1-Methyl-3-propyl-7-butylxanthine, a Novel Biochemical Modulator, Enhances Therapeutic Efficacy of Adriamycin

Yasuyuki Sadzuka,[1, 4] Ayano Iwazaki,[1] Tomomi Sugiyama,[1] Takayuki Sawanishi[2] and Ken-ichi Miyamoto[3]

[1]*School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422, [2]School of Pharmacy, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa 920-11 and [3]Department of Pharmacology and Pharmaceutics, Kanazawa University, 13-1 Takara-machi, Kanazawa 920*

We have screened xanthine derivatives for activity as novel biochemical modulators by assay of their inhibitory effect on adriamycin efflux from tumor cells. Strong inhibition of adriamycin efflux was shown by some xanthine derivatives with various alkyl or oxoalkyl substituents at the 1-, 3- and 7-positions. 1-Methyl-3-propyl-7-butylxanthine (XT-77), which had the greatest inhibitory effect on adriamycin efflux *in vitro* among the compounds tested, potentiated adriamycin-induced antitumor activity by causing an increase of adriamycin concentration in the tumor *in vitro*. Furthermore, XT-77 reduced the adverse drug reactions of adriamycin by decreasing the adriamycin concentrations in the heart and the liver. Thus, the combination of XT-77 with adriamycin not only increased the antitumor activity of adriamycin, but also decreased the adverse drug reactions.

Key words: Xanthine derivative — Biochemical modulation — Adriamycin — 1-Methyl-3-propyl-7-butylxanthine

In cancer chemotherapy, biochemical modulation is one of the most effective methods to improve the activity of known agents, and modulators have been found in recent years for cisplatin and 5-fluorouracil.[1–3] Caffeine, a methylxanthine derivative, inhibits DNA repair,[4, 5] and this agent has been suggested as a potential amplifier of the cytocidal effects of DNA-damaging antitumor agents (such as cisplatin) in culture.[6–8] We have reported that caffeine enhances the antitumor effect of adriamycin, without increasing its side effects; however this action is not due to an inhibitory effect on DNA repair, but rather, to a specific increase in adriamycin concentration in the tumor, brought about by the inhibition of adriamycin efflux from the tumor cells.[9–12] Furthermore, the major metabolites of caffeine did not have modulatory effects on the antitumor activity of adriamycin, the adriamycin concentration in the tumor *in vivo*, or the adriamycin efflux from the tumor cells *in vitro*.

In this study, we searched for effective biochemical modulators among xanthine derivatives (Fig. 1) by assay of their ability to inhibit the adriamycin efflux from Ehrlich ascites carcinoma cells. 1-Methyl-3-propyl-7-butylxanthine (XT-77) was found to enhance the antitumor activity of adriamycin and to reduce the adverse drug reactions. We also examined the mechanisms of these effects, and the structure-activity relationship.

MATERIALS AND METHODS

Reagents Adriamycin injection, 10 mg/vial (Adriacin), was purchased from Kyowa Fermentation Inc., Tokyo. The xanthine derivatives used were synthesized in our laboratory.[13] The other chemicals used in this study were of the highest purity available.

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

To examine the effects of xanthine derivatives on adriamycin efflux in Ehrlich ascites carcinoma cells, 1×10^7 cells/ml and 9.0 nmol/ml adriamycin was preincubated in the medium at 37°C for 30 min. After incubation, the medium was cooled on ice and centrifuged at $150g$ for 3 min. The cells were washed and resuspended in Eagle's MEM medium containing 10% fetal bovine serum. The cell suspension (1×10^7 cells/ml) was then incubated at 37°C for 180 min in the presence or absence of a xanthine derivative (100 nM). After incubation, the medium was cooled on ice and centrifuged at $150g$ for 3 min. The cells were washed and resuspended in ice-cold phosphate buffer (10 mM, pH 7.8). The suspension was mixed for 30 s with chloroform-methanol (4:1, v/v) and centrifuged ($1,200g$, 15 min). The concentration of adriamycin in the organic phase was determined with a fluorescence spectrophotometer (excitation, 470 nm; emission, 585 nm).

In addition, a cell suspension (1×10^7 cells/ml) containing 9.0 nmol/ml adriamycin was incubated at 37°C for 60 min in the presence or absence of a xanthine derivative (100 nM). Subsequent procedures were similar to those described above.

Effects of XT-77 on antitumor activity of adriamycin *in vivo* Ehrlich ascites carcinoma cells (5×10^5 cells/ani-

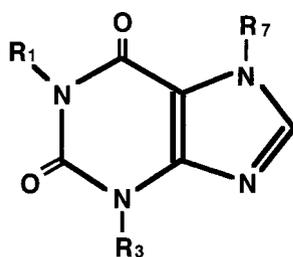---

[4] To whom correspondence should be addressed.

Fig. 1. The basic structure of 1,3,7-trialkylxanthines. In general, substituents have the following effects: R_1, increase in broncho-selectivity, increase in PDE IV inhibition and decrease in chronotropism and PDE III inhibition; R_3, increase in chronotropism and bronchoselectivity; R_7, increase in bronchoselectivity, decrease in chronotropism and PDE III inhibition.

Table I. Effects of Xanthine Derivatives (100 μM) on Efflux of Adriamycin in Ehrlich Ascites Carcinoma Cells

Drug	R_1	R_3	R_7	%
XT-10	Me	Pro	H	19.3[b]
XT-75	Me	Pro	Pro	27.9[c]
XT-136	Me	Pro	Oxopro	21.8[a]
XT-43	Pro	Pro	H	0.27
DPCPX	Pro	Pro	H (R_8: cPen)	29.7[a]
XT-56	Bu	Me	H	−6.8
XT-48	Bu	Bu	H	9.4
Denbufylline	Bu	Bu	Oxopro	48.9[a]
XT-80	Oxohex	Pro	H	−36.4[a]
XT-83	Oxohex	Pro	Pro	50.3[b]

Each value represents the percent inhibition compared with adriamycin alone at 180 min. Significant differences from the level of adriamycin alone are indicated by a) $P<0.05$, b) $P<0.01$ and c) $P<0.001$.
Abbreviations: Me, methyl(CH_3-); Pro, n-propyl($CH_3CH_2CH_2$-); Bu, n-butyl($CH_3CH_2CH_2CH_2$-); Oxopro, 2′-oxopropyl-(CH_3COCH_2-); Oxohex, 5′-oxohexyl($CH_3CO(CH_2)_4$-); cPen, cyclopentyl($\bigcirc$).

mal) were transplanted onto the backs of mice, and adriamycin (2.0 mg/kg/day×4 days) was intraperitoneally administered to groups consisting of 8 mice, at 10, 12, 14 and 16 days after inoculation. XT-77 (10 mg/kg/day×4 days) was intraperitoneally injected 11, 13, 15 and 17 days after tumor inoculation. The animals were killed by cervical dislocation on the 18th day after inoculation. The tumor, heart and liver were rapidly removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10 mM phosphate buffer (pH 7.8), and the adriamycin concentration was determined as previously described.

Effects of XT-77 on adverse reactions to adriamycin *in vivo* Groups of 5 or 6 mice were used. XT-77 (100 mg/kg/day, i.p.) was injected into normal mice once a day for 5 days. On the second day, at 3 h after the XT-77 injection, adriamycin (15 mg/kg, i.p.) was injected. The animals were killed by cervical dislocation on the day following the last administration of XT-77. The heart, liver, kidneys and lungs were then rapidly dissected out, washed with isotonic saline, and weighed. The lipid peroxide level and glutathione peroxidase (GSHpx) activity in each sample were assayed by using our method[14] and according to Hafeman et al.,[15] respectively.

Statistical analysis Statistical analysis was performed by using Student's t test and ANOVA.

RESULTS

Effect of variously substituted xanthine derivatives on adriamycin efflux from Ehrlich ascites carcinoma cells *in vitro* The inhibitory effects of xanthine derivatives with various alkyl or oxoalkyl substituents at the 1-, 3- and 7-positions (0.1 mM) on adriamycin efflux from the tumor cells after 180 min of incubation are shown in Table I. Alkyl chain elongation at the 1-position of 3-propylxanthine from methyl to propyl and to oxohexyl (XT-10, XT-43 and XT-80, respectively) decreased the inhibition of
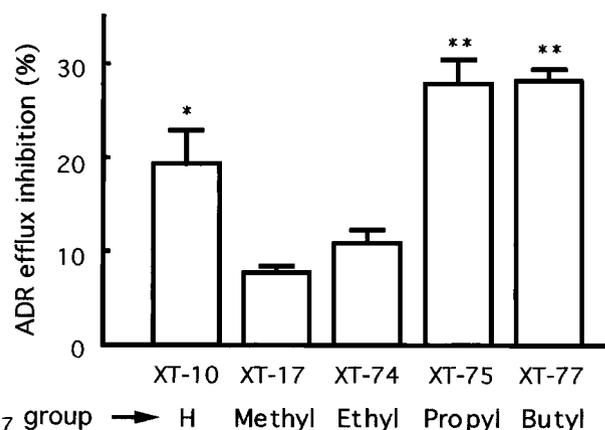

Fig. 2. Effects of xanthine derivatives on efflux of adriamycin in Ehrlich ascites carcinoma cells (180 min). Each column shows the percentage inhibition compared with the adriamycin-alone group in each experiment, and represents the mean±SD of three experiments. Significant differences from the adriamycin-alone level are indicated by ∗ $P<0.01$ and ∗∗ $P<0.001$.

the adriamycin efflux. The alkyl chain at the 3-position of 1-butylxanthine seemed to have little effect (methyl and butyl: XT-56 and XT-48, respectively). 7-Alkylxanthines (XT-75, XT-136, denbufylline, XT-83) were strong inhibitors of adriamycin efflux. The inhibitory effects of 1-methyl-3-propylxanthine with various 7-alkyl substituents (0.1 mM) are shown in Fig. 2. Inhibition increased with
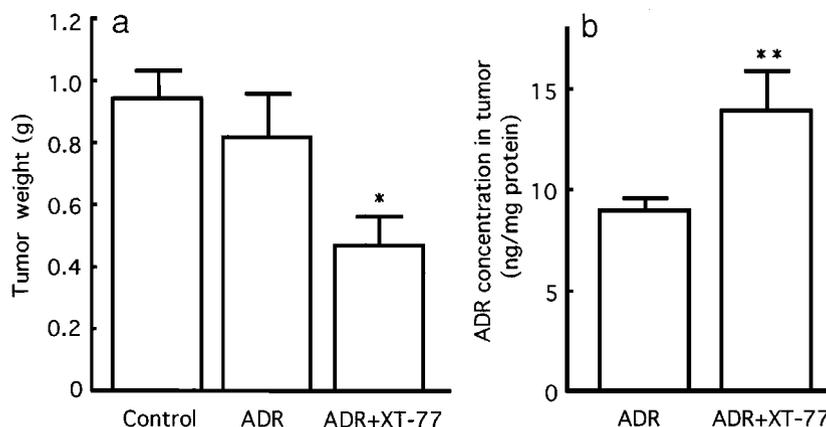
Fig. 3. Effects of XT-77 on adriamycin-induced changes in tumor weight (a) and adriamycin concentration in the tumor (b). Each column represents the mean±SD of eight mice. Significant differences from the adriamycin-alone level are indicated by ∗ $P<0.01$ and ∗∗ $P<0.001$.

increasing chain length, reaching about 30% for the 7-propyl compounds.

These xanthine derivatives did not influence adriamycin influx into the Ehrlich ascites carcinoma cells (data not shown).

Effect of 1-methyl-3-propyl-7-butylxanthine (XT-77) on adriamycin-induced decrease in tumor weight and adriamycin concentration in the tissues of solid Ehrlich ascites carcinoma-bearing mice The effects of XT-77 on adriamycin- induced changes in tumor weight are shown in Fig. 3. The tumor weight was significantly reduced ($P<0.01$) in the adriamycin plus XT-77 group as compared with the adriamycin-alone group, or the control group. XT-77 alone had no antitumor activity (data not shown). The combination treatment (adriamycin plus XT-77) also significantly enhanced the adriamycin concentration in the tumor, by 1.6-fold vs. the adriamycin-alone group ($P<0.001$). Similar effects of XT-77 were seen after adriamycin administration at 0.5 mg/kg/day×4 days (data not shown).

The adriamycin concentrations in the heart and liver are shown in Fig. 4. In the heart, the adriamycin concentration was decreased by 26% (compared with the adriamycin-alone group) by the combination with XT-77; it was decreased by 15% in the liver.

Effects of XT-77 on adverse reactions to adriamycin The effects of XT-77 on the adriamycin-induced increase in the lipid peroxide level and decrease in GSHpx activity, as indices of cardiotoxicity, are shown in Table II. In the heart, the lipid peroxide level in the adriamycin-alone group was elevated to 1.4-fold ($P<0.01$), whereas that in the adriamycin plus XT-77 group remained at the normal level ($P<0.01$ vs. adriamycin alone). GSHpx activity in the heart was reduced by 32% ($P<0.01$) by adriamycin ad-
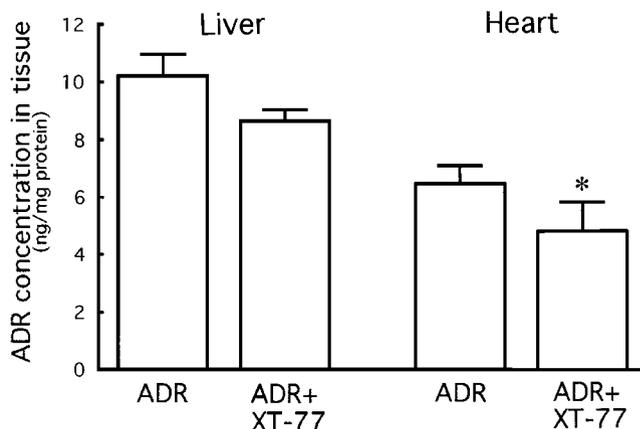

Fig. 4. Effects of XT-77 on adriamycin concentration in the liver and heart of mice. Each column represents the mean±SD of eight mice. Significant difference from the adriamycin-alone level is indicated by ∗ $P<0.01$.

ministration, but remained at the normal level in the adriamycin plus XT-77 group. In the liver, the lipid peroxide level increased 1.2-fold after adriamycin injection, but was normalized by the combination with XT-77. GSHpx activity in the liver was decreased 12% ($P<0.05$) by adriamycin, but the combination with XT-77 did not normalize this change. The lipid peroxide level in the bone marrow cells in the adriamycin-alone group increased 2.5-fold ($P<0.001$) from that of normal mice, and this increase was significantly suppressed by XT-77 ($P<0.05$ vs. adriamycin-alone group).

XT-77 alone had no significant effect on any of these parameters.

Table II.   Effects of XT-77 on the Lipid Peroxide (LPO) Level and GSHpx Activity in the Tissues of Mice

	Normal	XT-77	ADR	ADR + XT-77
Heart				
LPO	4.55±0.55	5.21±0.71	5.98±1.09[c]	4.28±0.95[f]
GSHpx	4.79±0.92	4.54±2.63	3.24±0.72[c]	4.72±0.82[f]
Liver				
LPO	0.829±0.115	0.808±0.233	0.965±0.308	0.617±0.139
GSHpx	208±15.2	208±15.3	181±25.4[b]	177±21.1[b]
Bone marrow				
LPO[a]	3.75±0.93	5.39±1.03	9.22±2.16[d]	6.12±2.28[e]

Each value represents the mean±SD of eight mice. Lipid peroxide level and GSHpx activity are expressed as nmol/mg protein (*a*) nmol/10^8 bone marrow cells) and unit/mg protein, respectively. Significant differences from the level of the normal group are indicated by *b*) $P<0.05$, *c*) $P<0.01$ and *d*) $P<0.001$. Significant differences from the level of the adriamycin-alone group are indicated by *e*) $P<0.05$ and *f*) $P<0.01$.

DISCUSSION

We have screened various xanthine derivatives for ability to inhibit the efflux of adriamycin from Ehrlich ascites carcinoma cells, with the aim of finding novel biochemical modulators. Strong inhibition of adriamycin efflux was shown by xanthine derivatives whose 1-position and 3-position, or 3-position and 7-position have the same alkyl substituents as caffeine, theobromine and theophylline.[12] In the case of fixed substituents at the 1-position and 3-position, a longer 7-alkyl chain resulted in more potent inhibition, and derivatives with the 7-propyl moiety showed strong inhibition of adriamycin efflux. 1-Methyl-3-propyl-7-butylxanthine (XT-77) appeared to be the optimum compound in this series.

In considering the usefulness of biochemical modulators, two potential problems must be considered: a) the effect of the modulator on the adverse effects of the antitumor agent, b) other effects of the modulators, namely the side effects of the modulator itself. The main pharmacological actions of xanthine derivatives include positive chronotropic activity, diuretic activity and tracheal relaxant activity as a consequence of elevation of cyclic AMP through inhibition of cyclic AMP phosphodiesterase (PDE).[16–19] The effects of various substituents on these actions of xanthine derivatives are as follows (see Fig. 1). It was reported that elongation of the alkyl chain at the 1-position of xanthine increased tracheal relaxant activity through greater inhibition of PDE IV activity and decreased chronotropism through reduced inhibition of PDE III activity. The action on PDE III activity was also influenced by the chain length at the 7-position. On the other hand, elongation of the chain length at the 3-position increased tracheal relaxant activity and chronotropism.[13, 20–25] In our study, the elongation of the chain length at the 7-position, known to decrease the pharmaco-

logical activity of xanthine derivatives, increased the inhibition of the adriamycin efflux from the tumor cells. These results are favorable for the use of xanthine derivatives as biochemical modulators. In particular, as the effect on the heart was reduced with elongation of the chain length at the 7-position, we expected that the use of xanthine derivatives in combination with adriamycin, which induces severe cardiotoxicity, might be feasible.

As regards the mechanism of action, xanthine derivatives used in this study did not have any effect on the time-dependent increase in adriamycin concentration in adriamycin-treated tumor cells, so they do not appear to affect the drug influx system(s). In addition, P-glycoprotein, multidrug resistance-associated protein (MRP) and GS-X pump are not overexpressed in this cell line,[12] and inhibitors of these pumps have no effect on the efflux of adriamycin in this cell line.[12] Furthermore, xanthine derivatives do not affect adriamycin metabolism.[10] The particular drug efflux system inhibited by XT-77 remains to be identified.

We next examined whether the effect of XT-77 *in vitro* would be reflected *in vivo*. The combination of adriamycin with XT-77 significantly decreased the tumor weight, compared with that in the adriamycin-alone group. Further, the adriamycin concentration in the tumor was significantly increased compared with that in the adriamycin-alone group, as expected. The effects of XT-77 are greater than those of caffeine.[9] On the other hand, the adriamycin concentration in the heart and the liver was rather decreased by the XT-77 combination, suggesting that a reduction of the adverse reactions to adriamycin should also be caused by XT-77. Such an effect is not seen with caffeine.

Next, we examined the changes in the lipid peroxide level and GSHpx activity in the heart, as indices of cardiotoxicity induced by adriamycin.[26–32] The combination of adriamycin with XT-77 significantly decreased the

adriamycin-induced increase in lipid peroxide level and restored the GSHpx activity in the heart to a normal level. Namely, XT-77 decreased adriamycin-induced cardiotoxicity. The beneficial effect of XT-77 was also confirmed by measuring creatine phosphokinase activity (data not shown). In the liver, the combination of adriamycin with XT-77 significantly decreased the adriamycin-induced lipid peroxidation, and a similar effect was seen in the bone marrow, another target tissue of adriamycin toxicity. Therefore, XT-77 should be useful to suppress the bone marrow toxicity induced by adriamycin. XT-77 alone caused no significant change in the lipid peroxide level or GSHpx activity in each tissue (data not shown).

As XT-77 had no effect on the NADPH-dependent microsomal lipid peroxidation in mouse liver, we considered that XT-77 did not have an antioxidative action. It seems likely that the reduction of the adriamycin-induced side effects by XT-77 was due to the XT-77-induced decrease in the adriamycin concentration in the normal tissues. The combination of caffeine with adriamycin increased the adriamycin concentration in the tumor, but did not affect the adriamycin concentrations in the normal tissues, suggesting that caffeine has different actions in the tumor and normal tissues.[9, 12] In contrast, the combination of XT-77

with adriamycin increased the adriamycin concentration in the tumor and decreased adriamycin concentrations in the normal tissues. It is possible that the decrease in the adriamycin concentration in the normal tissues may be related to the accumulation in the tumor. Another possibility is that the nature or type of drug transporters in the tumor cells might be different from that of the normal cells, or the concentrations of XT-77-sensitive transporters in the tumor cells might be greater than that in normal cells.

In conclusion, the combination of XT-77 with adriamycin not only increased the antitumor activity of adriamycin, but also decreased the side effects of adriamycin. Namely, it appears that XT-77 has excellent characteristics as a biochemical modulator. In addition, the tracheal relaxant activity and the chronotropic activity of XT-77 are relatively weak.

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