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Screening of biochemical modulator by tumor cell permeability of doxorubicin

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Abstract

We screened various food components for their ability to inhibit doxorubicin (DOX) permeability in tumor cells in vitro with the aim of finding novel modulators. Capsaicin did not change DOX permeability in the tumor cells, although the capsaicin derivatives gingerol and ferulic acid tended to promote DOX efflux. Combinations of these components with DOX were also not effective. In contrast, cucurbitacin E significantly promoted DOX influx into tumor cells and increased DOX concentration in tumor cells. Furthermore, combined cucurbitacin E significantly suppressed DOX efflux from tumor cells and was shown to maintain the DOX level in tumor cells. It was also confirmed that the combination of cucurbitacin E with DOX resulted in effective cytotoxicity for tumor cells in culture. Additionally, the combination of cucurbitacin E and DOX showed increased cytotoxicity when compared to each treatment alone. In vivo, DOX alone treatment did not change the time course of tumor size or tumor weight of M5076 ovarian sarcoma, compared to control levels. In contrast, the combination of cucurbitacin E with DOX resulted in decreased tumor size and tumor weight, compared to that in DOX alone group, indicating effective antitumor activity. In conclusion, the combination of cucurbitacin E with DOX may be an effective tool with treated application in the cancer chemotherapy.

Keywords: Cucurbitacin E; Doxorubicin; Cancer chemotherapy; Influx; Efflux

1. Introduction

Cancer chemotherapy has been applied to advanced tumors and intractable tumors, playing an important role in their treatment. However, there are many problems including the appearance of adverse reactions and acquisition of drug resistance (Chen et al., 1993; Kang and Perry, 1994). Cancer chemotherapy is clinically applied using multidrug combined therapy. During biochemical modulation, the pharmacodynamics of an antitumor agent are modulated by combination with another drug. Clinically, UFT drugs (tegafur + uracil) or 5-furorouracil + leucovorin therapies have been tried (Kubota et al., 1993; Bertino et al., 1977; O'Connel, 1988; Hidalgo et al., 1989; Ichinose et al., 1995). However, these therapies have severe adverse reactions that prevent them from increasing the therapeutic index (Bud et al., 1987).

Doxorubicin (DOX), an anthracycline antitumor agent, is clinically used for the treatment of a variety of malignancies whereas the clinical use of DOX is restricted by severe adverse reactions such as cardiotoxicity (Sazuka et al., 1989). For biochemical modulation, we have reported that combination of DOX with caffeine, a xanthine derivative, and theanine, a glutamate derivative, increased DOX-induced antitumor activity (Sadzuka et al., 1993, 1995a,b, 2000, 2001a,b; Sugiyama et al., 2004; Sugiyama and Sadzuka, 2003). It appears that these effects were mediated by increased DOX concentration in the tumors, through suppression of DOX efflux from tumor cells (Sugiyama and Sadzuka, 2003). As this mechanism differs from previous described mechanisms, we hypothesized that it could lead to discovery of a novel biochemical modulator.

In this study, we screened various compounds for their ability to inhibit DOX permeability in tumor cells in vitro with

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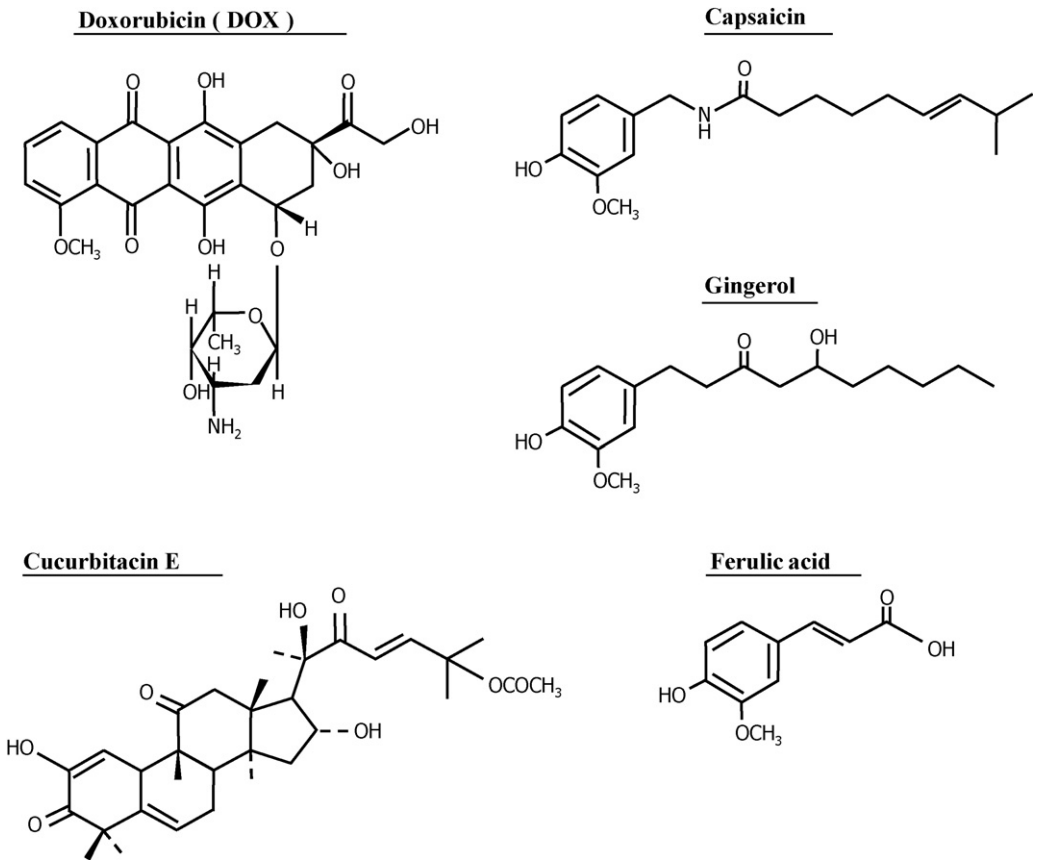

Fig. 1. Chemical structures of doxorubicin and combined compounds.

the aim of finding novel modulators. In clinical cancer therapy, many medicines are applied to multidrug combined therapy or to the decrease of antitumor agent-induced adverse reactions. However, the increased numbers of medications resulted in a decreased quality of life for the patients. In contrast, food and food components can be used to increase the activity of antitumor agents without increasing the number of medicines, that patients take. Therefore, we examined the effects of capsaicin, gingerol, ferulic acid and cucurbitacin E (Fig. 1), which are all food components, on DOX permeability in tumor cells and DOX-induced antitumor activity.

2. Materials and methods

2.1. Chemicals

DOX, 10 mg per vial (Adriacin), was purchased from Kyowa Fermentation, Inc. (Tokyo, Japan). Capsaicin (purity: 90%) and gingerol (purity: 98%) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Ferulic acid (purity: 99%) and cucurbitacin E (purity: 99%) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Eagle's minimum essential medium (MEM) and RPMI 1640 were obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). The drugs were dissolved in sterile isotonic saline. The other chemicals used in this study were of the highest purity available.

2.2. Effects of test compounds on the DOX concentration in Ehrlich ascites carcinoma cells and M5076 ovarian sarcoma cells (in vitro)

Ehrlich ascites carcinoma cells and M5076 ovarian sarcoma cells (1×10^6 cells per animal) were intraperitoneally transplanted into male ddY mice and C57BL/6 mice, respectively. Ascites fluid was collected on the 7th day (Ehrlich) and 14th day (M5076) after transplantation. The tumor cells were washed twice and then resuspended in RPMI 1640 medium containing 10% fetal bovine serum.

To examine the influx of DOX into tumor cells, cells (5×10^6 cells/ml medium) were incubated with 9.0 nmol/ml of DOX at 37 °C for 60 min in the presence or absence of test compounds.

To examine the effect of test compounds on the DOX efflux from M5076 ovarian sarcoma cells, cells were preincubated with 9.0 nmol/ml DOX in RPMI medium at 37 °C for 30 min. After incubation, the medium was cooled on ice and then centrifuged at $150 \times g$ for 3 min. The cells were washed and then resuspended in fresh medium. The resulting cell suspension (5×10^6 cells/ml) was incubated at 37 °C for 120 min in the presence or absence of test compounds.

In both systems, the medium was cooled on ice after incubation, and then centrifuged at $150 \times g$ 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 5.0 ml of

chloroform–methanol (4:1, v/v) and then centrifuged (1200 × *g*, 15 min). The concentration of DOX in the organic phase was determined. (Sadzuka et al., 1996; Sugiyama and Sadzuka, 1998)

2.3. Effect of cucurbitacin E on DOX cytotoxicity of M5076 ovarian sarcoma cells

M5076 ovarian sarcoma cell suspensions were seeded in a 96-well plate (Falcon), and then incubated at 37 °C for 24 h. After incubation, DOX was added to the cell suspension, and this suspension was incubated at 37 °C for 48 h in the presence or absence of cucurbitacin E. Afterwards, WST-8 was added to this cell suspension, and this suspension was then incubated at 37 °C for 3 h. The absorbance at 560 nm was calculated. The probability of cell survival without drug exposure was expressed as 100%. We determined the probability of cell survival in each sample.

2.4. Effects of cucurbitacin E on DOX-induced antitumor activity (in vivo)

Male BDF_1 mice (5 weeks old and weighing 20–25 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in a room maintained at 25 ± 1 °C and 55 ± 5% relative humidity, and were given free access to regular chow pellets and water.

M5076 ovarian sarcoma cells (5×10^5 cells per animal) were characterized and kindly provided by the Japanese Foundation for Cancer Research, and were transplanted onto the backs of BDF_1 mice. DOX (2.0 mg/(kg day) for 4 days) was administered intraperitoneally at 16, 18, 20 and 22 days after the inoculation. Cucurbitacin E (1.0 or 0.2 mg/(kg day) for 4 days) was intraperitoneally injected at 17, 19, 21 and 23 days post-inoculation. During the treatment, two perpendicular tumor diameters (*a*: long diameter; *b*: short diameter) were measured with calipers. Tumor size (Zou et al., 1994) was calculated as $ab^2/2$ and expressed in cm^3. The mice were killed by cervical dislocation on the 24th day, and then the solid tumors, livers and hearts were immediately removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10 mM phosphate buffer (pH 7.8). Each suspension was mixed for 60 s with 5.0 ml of chloroform-methanol (4:1, v/v) and then centrifuged (1200 × *g*, 15 min). The DOX concentration was determined as previously described (Sadzuka et al., 1996; Sugiyama and Sadzuka, 1998).

Table 1
Effects of capsaicin, gingerol and ferulic acid on DOX influx and efflux in Ehrlich ascites carcinoma cells

Agent	Influx (%)	Efflux (%)
DOX alone	100	100
DOX + capsaicin	98	104
DOX + gingerol	101	135
DOX + ferulic acid	99	123

Influx: data are expressed amount of DOX uptake for 60 min as a percentage to DOX alone group. Efflux: data are expressed amount of DOX release for 120 min as a percentage to DOX alone group.

2.5. Statistical analysis

Statistical analysis was carried out using Student's *t*-test and ANOVA. The data were analyzed by fitting the General Linear Model. The variance–covariance structure for the repeated measures data was assumed to have the compound symmetry structure.

3. Results

3.1. Effects of test compounds on the DOX concentration in Ehrlich ascites carcinoma cells (in vitro)

The effects of capsaicin, gingerol and ferulic acid on DOX influx and efflux in Ehrlich ascites carcinoma cells in vitro are shown in Table 1. All test compounds had no effects on DOX influx for 60 min, compared to that in control group. Furthermore, the DOX efflux level did not change after capsaicin

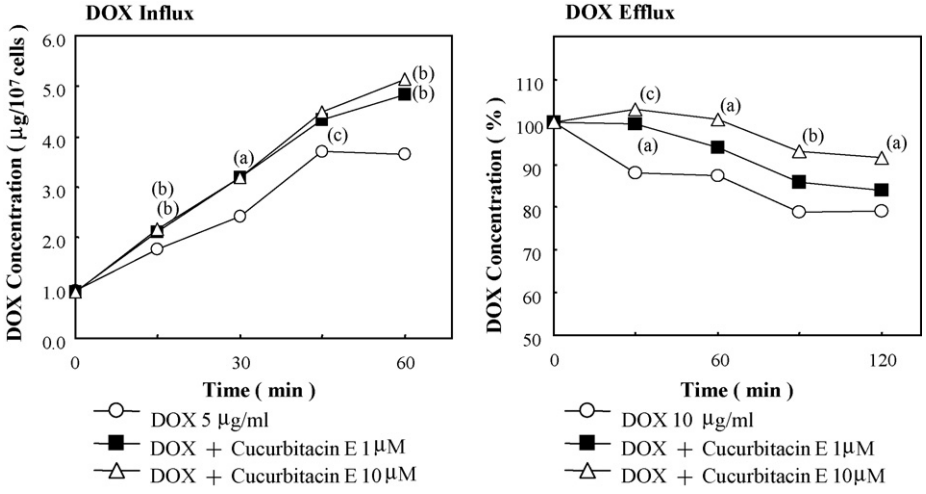

Fig. 2. Effects of cucurbitacin E on the membrane transport of DOX in Ehrlich ascites carcinoma cells. Each point represents the mean for four samples. Each S.D. is less than 10%. Significant differences from the level of the DOX alone group are indicated by (a) $P < 0.05$, (b) $P < 0.01$ and (c) $P < 0.001$.

treatment whereas it tended to be promoted by gingerol or ferulic acid treatment.

The effect of cucurbitacin E on DOX permeability in Ehrlich ascites carcinoma cells is shown in Fig. 2. In the DOX influx system, combined cucurubitacin E significantly increased the DOX level in carcinoma cells ($P<0.001$ by General Linear Model), specifically, DOX levels in cells treated with 1 and 10 μM cucurbitacin E for 60 min increased by 44% ($P<0.01$) and 56% ($P<0.01$), respectively, compared to control levels. Furthermore, cucurbitacin E was found to affect significantly the DOX efflux system ($P<0.001$ by General Linear Model). After 120 min incubation, DOX efflux was inhibited by 57% ($P<0.05$) in cells treated with 10 μM cucurbitacin E, compared to control level.

3.2. Effects of cucurbitacin E on the DOX concentration in M5076 ovarian sarcoma cells (in vitro)

The combined effects of cucurbitacin E on DOX permeability in M5076 ovarian sarcoma cells were examined. The addition of cucurbitacin E (1 and 10 μM) was shown to significantly promote on DOX influx and suppress DOX efflux. Specifically, combined cucurbitacin E increased DOX influx by 40% and decreased DOX efflux by 59%.

Next, we examined the effect of low concentrations of cucurbitacin E on the same systems. In the DOX influx system (Fig. 3(A)), cucurbitacin E (0.01 and 0.1 μM) combination significantly promoted DOX influx in M5076 ovarian sarcoma cells ($P<0.001$ by General Linear Model), and the increase for cell treated with 0.01 μM was 1.8-fold ($P<0.05$) of DOX alone group at 30 min after incubation. Thereafter, the DOX level gradually increased and reached a plateau after 90 min incubation. At this point, the DOX level in the cucurbitacin E combined group was still higher than in control group.

In the DOX efflux system (Fig. 3(B)), combined cucurbitacin E (0.01 and 0.1 μM) suppressed DOX efflux in M5076 ovarian sarcoma cells. The DOX concentration in tumor cells after 120 min incubation by combined cucurbitacin E was higher than

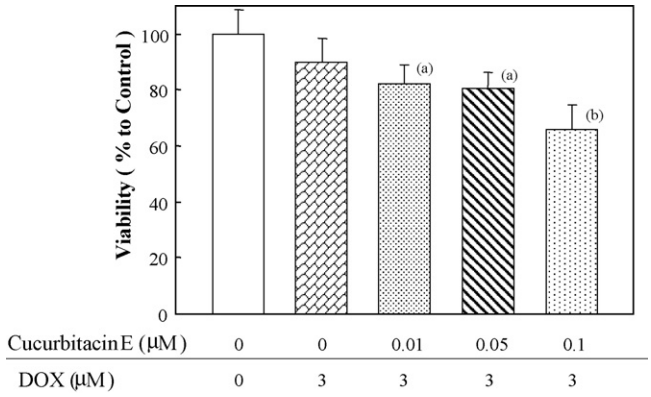

Fig. 4. Cytotoxic effects of DOX combined with cucurbitacin E in M5076 ovarian sarcoma cells. Each column represents the mean ± S.D. ($n=6$–8) of cell viability as a percentage of control samples. Significant differences from the level of the DOX alone group are indicated by (a) $P<0.05$ and (b) $P<0.001$.

that in cells treated with DOX alone: DOX efflux was inhibited by 17% ($P<0.01$) and 26% ($P<0.01$) in cells treated with 0.01 and 0.1 μM cucurbitacin E, respectively.

3.3. Effect of cucurbitacin E on DOX cytotoxicity in M5076 ovarian sarcoma cells

The survival ratio of tumor cells after single DOX (4.0 μM) treatment was suppressed by 30% ($P<0.001$), whereas cell survival after DOX (3.0 μM) treatment did not change. The survival ratio after single cucurbitacin E (0.1 μM) treatment was suppressed by 81% ($P<0.001$), whereas survival of cells treated with 0.01 and 0.05 μM cucurbitacin E did not differ from untreated cells (data not shown).

Next, the combination of cucurbitacin E with DOX was examined. Concentrations of cucurbitacin E and DOX were used that had no effect when applied individually. The survival ratio for cells receiving the combined treatment was significantly decreased, compared to treatment with DOX alone (Fig. 4). Specifically, combination of DOX with 0.01 and 0.05 μM cucurbitacin E suppressed cell survival ratios by

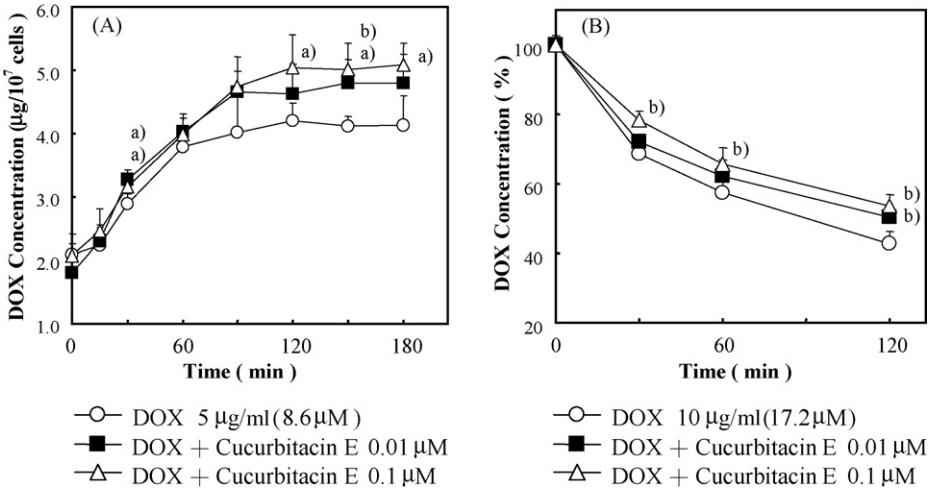

Fig. 3. Effects of cucurbitacin E on the membrane transport of DOX in M5076 ovarian sarcoma cells (A: DOX influx; B: DOX efflux). Each point represents the mean ± S.D. ($n=4$). Significant differences from the level of the DOX alone group are indicated by (a) $P<0.05$ and (b) $P<0.01$.

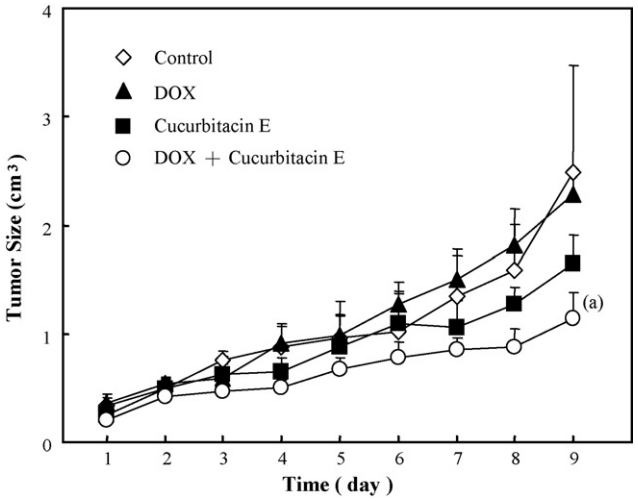

Fig. 5. Changes in tumor growth induced by administration of cucurbitacin E with DOX. During treatment, two perpendicular tumor diameters (*a*: long diameter; *b*: short diameter) were determined with calipers. The tumor size was calculated as $ab^2/2$ and expressed in cm^2. Each point represents the mean ± S.D. ($n = 4–5$). Significant difference from the level of the DOX alone group is indicated by (a) $P < 0.05$.

19% and 20%, respectively. Furthermore, combination of DOX (3.0 μM) + cucurbitacin E (0.1 μM) also significant affected the survival ratio, compared to individual DOX and cucurbitacin E, showing a 3.4-fold ($P < 0.001$) inhibition when compared to treatment with 3.0 μM DOX alone.

3.4. Effects of cucurbitacin E on the antitumor activity induced by DOX (in vivo)

The effects of cucurbitacin E on DOX-induced antitumor activity in M5076 ovarian sarcoma bearing mice are shown in Figs. 5 and 6. During test drug treatment, the tumor sizes gradually increased and this size in DOX + cucurbitacin E group significantly decreased from control level ($P < 0.001$ by General Linear Model). On the 9th day, the average tumor size in the control and DOX alone groups were shown to be 2.49 ± 0.98 and 2.28 ± 0.21 cm^3, respectively, showing that DOX treatment had no effect. In contrast, the tumor size in the cucurbitacin E (1.0 mg/kg) alone group was 72% (1.65 ± 0.27 cm^3) of that in control group. However, treatment with cucurbitacin E (0.2 mg/kg) did not reduce the average tumor size (data not shown). The combination of cucurbitacin E (0.2 mg/kg) with DOX decreased the average tumor size by 50% ($P < 0.05$, 1.14 ± 0.24 cm^3) of that in DOX alone group (Fig. 5).

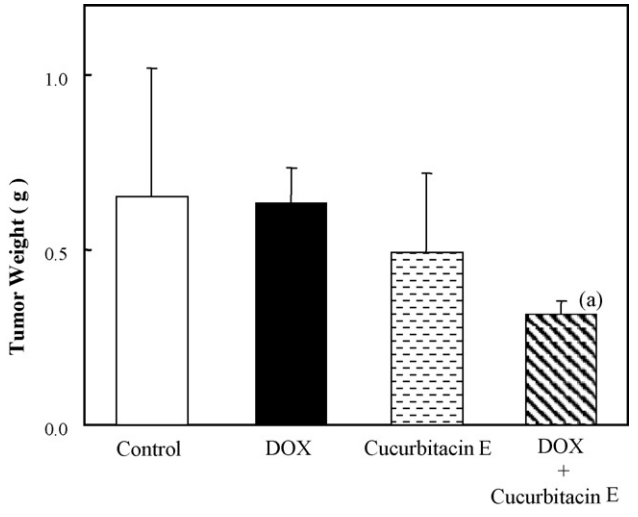

Fig. 6. Effect of cucurbitacin E on the changes of tumor weight induced by DOX. Each column represents the mean ± S.D. ($n = 4–5$). Significant difference from the level of the DOX alone group is indicated by (a) $P < 0.05$.

Table 2
Effects of cucurbitacin E on DOX concentrations in the tissues of M5076 tumor-bearing mice

Tissue	DOX concentration (ng/mg protein)	
	DOX	DOX cucurbitacin E
Tumor	1.69 ± 0.43	1.61 ± 0.25
Heart	15.48 ± 1.15	5.89 ± 1.88[a]
Liver	17.29 ± 3.14	16.59 ± 2.26
Lung	20.74 ± 6.26	10.09 ± 3.24

Each value showed the mean ± S.E. ($n = 4–5$).
[a] Significant difference from the level of the DOX alone group is indicated by $P < 0.05$.

As shown in Fig. 6, the average tumor weight in the DOX alone group (0.63 ± 0.10 g) was unchanged when compared to the control group (0.65 ± 0.37 g). However, the average tumor weight in the cucurbitacin E (1.0 mg/kg) alone group decreased to 76% of the control level. Furthermore, the combination of cucurbitacin E (0.2 mg/kg) with DOX decreased the average tumor weight by 50% ($P < 0.01$, 0.32 ± 0.04 g) of that in DOX alone group. In the normal tissues such as liver, heart and lung, the DOX concentration after combined cucurbitacin E and DOX treatment tended to decrease (Table 2). In the heart, the DOX concentration decreased by 38% ($P < 0.05$) of that in DOX alone group. However, there was no difference in tumor DOX concentration between mice treated with DOX alone or a combination of cucurbitacin E with DOX.

4. Discussion

Caffeine and theanine have been reported to increase DOX-induced antitumor activity (Sadzuka et al., 1993, 1995a,b, 2000, 2001a,b; Sugiyama et al., 2004; Sugiyama and Sadzuka, 2003). These compounds act by suppressing DOX efflux from tumor cells, increasing DOX concentration in the tumor, and inducing an increase of antitumor activity. Considering these mechanisms, we have screened other compounds in search of a novel modulator of DOX permeability in tumor cells.

Capsaicin is responsible for the "hot" taste of red pepper in *Capsaicum* plant. Capsaicin's active form promotes of energy metabolism and has bactericidal activity (Kawada et al., 1986; Surh and Lee, 1996). Furthermore, capsaicin enhances cell membrane fluidity at low concentrations and inhibits fluidity at high concentrations (Tsuchiya, 2001). In the present study, capsaicin did not change DOX permeability in Ehrlich ascites carcinoma cells. However, the capsaicin derivatives gingerol and ferulic

acid showed a tendency to promote DOX efflux. Combinations of these compounds with DOX were also not effective.

In contrast, cucurbitacin E provides the bitter taste to the squash family and is classified as a triterpenoid derivative (Jayaprakasam et al., 2003). Cucurbitacin derivatives have anti-hepatotoxic and anti-inflammatory effects that are mediated by pharmacological inhibition of COX-2. In the present study, cucurbitacin E significantly promoted DOX influx into tumor cells and increased DOX concentration in tumor cells. Furthermore, the combined cucurbitacin E significantly suppressed DOX efflux from tumor cells and was confirmed to maintain the DOX level in tumor cells.

Next, we studied the effects of cucurbitacin E in M5076 ovarian sarcoma cells, which have a low sensitivity to DOX (Talmadge et al., 1981). Namely, we would like to see if treatment with combined cucurbitacin E could increase DOX mediated antitumor activity in M5076 ovarian sarcoma. In DOX permeability in M5076 cells, cucurbitacin E had identical effects as those found in Ehrlich cells. Thus, cucurbitacin E promoted of DOX influx and suppressed DOX efflux in M5076 cells. These effects appeared when using a low concentration of cucurbitacin E (0.01 μM). In the DOX influx system, the DOX level in tumor cells reached a plateau after 90 min incubation, and combined treatment with cucurbitacin E-induced high DOX level. For this time course in influx system, the effects of cucurbitacin E appeared in the late stages of incubation, thus the action mechanism of cucurbitacin E was likely related DOX efflux. In the DOX influx system, DOX was taken into tumor cells at an early time and released from at later time points. In the early stages of incubation, the DOX concentration in tumor cells was lower than that in the medium and the simple diffusion rate was influx > efflux. In contrast, the DOX concentration in tumor cells at later time points was increased, and the rate was influx < efflux. It is therefore likely that the inhibitory effect of cucurbitacin E on DOX efflux also affected DOX influx, resulting in apparent promotion of DOX influx by cucurbitacin E.

It was confirmed that the combination of cucurbitacin E with DOX resulted in effective cytotoxicity for tumor cells. Additionally, the combination of cucurbitacin E with DOX showed increased cytotoxicity, compared to each treatment alone. We speculate that the increased cytotoxicity of cucurbitacin E + DOX was not only generated by the summation of individual cytotoxic effects, but also by a cucurbitacin E mediated increase in DOX concentration.

In vivo, DOX treatment by itself did not change in the time course of tumor size and tumor weight in M5076 ovarian sarcoma, compared to control levels. In contrast, the combination of cucurbitacin E with DOX was decreased tumor size and tumor weight, compared to that in DOX alone group, indicating effective antitumor activity.

The combined cucurbitacin E treatment showed a tendency for decreased DOX levels in normal tissues, compared to that of DOX only group. In particular, DOX level in the heart was significantly decreased by the combined treatment with cucurbitacin E, suggesting that cucurbitacin E may decrease DOX-induced cardiotoxicity. In contrast, the combination of cucurbitacin E with DOX did not change DOX levels in the tumor. Based on the reduction of tumor size and tumor weight, combined treatment with cucurbitacin E appeared to increase the DOX-induced antitumor activity in vivo. Furthermore, cucurbitacin E application was shown to sustain DOX concentration in tumor cells in vitro. We originally hypothesized that cucurbitacin E induced incremental increases in DOX activity were caused by increased DOX concentration in the tumor. However, the intratumorial DOX concentrations were similar between the DOX alone group and DOX + cucurbitacin E group at 48 h after treatment. Therefore, it is likely that cucurbitacin E increased DOX concentration in the tumor at earlier time points.

In conclusion, we have demonstrated that cucurbitacin E had sustained DOX concentrations in the M5076 ovarian sarcoma cells with low DOX sensitivity via the inhibition of DOX efflux. Furthermore, the combination of cucurbitacin E with DOX was shown to significantly enhance cytotoxicity in M5076 ovarian sarcoma cells. In vivo, these effects significantly increased DOX-induced antitumor activity against M5076 ovarian sarcoma cells. On the other hand, there was no effect on DOX concentration in normal tissues. The combination of cucurbitacin E with DOX may therefore be an effective tool with broad applications in cancer chemotherapy.

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