposed to various doses of THP and doxorubicin for 60 min at 37°C in the presence or absence of CAF. Following treatment the cells were centrifuged (500×g) and washed twice with phosphate buffered saline (PBS). After the final washing, the cells treated with THP alone or THP plus CAF were resuspended in RPMI 1640 medium supplemented with 10% FBS and 5×10^{-5} 2-mercaptoethanol and incubated for an additional 48 h at 37°C. The viability in control and treated cells was then assessed by trypan blue dye exclusion.

Macromolecular synthesis The incorporation into acid-insoluble material was determined after 60 min incubation of P388 cells (10^6 cells/ml) with [methyl-^{3}H] thymidine ([^{3}H]

Thd, 1 μCi, Amersham) or [5-^{3}H]uridine ([^{3}H]Urd, 1 μCi, NEN Products). The average of triplicate determinations was plotted, and the rate of incorporation was determined by linear regression and expressed as a percentage of control.

Anthracycline uptake P388 cells (1×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% FBS were treated *in vitro* with DOX, THP or EPI at concentration of 1 μg/ml in the presence or absence of CAF at 37°C. Treated cells were centrifuged ($500\times g$) and washed twice with PBS. Following the final washing, the cell pellet was resuspended in 50% ethanol-0.3 N hydrochloric acid, mixed thoroughly in a vortex mixer and centrifuged at $3,000\times g$. The content of anthracycline in the supernatant was then determined fluorimetrically[26] in an Hitachi spectrofluorometer (650-10S type). The excitation and emission wavelengths of 470 and 585 nm, respectively, were used for fluorometric analysis of anthracyclines.

Microfluorometric analysis P388 cells were exposed to THP (5 μg/ml) in the presence or absence of 10 mM CAF for 60 min at 37°C. The function and shapes of living cells were then analyzed by cytofluorometry combined with image analysis (ARGUS 100 computer system, Hamamatsu Hotonics, Japan). The excitation and emission wavelengths of 450-490 and 590 nm, respectively, were used for microfluorometric analysis of anthracyclines. Fluorescence intensity is expressed on the X-or Y-axis.

Antitumor activity studies P388 cells (10^6 cells in 0.1 ml saline) were injected intraperitoneally on day zero into BDF_1 mice weighing 20-22 g, and the drugs were administered on days 3, 6, and 9 by *i.p.* injection at a constant ratio of 0.1 ml/20 g body weight 24 h after tumor implantation. Each test group had 6 to 10 mice and values for the increase in life span (ILS) % to evaluate the antitumor effects were calculated from the mean survival time (MST) of treated animals relative to that of control animals.

Statistical analyses Statistical differences between mean values were analyzed by the Student's t-test; P values less than 0.01 were considered statistically very significant.

Results

Effect of CAF on anthracycline-induced cell killing

The effect of CAF on the cytotoxicity of anthracycline was examined. The cells were exposed to anthracycline alone or anthracycline plus CAF. The modulation in cytotoxicity was observed when anthracycline and CAF were given simultaneously. Figure 1 demonstrates the reduction in survival of P388 cells treated with 10 mM CAF for 60 min. Cotreatment with CAF resulted in a dramatic reduction in cell killing. For example, treatment with THP alone (0.05 μg/ml) resulted in a survival of about 50%, but the presence of 10 mM CAF caused an increase to about 98%. A reduction in cell killing due to CAF was also observed for DOX and EPI (data not shown). The modulatory effect of CAF after treatment with anthracycline was similar to that observed after cotreatment with CAF and anthracycline.

Effect of CAF on anthracycline-induced macromolecular synthesis inhibition

The inhibitory effect of anthracycline and the modulatory effect of CAF on the macromolecular synthesis of cells were examined. The results obtained are shown in Fig. 2. When P388 cells were incubated with THP or EPI (1 μg/ml) for 60 min, the inhibiton of the

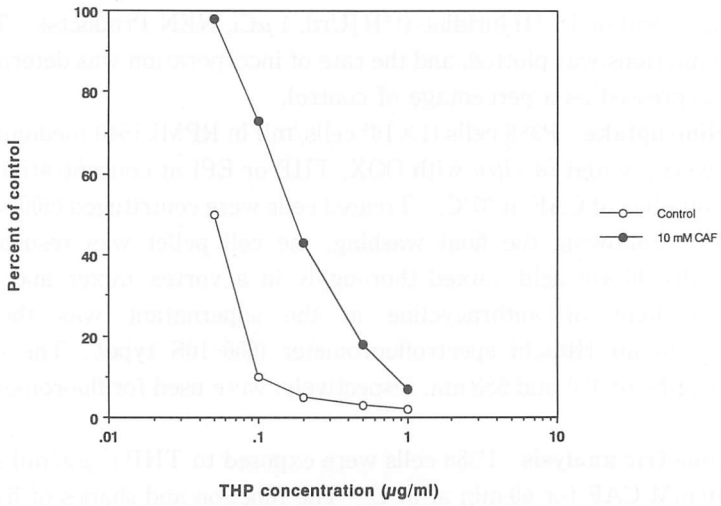

Fig. 1 The modification of anthracycline-induced cell killing by CAF. P388 cells were exposed to anthracycline in the presnce or absence of 10 mM CAF for 60 min. Then the cells were washed and reincubated in drug-free medium for 48 h. The quantitative assays for cytotoxicity were done by microscopic observation. The viability in treated cells was assessed by trypan blue dye exclusion.

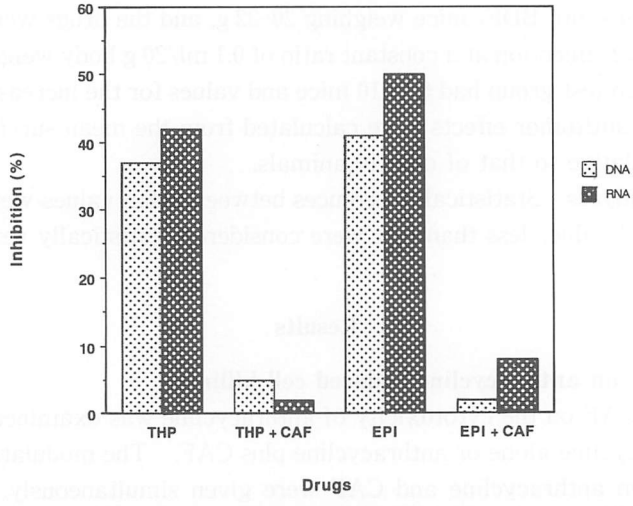

Fig. 2 Inhibition of macromolecular synthesis by anthracyclines and its modification by CAF
The cells were treated with anthracyclines (1 μg/ml) in the presence or absence of 10 mM CAF for 60 min at 37°C. Then the cells were labeled for 60 min with [^{3}H] Thd or [^{3}H] Urd (1 μCi). Radioactivity of the TCA-precipitant was determined after incorporation.

uptake of [^{3}H]Thd into the DNA of cells was about 37% and 41%. CAF significantly decreased the inhibitory effects of THP and EPI on the incorporation of [^{3}H]Thd, no effect of anthracyclines was seen in 10 mM CAF treated cells. Similar to the effect on DNA synthesis, a reduction in the effects of THP and EPI on the incorporation of [^{3}H]Urd into the RNA of cells was observed when the cells were simultaneously treated with CAF. Inhibitory

effects of THP and EPI on RNA synthesis were not observed in cells treated with CAF.

Effect of CAF on the uptake of anthracycline

The function and shapes of living cells were analyzed by cytofluorometry combined with image analysis (ARGUS 100 computer system), and the treatment by 10 mM CAF markedly reduced the sensitivity of anthracyclines on the cellular level (data not shown). These results demonstrated that CAF had a significant effect on its abillity for cellular accumulation.

The cells were treated in growth medium with CAF and the uptake of anthracycline was also determined fluorometrically. When CAF was added to growth medium before anthracycline exposure, a modification in drug uptake was observed (Fig. 3). The results were similar to the data obtained for the modification of drug uptake in cytofluorometric analysis. An increase in the amount of CAF caused inhibition of the anthracycline uptake. The rate of inhibition in cells treated with 10 mM CAF was found to be over half that without CAF.

Effect of CAF on the antitumor effect of anthracycline

The combination effects of CAF on the antitumor activity of anthracycline in mice bearing P388 ascites tumor were examined. The MST in each group was compared to that of control case. Results are shown in Fig. 4. CAF at the tested dose (100 mg/kg) did not inhibit tumor growth (dath not shown). A significant effect of CAF on the activity of doxorubicin was observed in mice bearing P388 ascites tumor treated with CAF. The MST of the P388 ascites tumor group in untreated mice was 11.2 days. The ILS of the P388 ascites tumor group administered doxorubicin (2 mg/kg) alone was 72%, whereas, that of the drug-combined groups of CAF (10 mg/kg) and DOX was ILS 39%. On the other hand, in contrast to these results, the reduction of the antitumor effect by CAF was not observed at the doses (5 mg/kg) of THP and EPI (data not shown).

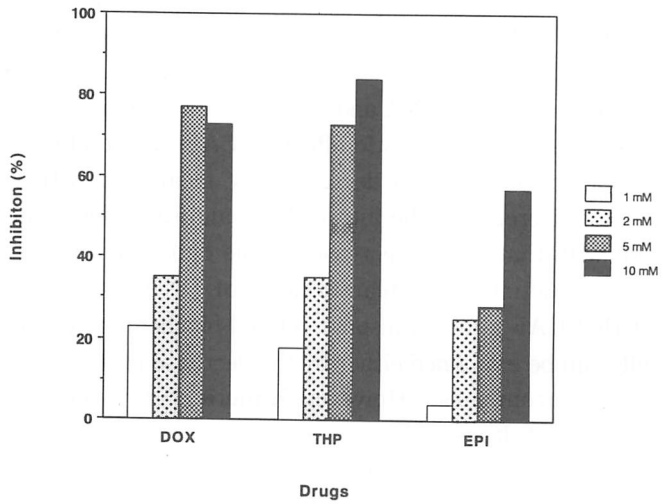

Fig. 3 Effect of CAF on the uptake of anthracycline in P388 cells. P388 cells were exposed to DOX, THP and EPI (1 μg/ml) in the presence or absence of CAF (1-10 mM) for 60 min at 37°C. The anthracycline content was determined fluorometrically.

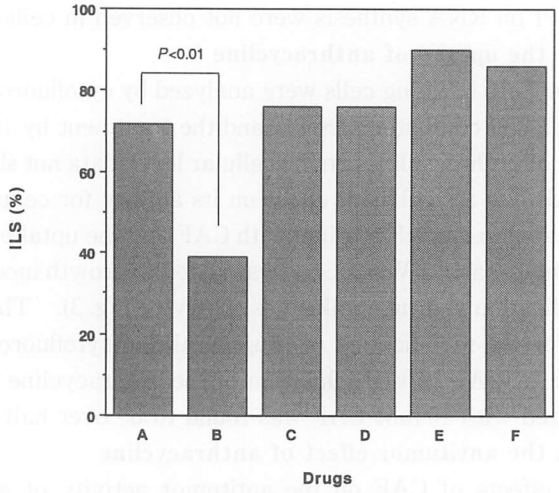

Fig. 4 Effect of CAF on the increase in life span induced by anthracyclines. Mice were treated *i.p.* with 10^6 cells of P388 leukemia on day 0, and drugs were given *i.p.* on day 3, 6 and 9. CAF (100 mg/kg) was given 5 min before adminintration of anthracyclines. Each bar represents the mean value (n=6-10). A: DOX 2 mg/kg, B: DOX plus CAF, C: THP 5 mg/kg, D: THP plus CAF, E: EPI 5 mg/kg, F: EPI plus CAF.

Discussion

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.

As our data indicate, CAF is relatively nontoxic, and even at millimolar concentrations it does not immediately affect cell viability. In the present study, it was found that CAF decreases the cytotoxicity of anthracyclines in P388 leukemia cell culture. That is to say, the data demonstrate that the modulation of the anthracyclines cytotoxicity is dependent on the concentration of CAF. The cytotoxic mechanism of anthracyclines has been shown to cause or to be associated with inhibition of nucleic acid synthesis[27]. The inhibitory effects of THP and EPI on the synthesis of DNA and RNA in cells were markedly reduced by 10 mM CAF. These data suggest an antagonistic effect of CAF on the efficacy of THP *in vitro*. The uptake of anthracycline was also decreased by treatment with CAF. The present results indicate that the decrease in the intracellular anthracycline level in the presence of CAF correlated well with that of the decreases in the cytotoxic effect. This phenomenon may account for the reduction of cellular toxicity of DNA-intercalating drugs by CAF. These data suggest that CAF alters transport-related biochemical processes. The reduced accumulation in cells can be explained either by the decrease in the uptake of the drug or by the increase in the efflux from cells. However, Kimura *et al.*[25] reported that CAF did not have effects on the efflux of drugs.

CAF also decreased the survival time of the P388 ascites tumor-bearing mice treated with DOX. Accordingly, the antitumor activity of DOX in combination with CAF in mice appears to correlate well with the *in vitro* antagonistic activity of CAF on the cytogenetic damage caused by DOX. The observation that CAF modifies the antitumor effect of DOX

in mice is remarkable. In contrast, CAF has little effect on the enhancement of ILS caused by THP and EPI.

Recently, intercalating drugs have been shown to interfere with DNA topoisomerase II, which relates to the drug cytotoxic effect[28,29]. Thus it is possible that the CAF use here may affect the activity of this nuclear enzyme. Monomeric forms of both CAF and intercalating drugs interact with single- and double-stranded nucleic acids[30,31]. These interactions with DNA may cause the release of anthracycline from the DNA. The releasing of the drug from DNA molecules by CAF may diminish damage to the DNA, thereby reducing the cytotoxicity of anthracyclines.

Further studies are needed to elucidate the mechanism of interactions between CAF and intercalating drugs.

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