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Enhancement of doxorubicin concentration in the M5076 ovarian sarcoma cells by cucurbitacin E co-treatment

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ABSTRACT

Cucurbitacin E increases the doxorubicin (DOX) level in M5076 ovarian sarcoma via suppressed DOX efflux in vitro. An increase in DOX induced antitumor activity by cucurbitacin E in vivo has been reported previously. This paper attempts to clarify the mechanism of cucurbitacin E induced increments in the antitumor activity of DOX. MK-571, a multidrug resistance associated protein (MRP) inhibitor, significantly suppressed DOX efflux from M5076 ovarian sarcoma cells. The combination of cucurbitacin E with MK-571 also inhibited DOX efflux, whereas the efficacy was the same in each treatment. Namely, the inhibition of DOX efflux by cucurbitacin E was expected to be related to MRP. In contrast, it appeared that the effect of cucurbitacin E on DOX permeability did not relate to P-gp. The cucurbitacin E co-treatment significantly increased DOX concentration in the tumor within a short time after DOX administration, whereas the same treatment decreased the DOX concentration in normal tissues. The different effects of cucurbitacin E between tumor and normal tissues was speculated to be related to differences in DOX transport system on cell membrane. In DOX therapy, cucurbitacin E co-treatment was expected to increase DOX induced antitumor activity without an increase in adverse reactions due to DOX.

1. Introduction

Biochemical modulation has been playing an increasingly important role in cancer chemotherapy (Bertino et al., 1977; Kubota et al., 1993; O'Connel, 1988; Hidalgo et al., 1989; Ichinose et al., 1995). Biochemical modulation is the term used when the pharmacological properties of an effector (antitumor agent) are improved by a modulator (combined drug) with no antitumor activity. From the point of membrane transport in tumor cells, it was reported that the utilization of caffeine, theanine, and anserine as food components affected doxorubicin (DOX)-induced antitumor activity (Sadzuka et al., 1993, 1995a,b, 1996, 2000, 2001a,b; Sugiyama and Sadzuka, 1998, 2003; Sugiyama et al., 2004). These components were shown to affect DOX transport across the tumor cell membrane, inhibit DOX efflux, increase the intracellular DOX concentration in the tumor, and enhance DOX induced antitumor activity.

In a previous paper, cucurbitacin E (the bitter tasting component of squash fruit) increased the DOX concentration in tumor cells in vitro (Sadzuka et al., 2008). Furthermore, in vivo, the combination of cucurbitacin E with DOX had significant antitumor activity, suggesting that cucurbitacin E is useful as a modulator. However, the increase in DOX induced antitumor activity by cucurbitacin E was not clarified in detail.

The DOX permeability of the tumor cell membrane was indicated to be due to simple diffusion or other transporters in previous reports (Skovsgaard, 1977a,b, 1978a,b; Siegfried et al., 1985; Slapak et al., 1992). In multidrug resistant tumor cells, some transporters, such as P-glycoprotein (P-gp), MRP/GS-X pump, or BCRP were confirmed to be overexpressed, and it appeared that DOX was transported via these transporters (Skovsgaard, 1977a,b, 1978a,b; Siegfried et al., 1985). The suppression of DOX efflux by cucurbitacin E in M5076 ovarian sarcoma cells was reported to be greater than that in Ehrlich ascites carcinoma cells (Sadzuka et al., 2008). Thus, it was speculated that the attack point of cucurbitacin E was related to the tumor sensitivity, as M5076 ovarian sarcoma is a low sensitivity tumor. It was reported that M5076 ovarian sarcoma cells overexpress the MRP/GS-X pump (Sadzuka et al., 2000; Sugiyama and Sadzuka, 2003). Thus, MRP was investigated as the attack point of cucurbitacin E. This paper examined the activity of cucurbitacin E against MRP, and the utilization of food components in the antitumor activity of DOX, leading to improvement in Quality of Life for patients treated with DOX.

Abbreviations: DOX, doxorubicin; GSH, glutathione; MRP, multidrug resistance associated protein.

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2. Materials and methods

2.1. Chemicals

DOX, 10 mg/vial (Adriacin), was purchased from Kyowa Fermentation, Inc. (Tokyo, Japan). Verapamil was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). MK-571 and cucurbitacin E (purity: 99%) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). RPMI 1640 was 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. DOX influx or efflux system in M5076 ovarian sarcoma cells (in vitro)

M5076 ovarian sarcoma cells, P388 leukemia cells, and DOX resistant P388 (P388/DOX) leukemia cells (1×10^6 cells/animal) were characterized and kindly provided by Japanese Foundation for Cancer Research. These tumor cells were intraperitoneally transplanted into male C57BL/6 (M5076) mice and DBA/2 mice (P388 or P388/DOX). Ascites fluid was collected on the 14th day (M5076) or 7th day (P388 or P388/DOX) and after transplantation. The tumor cells were washed twice and then resuspended in RPMI 1640 medium containing 10% fetal bovine serum.

To examine the effect of cucurbitacin E on DOX influx in M5076 ovarian sarcoma cells in ATP-depletion conditions, cells were preincubated with sodium azide (10 mM) and 2-deoxy-D-glucose (10 mM) in RPMI medium at 37 °C for 20 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 PBS (-). The resulting cell suspension (5×10^6 cells/ml) was incubated with 0.2 μg/ml DOX in the presence or absence of 0.1 μM cucurbitacin E at 37 °C for 15 min. 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 ($1200 \times g$, 15 min). The concentration of DOX in the organic phase was determined. (Sadzuka et al., 1996; Sugiyama and Sadzuka, 1998). The DOX concentration in the tumor cell immediately after DOX addition was expressed as the initial level (time; 0 min).

In efflux system, M5076 ovarian sarcoma cells, P388 leukemia cells and P388/DOX leukemia cells were incubated with DOX at 37 °C for 30 min. After incubation, the cells were washed and incubated in the presence or absence of test drug at 37 °C for 120 min.

To examine the effect of MRP inhibitor on DOX efflux from M5076 ovarian sarcoma cells, the same procedure was performed using MK-571 instead of ATP depletion inducers. To examine the effect of P-gp inhibitor on DOX efflux from P388 or P388/DOX leukemia cells, the same procedure was performed using verapamil instead of ATP depletion inducer.

2.3. Effects of cucurbitacin E on DOX concentration in each tissue (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/animal) were transplanted into the backs of BDF_1 mice. DOX (2.0 mg/kg, i.p.) was administered intraperitoneally at 22 days after the inoculation. Cucurbitacin E (1.0 mg/kg, i.p.) was injected intraperitoneally at 0 or 6 h after DOX administration. At 8 h after DOX administration, the mice were sacrificed by cervical dislocation, and then the solid tumors, livers, and hearts were removed immediately. 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 ($1200 \times g$, 15 min). The DOX concentration was determined as described previously (Sadzuka et al., 1996).

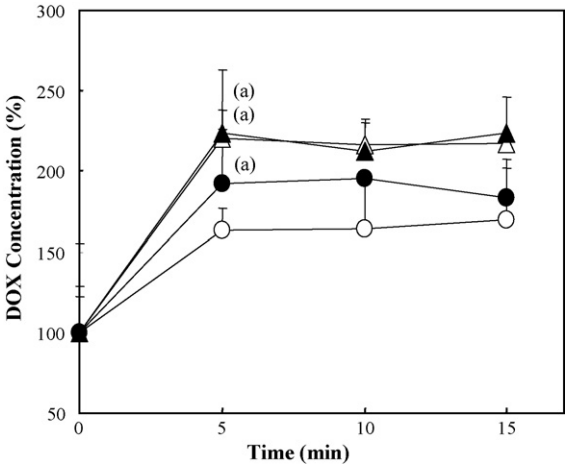

Fig. 1. Effect of energy inhibition on the transport of DOX in M5076 ovarian sarcoma cells. M5076 ovarian sarcoma cells were incubated at 37 °C with or without energy inhibitor. After 20 min, the cells were washed and incubated with 0.2 μg/ml DOX in the presence or absence of 0.1 μM cucurbitacin E. Data are expressed amount of DOX as a percentage to inhibition 0 min in each group. Each point represents the mean ± SD ($n = 4$). Significant differences from the level of the DOX alone group are indicated by (a) $P < 0.01$. (○): DOX, (●): DOX + cucurbitacin E, (△): DOX (ATP depletion), (▲): DOX + cucurbitacin E (ATP depletion).

2.4. Statistical analysis

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

3. Results

3.1. Effect of cucurbitacin E on DOX influx under ATP depletion

The effect of cucurbitacin E on DOX influx system under ATP-depletion conditions is shown in Figs. 1 and 2. The DOX influx level increased remarkably for 5 min and then reached a plateau. Following ATP depletion, the DOX influx level increased significantly. The DOX level under normal conditions was 1.6-fold higher than the initial level (time: 0 min), whereas its level under ATP-depletion conditions reached 2.2-fold ($P < 0.01$) of the initial level. Under normal conditions, the DOX influx level in tumor cells showed a tendency to be promoted by a cucurbitacin E co-treatment, whereas the DOX influx level in the combined cucurbitacin E group under ATP-depletion conditions was shown to be same as in the DOX alone group following ATP depletion. Thus, the effect of cucurbitacin E on DOX influx was inhibited by ATP-depletion conditions.

3.2. Effect of cucurbitacin E or MRP inhibitor on DOX permeability in M5076 ovarian sarcoma cells

The effect of cucurbitacin E or MK571 on DOX efflux system in M5076 ovarian sarcoma cells is shown in Fig. 3 as the amount of DOX released over 120 min. This level in the DOX alone group was 1.14 ± 0.09 μg/10^7 cells, whereas the combination of DOX with

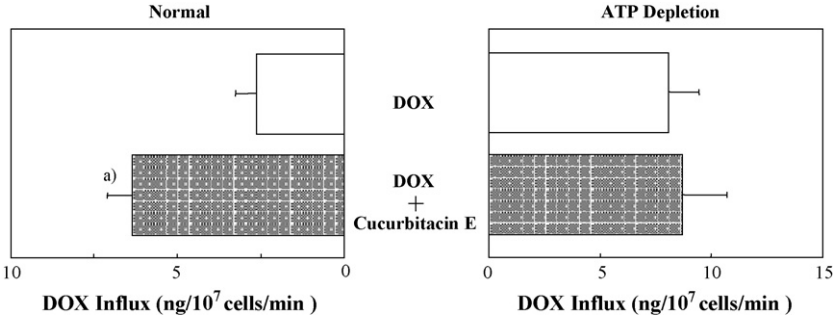

Fig. 2. Effect of cucurbitacin E with or without energy inhibition on DOX influx rate in M5076 ovarian sarcoma cells. The DOX influx rate was calculated using the amount of DOX influx over 5 min. Each column represents the mean ± SD ($n = 4$). Significant differences from the DOX alone group are indicated by (a) $P < 0.01$. DOX: 0.2 μg/ml, Cucurbitacin E: 0.1 μM.

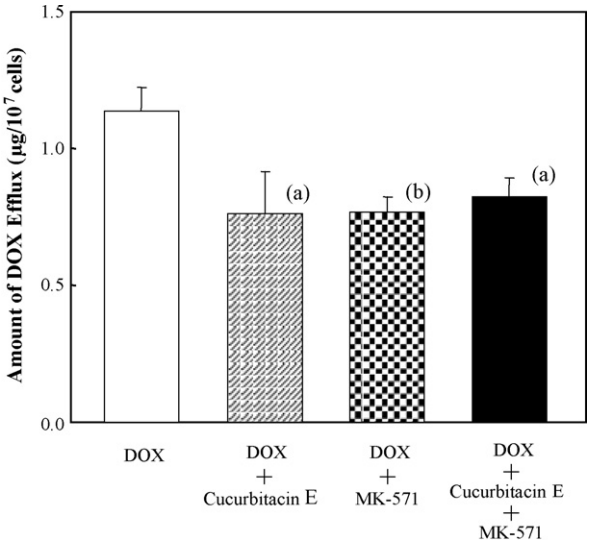

Fig. 3. Effect of an inhibitor of MRP on DOX efflux from M5076 ovarian sarcoma cells. M5076 ovarian sarcoma cells were incubated with DOX at 37 °C for 30 min. After incubation, the cells were washed and incubated in the presence or absence of drug at 37 °C for 120 min. Data are expressed as the amount of DOX released over 120 min. Each column represents the mean ± SD ($n = 4$). Significant differences from the level of the DOX alone group are indicated by (a) $P < 0.01$ and (b) $P < 0.001$. DOX: 10 μg/ml, Cucurbitacin E: 0.1 μM, MK-571: 1.0 μM.

cucurbitacin E or MK571 significantly decreased the DOX efflux level by 33.2 ± 10.9% ($P < 0.01$) and 32.4 ± 2.8% ($P < 0.001$), respectively, compared to that in the DOX alone group. Furthermore, the combination of cucurbitacin E + MK571 significantly suppressed DOX efflux by 27.6 ± 5.2% ($P < 0.01$). This suppressed ratio was at the same level as each single treatment.

3.3. Effect of cucurbitacin E on DOX permeability in P388 and P388/DOX leukemia cells

The effect of cucurbitacin E on DOX permeability in P388 leukemia cells is shown in Fig. 4. In the DOX influx system, the DOX concentration in P388 cells increased gradually and reached 2.31 ± 0.26 μg/10^7 cells after 90 min incubation in DOX alone conditions. However, the addition of cucurbitacin E at each concentration did not increase the DOX concentration in P388 cells compared to the level in DOX alone group. Namely, it appeared that cucurbitacin E co-treatment did not change DOX influx. In the DOX efflux system, DOX was rapidly released from P388 cells for 15 min and this level at 60 min was 60% of that at 0 min. There was no change in DOX efflux level by the cucurbitacin E co-treatment, compared to that in the DOX alone group. Thus, the suppression effect on DOX efflux by cucurbitacin E was not shown in P388 leukemia cells.

The effects of cucurbitacin E and verapamil on DOX permeability in P388/DOX cells were examined. In Fig. 5, the time course of DOX influx in the combined cucurbitacin E group was shown to be similar with that in the DOX alone group. In contrast, the DOX level in the combined verapamil group significantly increased from 60 min incubation, and the DOX level was 2.1-fold ($P < 0.01$) higher than in the DOX alone group at 90 min. Furthermore, in the DOX efflux system, cucurbitacin E co-treatment did not have a significant effect. On the other hand, verapamil co-treatment suppressed DOX efflux and the suppression ratio of the DOX concentration in P388/DOX cells was shown to be 31.3% ($P < 0.05$) at 120 min.

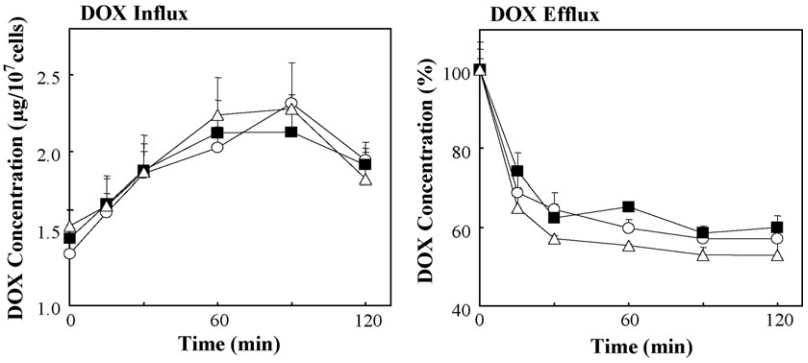

Fig. 4. Effect of cucurbitacin E on the membrane transport of DOX in P388 leukemia cells. In influx system, P388 leukemia cells were incubated with DOX at 37 °C for 120 min in the presence of cucurbitacin E. In efflux system, P388 leukemia cells were incubated with DOX at 37 °C for 30 min. After incubation, the cells were washed and incubated in the presence or absence of cucurbitacin E at 37 °C for 120 min. Each point represents the mean ± SD ($n = 4$). (○): DOX, (5 μg/ml), (■): DOX + cucurbitacin E (0.01 μM), (△): DOX + cucurbitacin E (0.1 μM).

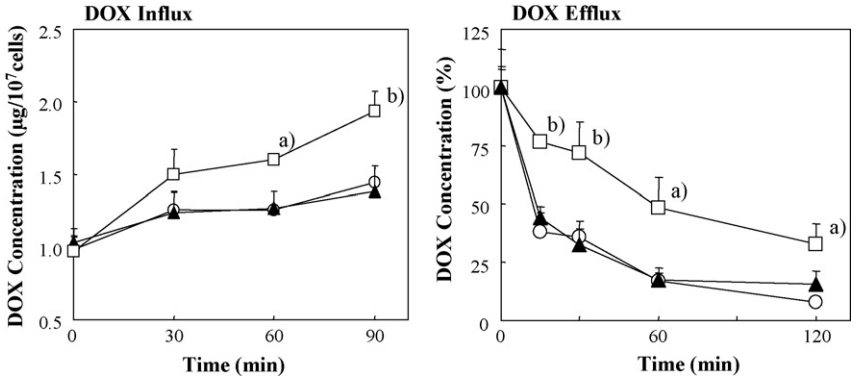

Fig. 5. Effects of cucurbitacin E and verapamil on the membrane transport of DOX in P388/DOX cells. In influx system, P388/DOX leukemia cells were incubated with 10 μg/ml DOX at 37 °C for 90 min in the presence of 1.0 μM cucurbitacin E or 10 μM verapamil. In efflux system, P388/DOX leukemia cells were incubated with DOX at 37 °C for 30 min. After incubation, the cells were washed and incubated in the presence or absence of 1.0 μM cucurbitacin E or 10 μM verapamil at 37 °C for 120 min. Each point represents the mean ± SD ($n = 4$). Significant differences from the level of the DOX alone group are indicated by (a) $P < 0.05$ and (b) $P < 0.01$. (○): DOX, (▲): DOX + cucurbitacin E, (□): DOX + verapamil.

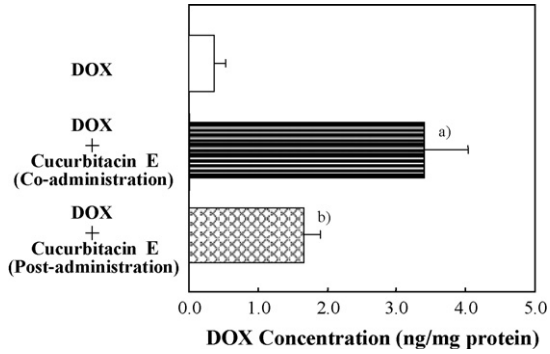

Fig. 6. Effects of cucurbitacin E on DOX concentration in tumors in M5076 ovarian sarcoma-bearing mice at 8 h after DOX administration. DOX (2.0 mg/kg) was injected intraperitoneally, and cucurbitacin E (1.0 mg/kg) was administered simultaneously or at 6 h after DOX treatment. Each column represents the mean ± SD ($n = 4$). Significant differences from the level of the DOX alone group are indicated by (a) $P < 0.05$ and (b) $P < 0.01$.

3.4. The effect of cucurbitacin E on DOX concentration in mice tissues

After the administration of DOX and cucurbitacin E, the DOX concentrations in some tissues in M5076 ovarian sarcoma-bearing mice are shown in Figs. 6 and 7. In the tumor, the DOX concentration in the DOX alone group was 0.37 ± 0.15 ng/mg protein, whereas this level was increased significantly by cucurbitacin E co-treatment. Thus, the DOX level in simultaneous treatment with DOX and cucurbitacin E was shown to be 9.2-fold higher ($P < 0.05$, 3.41 ± 0.62 ng/mg protein) than the DOX alone level, and the level in the post treatment of cucurbitacin E group was 4.5-fold higher than the DOX alone level (Fig. 6).

In contrast, the DOX concentration in normal tissues showed a tendency to decrease following cucurbitacin E co-treatment. DOX concentrations in the simultaneous treatment group were shown to be 36.4% and 69.1% ($P < 0.001$) in the heart and liver, compared to that in the DOX alone group, respectively. Furthermore, the DOX concentration in the heart and liver decreased in the post-treatment period following cucurbitacin E treatment (Fig. 7).

4. Discussion

It was reported that DOX permeability in M5076 ovarian sarcoma was regulated by simple diffusion, energy-dependent pathways, and Na^+ dependent transporters (Sadzuka et al., 2001a,b; Skovsgaard, 1977a,b, 1978a,b; Siegfried et al., 1985). In a previous paper, cucurbitacin E was shown to increase the DOX level in M5076 ovarian sarcoma cells via the suppression of DOX efflux in vitro (Sadzuka et al., 2008). Furthermore, it has been reported that cucurbitacin E co-treatment increased DOX-induced antitumor activity in vivo, whereas the DOX concentration in tumors did not increase in vivo. This paper attempted to clarify the mechanism of the cucurbitacin E induced increase in the antitumor activity of

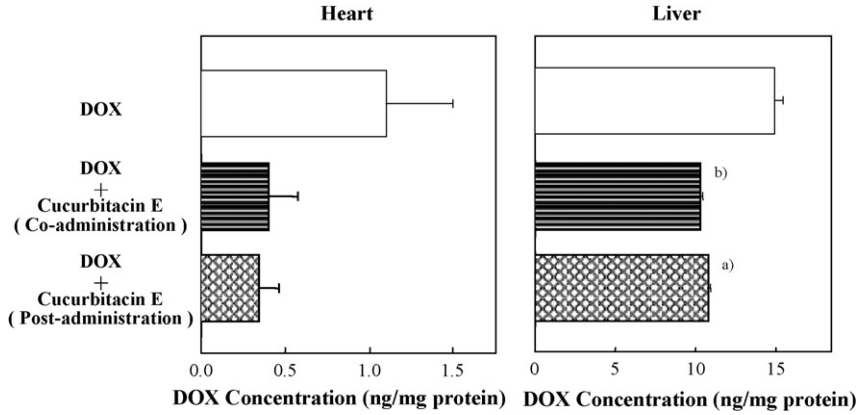

Fig. 7. Effects of cucurbitacin E on DOX concentrations in normal tissues in M5076 ovarian sarcoma-bearing mice at 8 h after DOX administration. DOX (2.0 mg/kg) was injected intraperitoneally, and cucurbitacin E (1.0 mg/kg) was administered simultaneously or at 6 h after DOX treatment. Each column represents the mean ± SD ($n = 4$). Significant differences from the level of the DOX alone group are indicated by (a) $P < 0.01$ and (b) $P < 0.001$.

DOX. Treatments of sodium azide or 2-deoxy-D-glucose lead to ATP depletion and thus the arrest of the DOX efflux (Slapak et al., 1992). Under ATP depletion, cucurbitacin E did not affect DOX influx system, whereas DOX influx in normal medium was confirmed to be promoted by cucurbitacin E co-treatment.

There are many reports on the cell membrane transport of anthracyclines and it was reported that this transport was connected with active transporter mechanisms (Skovsgaard, 1977a,b, 1978a,b; Siegfried et al., 1985). In multidrug resistant tumor cells, active transporters of DOX were shown previously to be connected with P-gp, the MRP/GS-X pump, and BCRP in the ABC transporter family (Gottesman et al., 2002; Goldstein et al., 1989; Kool et al., 1997, 1999; Kruh and Belinsky, 2003; Nooter et al., 1995; Litman et al., 2000; Doyle and Ross, 2003). In a previous paper, the effect of cucurbitacin E on M5076 ovarian sarcoma (low sensitivity on DOX) was reported to be stronger than that on Ehrlich ascites carcinoma (high sensitivity) (Sadzuka et al., 2008). The overexpression of MRP was expected as a factor in the low sensitivity of M5076 ovarian sarcoma cells to DOX (Marbeuf-gueye et al., 1998; Heijn et al., 1997; Kuo et al., 1998; Decorti et al., 2001). The overexpression of MRP5 was confirmed in M5076 ovarian sarcoma cell membranes (Sadzuka et al., 2001a,b; Sugiyama and Sadzuka, 2003). The connection with MRP and the effect of cucurbitacin E were examined. MK-571, as an MRP inhibitor (Gekeler et al., 1995), significantly suppressed DOX efflux from M5076 ovarian sarcoma cells. Furthermore, the combination of cucurbitacin E with MK-571 inhibited DOX efflux, whereas the efficacy of these treatments was the same level. It is considered that the combination shows synergistic effect on DOX efflux when the attack point of cucurbitacin E differs from that of MK-571. However, as the combined effect was at the same level in each group, the attack point of cucurbitacin E on DOX efflux was considered to be the same as that of MK-571. Namely, it is speculated that the inhibition of DOX efflux by cucurbitacin E was expected to be connected with MRP. However, other possible mechanisms are able to be considered.

Next, the effect of cucurbitacin E on P-gp was examined. Cucurbitacin E did not change the DOX permeability of P388 leukemia cells. Furthermore, in P388/DOX with the overexpression of P-gp, cucurbitacin E was shown not to affect DOX permeability. Verapamil, as a P-gp inhibitor, significantly inhibited DOX efflux from P388/DOX leukemia cells. Thus, it appeared that the effect of cucurbitacin E on DOX permeability was not related to P-gp.

In a previous paper, cucurbitacin E increased DOX induced antitumor activity, whereas the DOX concentration in the tumor did not increase following cucurbitacin E co-treatment on the 2nd day after the last treatment in vivo. In the present study, the DOX concentration was examined at short time after DOX administration. The cucurbitacin E co-treatment significantly increased the DOX concentration in the tumor, whereas the same treatment decreased the DOX concentration in normal tissues. It was expected that the different effects of cucurbitacin E between the tumor and normal tissues was a result of differences in the expression of transporters in the cell membranes of tumor cells and normal cells. In the transport system of DOX efflux in M5076 ovarian sarcoma cells, GS-DOX conjugate was produced by the binding of DOX and glutathione (GSH), which was then transported by the MRP/GS-X pump complex (Sugiyama and Sadzuka, 2003; Priebe et al., 1998; Zhang et al., 1999). MRP is expressed in many normal tissues and is comprised of MRP1-MRP9 as members of the MRP family (Borst et al., 1999; Bera et al., 2002). The expression of MRP subfamily members is known to differ in tumor cells and other tissues (Nooter et al., 1995). Namely, the different effects of cucurbitacin E between the tumor and normal tissues were speculated to be a result of differences in the MRP subtypes. The cucurbitacin E-enhanced antitumor activity of DOX in a previous paper was suggested to be related to the increased concentration of DOX in the tumor shortly after cucurbitacin E administration.

In conclusion, it was suggested that the suppression of DOX efflux by cucurbitacin E in vitro induced an increase in DOX concentration in the tumor, which increased the DOX antitumor activity. Furthermore, it was speculated that the attack point of cucurbitacin E was an MRP. In DOX therapy, combined treatment with cucurbitacin E is expected to increase DOX-induced antitumor activity without an increase in adverse reactions to DOX.

References

Bera, T.K., Iavarone, C., Kumar, V., Lee, S., Lee, B., Pastan, I., 2002. MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proc. Natl. Acad. Sci. 99, 6997–7002.

Bertino, R., Sawicki, W.I., Lindquist, C.A., Gupta, V.S., 1977. Schedule dependent antitumor effects of methotrexate and 5-fluorouracil. Cancer Res. 37, 327–328.

Borst, P., Evers, R., Kool, M., Wijnholds, J., 1999. The multidrug resistance protein family. Biochim. Biophy. Acta 1461, 347–357.

Decorti, G., Rosati, A., Candussio, L., Giraldi, T., Klugmann, F.B., 2001. Characterization of multidrug transporters in a normal renal tubular cell line resistant to doxorubicin. Multidrug transporters in the LLC-PK_1 cell line and its resistant counterpart. Biochem. Pharmacol. 61, 61–66.

Doyle, L.A., Ross, D.D., 2003. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 22, 7340–7358.

Gekeler, V., Ise, W., Sanders, K.H., Ulrich, W.R., Beck, J., 1995. The leukotriene LTD_4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem. Biophys Res. Commun. 208, 345–352.

Goldstein, L.J., Galski, H., Fojo, A., Willingham, M., Lai, A., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G.M., 1989. Expression of a multidrug resistance gene in human cancers. J. Natl. Cancer Inst. 81, 116–124.

Gottesman, M.M., Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2, 48–58.

Heijn, M., Hooijberg, J.H., Scheffer, G.L., Szabo, G., Westerhoff, H.V., Lankelma, J., 1997. Anthracyclines modulate multidrug resistance protein (MRP) mediated organic anion transport. Biochim. Biophys. Acta 1326, 12–22.

Hidalgo, O.F., Gonzalez, F., Gil, A., Campbell, W., Barrajon, E., Lacave, A.J., 1989. 120 Hours simultaneous infusion of cisplatin and fluorouracil in metastatic breast cancer. Am. J. Clin. Oncol. 12, 397–401.

Ichinose, Y., Takahashi, N., Yano, T., 1995. A phase III trial of oral tegafur and uracil plus cisplatin in patients with inoperable nonsmall cell lung cancer. Cancer 75, 2677–2680.

Kool, M., de Haas, M., Scheffer, G.L., Scheper, R.J., Van Eijk, M.J.T., Juijin, J.A., Baas, F., Borst, P., 1997. Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res. 57, 3537–3547.

Kool, M., van der Linden, M., de Haas, M., Baas, F., Borst, P., 1999. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissue and cancer cells. Cancer Res. 59, 175–182.

Kruh, G.D., Belinsky, M.G., 2003. The MRP family of drug efflux pumps. Oncogene 22, 7537–7552.

Kubota, Y., Hosaka, M., Fukushima, S., Kondo, I., 1993. Prophylactic oral UFT therapy for superficial bladder cancer. Cancer 71, 1842–1845.

Kuo, M.T., Bao, J., Furuichi, M., Yamane, Y., Gomi, A., Savaraj, N., Masuzawa, T., Ishikawa, T., 1998. Frequent coexpression of MRP/GS-X pump and γ-glutamylcysteine synthetase mRNA in drug-resistant cells, untreated tumor cells, and normal mouse tissue. Biochem. Pharmacol. 55, 605–615.

Litman, T., Brangi, M., Hudson, E., Fetsch, P., Abati, A., Ross, D.D., Miyake, K., Resau, J.H., Bates, S.E., 2000. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J. Cell Sci. 113, 2011–2021.

Marbeuf-gueye, C., Broxterman, H.J., Dubru, F., Priebe, W., Garnier-Suillerot, A., 1998. Kinetics of anthracycline efflux from multidrug resistance protein-expressing cancer cells compared with P-glycoprotein- expressing cancer cells. Mol. Pharmacol. 53, 141–147.

Nooter, K., Westerman, A.M., Flens, M.J., Zaman, G.J.R., Scheper, R.J., van Wingerden, K.E., Burger, H., Oostrum, R., Boersma, T., Sonneveld, P., Gratama, J.W., Kok, T., Eggermont, A.M.M., Bosman, F.T., Stoter, G., 1995. Expression of a multidrug resistance-associated protein (MRP) gene in human cancers. Clin. Cancer Res. 1, 1301–1310.

O'Connel, M.J., 1988. A phase III trial of 5-fluorouracil and leucovolin in the treatment of advanced colorectal cancer. Cancer 63, 1026–1030.

Priebe, W., Krawczyk, M., Kuo, M.T., Yamane, Y., Savaraj, N., Ishikawa, T., 1998. Doxorubicin- and daunorubicin- glutathione conjugates, but not unconjugated drugs, competitively inhibit leukotriene C_4 transport mediated by MRP/GS-X pump. Biochem. Biophys Res. Commun. 247, 859–863.

Sadzuka, Y., Mochizuki, E., Takino, Y., 1993. Caffeine modulates the antitumor activity and toxic side effects of adriamycin. Jpn. J. Cancer Res. 84, 348–353.

Sadzuka, Y., Mochizuki, E., Iwasaki, A., Hirota, S., Takino, Y., 1995a. Caffeine enhances adriamycin antitumor activity in Ehrlich ascites carcinoma-bearing mice. Biol. Pharm. Bull. 18, 159–161.

Sadzuka, Y., Mochizuki, E., Takino, Y., 1995b. Mechanism of caffeine modulation of the antitumor activity of adriamycin. Toxicol. Lett. 7, 39–49.
Sadzuka, Y., Sugiyama, T., Miyagishima, A., Nozawa, Y., Hirota, S., 1996. The effects of theanine, as a novel biochemical modulator, on the antitumor activity adriamycin. Cancer Lett. 105, 203–209.
Sadzuka, Y., Sugiyama, T., Sonobe, T., 2000. Efficacies of tea components on doxorubicin induced antitumor activity and reversal of multidrug resistance. Toxicol. Lett. 114, 155–162.
Sadzuka, Y., Sugiyama, T., Tanaka, K., Sonobe, T., 2001a. Inhibition of glutamate transporter by theanine enhances the therapeutic efficacy of doxorubicin. Toxicol. Lett. 121, 89–96.
Sadzuka, Y., Sugiyama, T., Suzuki, Sonobe, T., 2001b. Enhancement of the activity of doxorubicin by inhibition of glutamate transporter. Toxicol. Lett. 123, 159–167.
Sadzuka, Y., Hatakeyama, H., Daimon, T., Sonobe, T., 2008. Screening of biochemical modulator by tumor cell permeability of doxorubicin. Int. J. Pharm. 354, 63–69.
Skovsgaard, T., 1977a. Transport and binding of daunorubicin, adriamycin and rubidazone in Ehrlich ascites tumor cells. Biochem. Pharmacol. 26, 215–222.
Skovsgaard, T., 1977b. Carrier-mediated transport of daunorubicin, adriamycin, and rubidazone in Ehrlich ascites tumor cells. Biochem. Pharmacol. 26, 1221–1227.
Skovsgaard, T., 1978a. Mechanisms resistance to daunorubicin in Ehrlich ascites tumor cells. Cancer Res. 38, 1785–1791.
Skovsgaard, T., 1978b. Mechanism of cross-resistance between vincristine and daunorubicin in Ehrlich ascites tumor cells. Cancer Res. 38, 4722–4727.
Siegfried, J.M., Burke, T.G., Tritton, T.R., 1985. Cellular transport of anthracyclines by passive diffusion: Implications for drug resistance. Biochem. Pharmacol. 34, 593–598.
Slapak, C.A., Lecerf, J.M., Daniel, J.C., Stuart, B.L., 1992. Energy-dependent accumulation of daunorubicin into subcellular compartments of human leukemia cells and cytoplasts. J. Biol. Chem. 267, 10638–10644.
Sugiyama, T., Sadzuka, Y., 1998. Enhancing effects of green tea components on the antitumor activity of adriamycin against M5076 ovarian sarcoma. Cancer Lett. 133, 19–26.
Sugiyama, T., Sadzuka, Y., 2003. Theanine and glutamate transporter inhibitors enhance the antitumor efficacy of chemotherapeutic agents. Biochim. Biophys. Acta 1653, 47–59.
Sugiyama, T., Sadzuka, Y., Sonobe, T., 2004. Theanine, a specific glutamate derivative in green tea, reduces the adverse reactions of doxorubicin by changing the glutathione level. Cancer Lett. 212, 177–184.
Zhang, K., Yang, E.B., Wong, K.P., Mack, P., 1999. GSH, GSH-related enzymes and GS-X pump in relation to sensitivity of human tumor cell lines to chlorambucil and adriamycin. Int. J. Oncol. 14, 861–867.