Jpn. J. Cancer Res. 86, 594–599, June 1995

Effects of Methylxanthine Derivatives on Adriamycin Concentration and Antitumor Activity

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We studied the mechanism whereby caffeine acts as a biochemical modulator of adriamycin, and examined various methylxanthine derivatives to determine whether they would be of value as biochemical modulators. In an *in vitro* study of adriamycin efflux in Ehrlich ascites carcinoma cells, theophylline, pentoxifylline, and theobromine inhibited this efflux, while caffeine metabolites did not. The effects of several methylxanthine derivatives on the antitumor activity of adriamycin and on adriamycin concentration in tissue were also examined in CDF_1 tumor-bearing mice. Theobromine, which inhibited adriamycin efflux *in vitro*, increased the antitumor activity of adriamycin and the concentration of adriamycin in tumors. The caffeine metabolites, which had no effect on the adriamycin efflux, did not increase antitumor activity. These results suggest that the metabolism of caffeine may weaken its effect as a biochemical modulator, and that pentoxifylline and theobromine would be of value as biochemical modulators of adriamycin.

Key words: Adriamycin — Adriamycin efflux — Biochemical modulation — Caffeine — Theobromine

Chemotherapeutic agents are widely used in tumor therapy. However, with most of these agents, there are serious problems in terms of side effects and the appearance of resistant cells. Accordingly, many studies have been carried out with biochemical modulators, which can suppress the side effects or enhance the antitumor effects of the chemotherapeutic drugs.

Iliakis *et al.*[1] reported that caffeine seems to have an inhibitory effect on DNA repair, and Tsuchiya *et al.*[2-4] suggested that this agent is a potential enhancer of antitumor agents. Most of these reports showed that the best *in vitro* combination of caffeine with an antitumor agent was with cisplatin. However, it is clear that the *in vivo* specificity of these antitumor agents is different from that *in vitro*, and it has been shown that, *in vivo*, the antitumor activity of adriamycin in combination with caffeine is superior to that of cisplatin.[5] We have previously reported that caffeine enhanced only the antitumor effect of adriamycin, without increasing its side effects; this action was not due to the inhibitory effect on DNA repair, but was due, rather, to a specific increase in adriamycin concentration in the tumor, brought about by inhibition of adriamycin efflux from the tumor cells.[6]

Therefore, to determine the mechanism whereby caffeine influences adriamycin influx and efflux, in this study we investigated the inhibitory effects of caffeine metabolites on adriamycin efflux and influx in Ehrlich ascites carcinoma cells. We also investigated whether some methylxanthine derivatives that inhibit adriamycin efflux also enhance the antitumor activity of adriamycin and inhibit adriamycin efflux in tumor tissues *in vivo*.

MATERIALS AND METHODS

Reagents Caffeine, theophylline, and theobromine were purchased from Wako Pure Chemical Industries Ltd. (Tokyo). Pentoxifylline, 1,3,9-trimethylxanthine (isocaffeine), 1,3,7-trimethyluric acid, 1,7-dimethylxanthine and 7-methylxanthine were obtained from Sigma Chemical Co. (St. Louis, MO). Adriamycin injection, 10 mg/vial (Adriacin) was purchased from Kyowa Fermentation Inc. (Tokyo). The drugs were dissolved in sterile isotonic saline.

Animals Male CDF_1 mice, 5 weeks old and weighing 20–25 g, were obtained from Japan SLC (Hamamatsu). The animals were housed in a room maintained at 25 ± 1°C with 55 ± 5% relative humidity and were given free access to regular chow pellets and water.

Effects of drugs on adriamycin concentration in Ehrlich ascites carcinoma cells *in vitro* Ehrlich ascites carcinoma (1×10^6 cells/animal) was intraperitoneally transplanted to CDF_1 mice. The ascites were collected on the 7th day after transplantation. The ascites carcinoma cells were washed twice, and resuspended in Eagle's MEM medium containing 10% fetal bovine serum.

To examine the effects of drugs on adriamycin concentration in Ehrlich ascites carcinoma cells, medium containing 5×10^6 cells/ml and 10 μg/ml adriamycin was incubated at 37°C for 60 min in the presence or absence of caffeine (100 n*M*). After incubation, the medium was cooled on ice and centrifuged at 1,000 rpm for 3 min. The cells were washed and resuspended with ice-cold phosphate buffer (pH 7.8), then mixed for 30 s with

chloroform-methanol (4:1, v/v) and centrifuged. The concentration of adriamycin in the organic phase was determined with a fluorescence spectrophotometer (excitation, 470 nm; emission, 585 nm).

For examining adriamycin efflux from the cells, medium containing cell suspension (5×10^6 cells/ml) and 10 μg/ml adriamycin was preincubated at 37°C for 1 h. After incubation, the medium was cooled on ice and centrifuged at 1,000 rpm for 3 min. The cells were washed and resuspended in Eagle's MEM medium containing 10% fetal bovine serum. The cell suspension (5×10^6 cells/ml) was then incubated at 37°C for 180 min in the presence or absence of drugs (100 n*M*). Subsequent experiments were carried out in a manner similar to that employed for examining the uptake of adriamycin.

Animal experiments For the study of survival rate in tumor-bearing animals treated with adriamycin, Ehrlich ascites carcinoma (1×10^6 cells/animal) was intraperitoneally transplanted to groups of male CDF_1 mice, each group consisting of 10 mice. Adriamycin (0.5 mg/kg/day×5 days or 2.0 mg/kg/day×5 days) was administered intraperitoneally to mice at 1, 3, 5, 7, and 9 days after inoculation, and the drugs were injected intraperitoneally 2, 4, 6, 8, and 10 days after tumor inoculation. The numbers of living and dead animals were noted daily and the survival rate was calculated.

For the study of antitumor activity and adriamycin concentration, Ehrlich ascites carcinoma (5×10^6 cells/animal) was transplanted onto the backs of the mice and adriamycin (2.0 mg/kg/day×4 days) was administered intraperitoneally to groups consisting of 5–6 mice, 10, 12, 14, and 16 days after inoculation. The other drugs were injected intraperitoneally 11, 13, 15, and 17 days after tumor inoculation. The animals were killed by cervical dislocation on the day after the last day of drug administration. The livers, hearts, and tumors were rapidly removed and weighed. The tissue samples were homogenized in 10 volumes (w/v) of 10 m*M* phosphate buffer (pH 7.8), and adriamycin concentration was determined as above.

In both these studies, the drugs tested were caffeine, pentoxifylline, theobromine, and 1,3,7-trimethyluric acid; the dose was 10 mg/kg/day×4 days.

Statistical analysis Statistical analyses were performed by using Student's *t* test and the *U* test.

RESULTS

Effects of methylxanthine derivatives on intracellular concentration of adriamycin in Ehrlich ascites carcinoma cells Fig. 1 shows the effects of caffeine (100 n*M*) on adriamycin efflux in Ehrlich ascites carcinoma cells. As soon as incubation was began, the concentration of adriamycin in the cells began to decrease. After 60 min, there were no significant differences between the adriamycin alone and adriamycin plus caffeine groups in terms of the inhibition of adriamycin efflux. However, 180 min later, caffeine significantly inhibited the efflux of adriamycin by 35.4% ($P<0.05$) compared to the adriamycin alone group.

Table I shows the effects of the other methylxanthines tested on adriamycin efflux from Ehrlich ascites carcinoma cells. Theobromine, theophylline, and pentoxifyl-

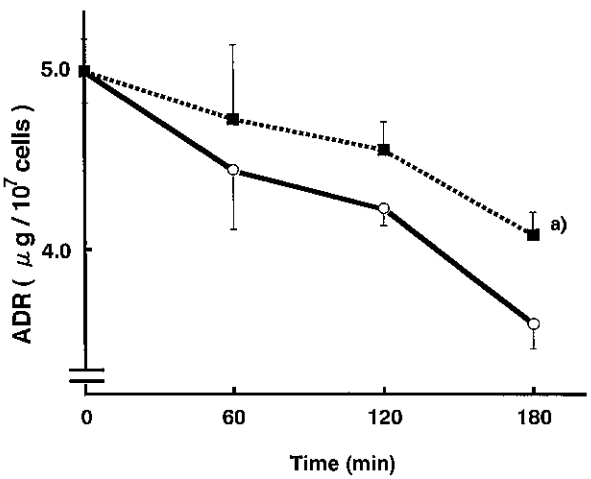

Fig. 1. Effects of caffeine on the time course of adriamycin (ADR) concentration in Ehrlich ascites carcinoma cells (efflux). Significant differences from the adriamycin alone level are indicated by a) $P<0.05$. ○, Without caffeine; ■, with caffeine.

Table I. Effects of Methylxanthines on Efflux of Adriamycin in Ehrlich Ascites Carcinoma Cells

Drug	Adriamycin concentration ($\mu g/1\times10^7$ cells)	
Adriamycin alone	3.59±0.14	(0)
Caffeine	4.08±0.12[a)]	(35.4)
Theobromine	4.07±0.12[b)]	(34.6)
Theophylline	4.18±0.27[a)]	(42.5)
Pentoxifylline	4.18±0.15[b)]	(42.5)
Isocaffeine	3.78±0.26	(13.8)
1,3,7-Trimethyluric acid	3.23±0.20	(−25.7)
1,7-Dimethylxanthine	2.99±0.12[a)]	(−43.0)
7-Methylxanthine	3.11±0.19	(−34.4)

Each value represents the mean±SD at 180 min. At 0 min, the adriamycin concentration was 4.98±0.18 $\mu g/1\times10^7$ cells with all drugs. Numbers in parentheses indicate percent inhibition compared with adriamycin alone. Significant differences from the level of adriamycin alone are indicated by *a*) $P<0.05$ and *b*) $P<0.01$.

line significantly inhibited the efflux of adriamycin, by 34.6% ($P<0.01$), 42.5% ($P<0.05$), and 42.5% ($P<0.01$), respectively, compared to the adriamycin alone group. With 1,7-dimethylxanthine, this level was significantly decreased compared to that of the adriamycin alone group ($P<0.05$).

Fig. 2 shows the effects of caffeine, theobromine, and pentoxifylline on adriamycin influx in Ehrlich ascites carcinoma cells. In the control group, the concentration of adriamycin in the cells increased immediately after incubation began, reaching a plateau 30 min later, the plateau being maintained until 60 min. Caffeine and the other methylxanthines did not show any effect on adriamycin influx.

Effects of methylxanthine derivatives on the reduction in tumor weight induced by adriamycin The effects of the methylxanthine derivatives on the adriamycin-induced changes in tumor weight are shown in Fig. 3. The mean tumor weight in the control group was 2.35±0.32 g. The adriamycin alone group showed a 20% reduction of tumor weight compared to the control level, and caffeine combined with adriamycin enhanced by 2.1-fold ($P<0.05$) the efficacy of the adriamycin alone group. Pentoxifylline and theobromine combined with adriamycin increased by 3.1-fold ($P<0.01$) and 3.2-fold ($P<0.001$), respectively, the efficacy of the adriamycin alone group;

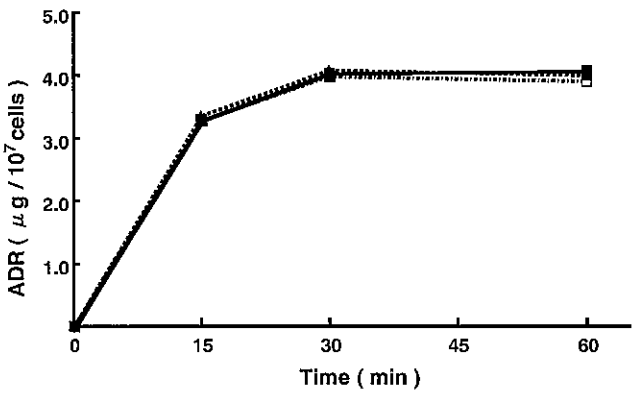

Fig. 2. Effects of caffeine, theobromine, and pentoxifylline on the time course of adriamycin (ADR) concentration in Ehrlich ascites carcinoma cell (influx). Each point represent the mean value of three independent experiments, each with duplicate data points with no more than 10% variation between them. ○, Without methylxanthines; ■, caffeine; □, pentoxifylline; △, theobromine.

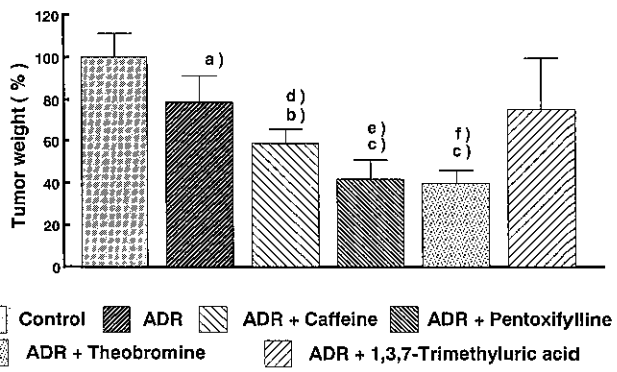

Fig. 3. Effects of test drugs on changes in tumor weight induced by adriamycin (ADR). Significant differences from the control level are indicated by a) $P<0.05$, b) $P<0.01$, and c) $P<0.001$. Significant differences from the adriamycin only level are indicated by d) $P<0.05$, e) $P<0.01$, and f) $P<0.001$.

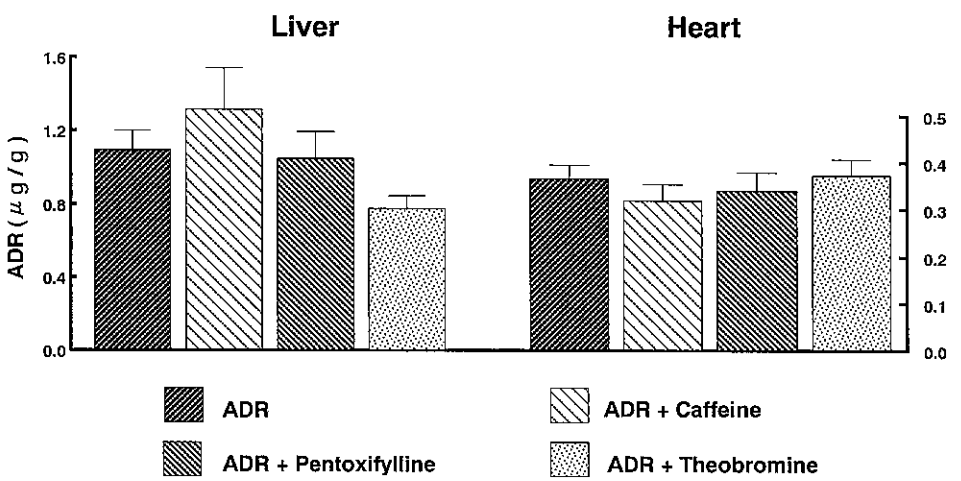

Fig. 4. Effects of test drugs on adriamycin (ADR) concentration in the livers and hearts of mice.

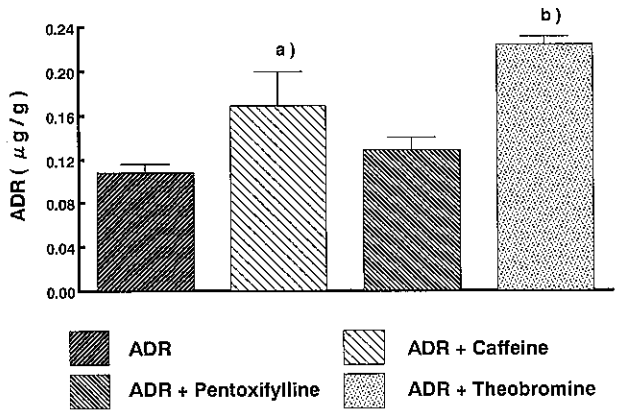

Fig. 5. Effects of test drugs on adriamycin (ADR) concentration in mouse tumors. Significant differences from the adriamycin only level are indicated by a) $P<0.05$, and b) $P<0.01$.

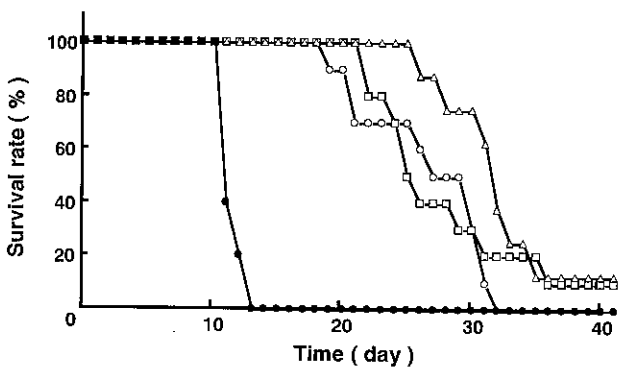

Fig. 6. Effects of test drugs on adriamycin-induced prolongation of survival. ●, Control; ○, adriamycin; □, adriamycin +pentoxifylline; △, adriamycin+theobromine.

1,3,7-trimethyluric acid combined with adriamycin did not show any enhancing effect.

The effects of the methylxanthine derivatives on the concentrations of adriamycin in heart and liver are shown in Fig. 4. In the heart, there were no significant differences in the concentration of adriamycin between the adriamycin alone and the combined drug groups. In the liver, although the combination with caffeine increased the concentration of adriamycin and the combination with theobromine decreased the concentration, the differences were not significant.

The effects of methylxanthine derivatives on the concentration of adriamycin in the tumor are shown in Fig. 5. The concentration of adriamycin in the adriamycin alone group was 0.108 ± 0.008 μg/g tumor. Pentoxifylline combined with adriamycin slightly increased this concentration, whereas the combination of caffeine or theobromine with adriamycin significantly enhanced the concentration of adriamycin in the tumor, by 1.6-fold ($P<0.05$) and 2.1-fold ($P<0.01$), respectively, compared to the adriamycin alone group. The combination with 1,3,7-trimethyluric acid did not increase the concentration of adriamycin (data not shown).

Effects of methylxanthine derivatives on survival of mice transplanted with Ehrlich ascites carcinoma cells and treated with adriamycin Fig. 6 shows the effects of methylxanthine derivatives on the survival of mice that received adriamycin 0.5 mg/kg/day×5 days. The median survival in the control group was 10.6 days. The survival of mice treated with adriamycin alone was markedly prolonged (25.8 days) compared to the control, and the combination of theobromine with adriamycin prolonged the survival by 36.2% ($P<0.05$) compared to the survival in the adriamycin alone group. However, pentoxifylline did not influence the antitumor activity of adriamycin. In mice that received adriamycin 2.0 mg/kg day×5 days, there was no prolongation in combination with any of these drugs (data not shown).

DISCUSSION

In a study of biochemical modulators, caffeine showed a specific strong enhancement of the effect of cisplatin in culture.[7] Caffeine has also been shown to enhance the antitumor effect of adriamycin *in vivo*, by inducing specific increases in the adriamycin concentration in the tumor, this being due to the inhibition of adriamycin efflux from tumor cells.[6] This effect has been clearly observed both *in vivo* and *in vitro*, and it may be one of the mechanisms whereby caffeine enhances the antitumor activity of adriamycin. Accordingly, to examine further this question, we investigated the effect of methylxanthine derivatives on adriamycin influx and efflux in Ehrlich ascites carcinoma cells.

In regard to adriamycin influx, none of the methylxanthine derivatives we tested changed the time course of increase in adriamycin concentration compared to the adriamycin alone group. In regard to adriamycin efflux, on the other hand, there were differences in the effects of these derivatives.

The major metabolites of caffeine, 7-methylxanthine and 1,3,7-trimethyluric acid, did not inhibit adriamycin efflux compared with the adriamycin alone group. Moreover, 1,7-dimethylxanthine, the major caffeine metabolite in humans,[8] promoted adriamycin efflux. These findings suggest that the metabolism of caffeine may weaken its effect in inhibiting adriamycin efflux. Caffeine, theophylline, theobromine, and pentoxifylline, however, inhibited adriamycin efflux in Ehrlich ascites carcinoma cells.

These effects were also observed in P388 leukemic cells (data not shown). It was therefore clear that these methylxanthine derivatives had different effects on adriamycin efflux.

The results with theophylline, theobromine, and pentoxifylline, were similar to those for caffeine, suggesting that these drugs are possible enhancers of antitumor agents *in vivo*.

In regard to the antitumor activity of adriamycin, we examined the effects of the methylxanthine derivatives *in vivo*; caffeine, theobromine, and pentoxifylline, which inhibited adriamycin efflux, and 1,3,7-trimethyluric acid, which did not. Theophylline, the most typical of the methylxanthines, has been widely used in the treatment of bronchial asthma. However, because therapeutic blood levels are close to levels that cause side effects, the drug level must be monitored. For this reason, we believe that theophylline is not suitable for use as a modulator, and we omitted it from the *in vivo* study. The combinations of adriamycin with caffeine, theobromine, and pentoxifylline, which inhibited adriamycin efflux *in vitro*, enhanced adriamycin antitumor activity compared with the adriamycin alone group, showing that these drugs should be of value as biochemical modulators. The combination with 1,3,7-trimethyluric acid, which had no inhibitory effect on adriamycin efflux *in vitro*, did not increase the antitumor activity of adriamycin compared with the adriamycin alone group, suggesting that caffeine, when metabolized, did not enhance the antitumor activity of adriamycin *in vivo*. All the drugs that inhibited adriamycin efflux *in vitro* enhanced the antitumor activity of adriamycin *in vivo*, while those drugs that had no effect *in vitro* also had no effect *in vivo*; i.e., there was a correlation between the *in vitro* and *in vivo* findings. These results suggest that caffeine is effective as a biochemical modulator and that our *in vitro* method is useful for the screening of biochemical modulators.

In our investigation of tissue adriamycin concentration in carcinoma-bearing mice, we found that the drug combinations increased adriamycin concentration in the tumor, compared to that in the adriamycin alone group. This effect was correlated with antitumor activity, again suggesting that these drugs are useful biochemical modulators of adriamycin. To our knowledge, there is no report of the effects of theobromine in enhancing the antitumor activity of adriamycin by increasing adriamycin concentration in tumor tissue, either *in vivo* or *in vitro*. Therefore, we consider that these results will also be of value in the study of biochemical modulation.

However, pentoxifylline, which enhanced the antitumor activity of adriamycin and inhibited adriamycin efflux *in vitro*, did not enhance the tissue concentration of adriamycin. These conflicting findings between the *in vivo* and *in vitro* studies indicate that the effect of pentoxifylline in increasing the antitumor activity of adriamycin may be due to other mechanisms. It has been reported that pentoxifylline has effects on tumor necrosis factor, interleukin,[9–12] erythrocytes, blood platelets, and fibrinogen,[13–15] and these actions may influence the *in vivo* antitumor activity of adriamycin.

Interestingly, none of these drugs had any influence on adriamycin concentration in heart and liver, suggesting that they did not increase the side effects of adriamycin.

We examined the prolonging effects of the combinations of adriamycin with theobromine and pentoxifylline on the survival of ascites tumor-bearing mice. The combination of theobromine with adriamycin 0.5 mg/kg/day $\times 5$ days enhanced the antitumor activity of adriamycin. Combinations of these drugs with adriamycin 2.0 mg/kg/day $\times 5$ days did not enhance the antitumor effect of adriamycin (data not shown), in accordance with previous findings for caffeine.[16] When adriamycin was administered at high concentrations *in vivo*, the combinations with pentoxifylline, theobromine, and caffeine had no effect. We speculate that, because adriamycin was administered intraperitoneally, it acted directly on the ascites tumor cells. Thus, the activity of this antitumor agent in such tumors could be greater than that in solid tumors, and the effect on the antitumor activity of a combined drug would be difficult to clarify. In this study, pentoxifylline had no effect on the prolongation of survival. The administration dose and schedule we used, which were similar to those reported for caffeine,[5] may not be suitable. Pentoxifylline has been shown to modulate the antitumor activity of adriamycin in solid tumors, and pentoxifylline has been reported to enhance the antitumor activity of cisplatin,[17, 18] to improve resistance to adriamycin,[19] and to inhibit *in vitro* DNA biosynthesis, in combination with vincristin.[20]

We found that combinations of caffeine, pentoxifylline, and theobromine with adriamycin significantly increased the antitumor activity of adriamycin. In particular, we found that the combination with theobromine, which has not previously been reported as a biochemical modulator, had the greatest effect in increasing adriamycin concentration and antitumor activity. Therefore, among the methylxanthine derivatives, theobromine appears to have the most promise as a biochemical modulator. Both the *in vitro* and *in vivo* studies suggested that adriamycin accumulated because its efflux was inhibited by the drugs with which it was combined. This effect in increasing adriamycin concentration was not found in healthy tissue, but only in tumor tissue, indicating that the drugs that produce these effects should be valuable as biochemical modulators of adriamycin. We consider that the increase in adriamycin concentration brought about by caffeine was not due to the metabolism of caffeine, and that some other action was responsible for this increase.

Caffeine has an inhibitory effect on DNA repair.[1] To exert this effect, the level of caffeine required is of the order of m*M*; however, in our study, the caffeine level was of the order of n*M*. There is no report of any action of caffeine at this latter level, except for its inhibition of adriamycin efflux.[6] We assume that this inhibitory action of caffeine is one of the mechanisms by which it modulates the antitumor activity of adriamycin. However, this action alone is not sufficient to explain the effects found here. Further studies are needed to examine other possible mechanisms for this antitumor activity.

(Received November 30, 1994/Accepted March 28, 1995)

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