Effects of Glutamate Transporter Inhibitors on the Antitumor Activity of Doxorubicin

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ABSTRACT
Dihydrokainate, a glutamate transporter inhibitor, was previously found to be a useful modulator of antitumor activity of doxorubicin (DOX). Dihydrokainate prevented an efflux of DOX by inhibiting the uptake of glutamate by tumor cells. We examined the potential of glutamate transporter inhibitors as modulators of DOX activity. We observed a significant reduction in the uptake of glutamate by other inhibitors and a similar effect on DOX efflux in M5076 ovarian sarcoma cells. However, in vivo, the tissue distribution of each isoform is different, and glutamate transporter inhibitors with different affinities for each isoform affected tumors and normal tissues differently. 

INTRODUCTION
In clinical cancer chemotherapy, there are many problems with regard to low sensitivity to antitumor drugs and drug resistance to long-term treatment. Thus, we emphasize the enhancement of antitumor activity to these tumors is more important. We previously confirmed that DHK,2 a glutamate transporter inhibitor, prevented the efflux of DOX via a novel mechanism similar to theanine (1). The mechanism involves reducing glutamate uptake via inhibition of the glutamate transporter, intracellular glutathione synthesis, GS-DOX conjugate, and subsequent extracellular transport of GS-DOX by the multidrug resistant associated protein/GS-X pump (2). We have shown that DHK keeps DOX in tumor cells and enhances its antitumor activity (1). Five types of glutamate transporter isoforms have been identified (3–8), and several are expressed in tumor cells (9, 10). The inhibitors for them have different affinity to each isoform (7, 11–14). To clarify the therapeutic potential of glutamate transporter inhibitors, we examined their effects as a modulator, from the view point of enhancing the antitumor activity of DOX.

MATERIALS AND METHODS

Chemicals. DOX (10 mg/vial; Adriacin), was purchased from Kyowa Fermentation, Inc. (Tokyo, Japan). DHK, AAD, and SOS were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). t-L-[14C]Glutamate (9.62 MBq/μmol) was purchased from Amersham (Tokyo, Japan). Scintisol Ex-H was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). The drugs were dissolved in sterile isotonic saline. The other chemicals used in this study were of the highest purity available.

Animals. Male C57BL/6, DBA/2, and BDF1 mice (5 weeks of age; weight, 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 they were given free access to regular chow pellets and water.

Effects of Glutamate Transporter Inhibitors on the DOX Concentration in M5076 Ovarian Sarcoma Cells in Vitro.
M5076 ovarian sarcoma cells (1 × 106 cells/animal) were i.p. transplanted into male C57BL/6 mice. Ascites fluid was collected on the 14th day after transplantation. The sarcoma cells were washed twice and then resuspended in RPMI 1640 containing 10% fetal bovine serum. To examine the influx of DOX into M5076 ovarian sarcoma cells, cells (5 × 106 cells/ml medium) were incubated with 9.0 nmol/ml DOX at 37°C for 60 min in the presence or absence of inhibitors (100 μM).

To examine the effect of inhibitors on the DOX efflux from M5076 ovarian sarcoma cells, cells were preincubated with 9.0 nmol/ml DOX in the medium at 37°C for 30 min. After incubation, the medium was cooled on ice and then centrifuged at 150 × g for 3 min. The cells were washed and then resuspended in fresh medium. The resuspended cell suspension (106 cells/ml) was incubated at 37°C for 120 min in the presence or absence of inhibitors (100 μM).

In both systems, after incubation, the medium was cooled on ice and then centrifuged at 150 × 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 with a fluorescence spectrophotometer (excitation wavelength, 470 nm; emission wavelength, 585 nm).

Effects of Glutamate Transporter Inhibitors on Glutamate Uptake by M5076 Ovarian Sarcoma Cells in Vitro. M5076 ovarian sarcoma cells and P388 leukemia cells (5 × 10⁶ cells/ml medium) were suspended in incubation buffer (125 mM NaCl, 4.5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, and 5.0 mM glucose (pH 7.4)) and then preincubated at 37°C for 5 min (15). The cells were then incubated with 1.0 μM glutamate (containing 0.1 μM [14C]glutamate) and inhibitors (100 μM) at 37°C for 30 min. The uptake of glutamate was stopped by adding ice-cold PBS (pH 7.0). After that, the cells were centrifuged at 200 × g for 5 min, washed twice with PBS, and then dissolved in 400 μl of 1.0 N NaOH. Aliquots of this solution (200 μl) were dissolved in 5.0 ml of Scintisol Ex-H, and then the radioactivity was determined with a liquid scintillation counter.

Effects of Glutamate Transporter Inhibitors on the Antitumor Activity Induced by DOX. M5076 ovarian sarcoma cells (5 × 10⁶ cells/animal) were transplanted onto the backs of BDF 1 mice. DOX (2.0 mg/kg/day for 4 days) was administered i.p. at 18, 20, 22, and 24 days after the inoculation. Glutamate transporter inhibitors (10 mg/kg/day for 4 days) were i.p. injected at 19, 21, 23, and 25 days after the inoculation. The mice were killed by cervical dislocation on the 26th 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 described previously.

Statistical Analysis. Statistical analysis was performed with Student’s t test and ANOVA.

RESULTS

Effects of Glutamate Transporter Inhibitors on DOX Concentration in M5076 Ovarian Sarcoma Cells in Vitro. The effect of DHK on the membrane transport of DOX in M5076 ovarian sarcoma cells is shown in Fig. 1.

The concentration of DOX in the sarcoma cells increased gradually with the influx, and the DHK(100 μM) group tended to show an increase in the uptake of DOX as compared with the DOX-alone group. In contrast, DHK inhibited the DOX efflux from M5076 ovarian sarcoma cells. After 120 min, the concentration of DOX remaining in the DHK (100 μM) group (1.72 ± 0.05 μg/10⁷ cells) was significantly increased as compared with that in the DOX-alone group (1.39 ± 0.13 μg/10⁷ cells; P < 0.01).

It was found that AAD or SOS (100 μM) inhibited the efflux of DOX as well as did DHK (by 16.7% and 17.1%, respectively).

Effects of Glutamate Transporter Inhibitors on L-[14C]Glutamate Uptake by M5076 Ovarian Sarcoma Cells. The effects of glutamate transporter inhibitors on the intracellular uptake of L-[14C]glutamate by M5076 ovarian sarcoma cells are shown in Fig. 2.

The inhibitors significantly prevented the uptake of glutamate by the cells, with SOS and AAD showing remarkable inhibition of 54.6% and 39.5%, respectively (Fig. 2A). Both SOS and AAD (1–1000 μM) inhibited the uptake in a concentration-dependent manner (Fig. 2B).

Effects of Glutamate Transporter Inhibitors on the Antitumor Activity of DOX against M5076 Ovarian Sarcoma. The effects of glutamate transporter inhibitors on the antitumor activity of DOX on M5076 tumor-bearing mice are shown in Fig. 3.

It was shown that DOX alone did not reduce tumor growth, but the combination of DOX and AAD or SOS reduced tumor weight. In particular, SOS significantly increased the antitumor activity of DOX and reduced the tumor weight to 31.8% (P < 0.01) as compared with the control. There was no change in tumor weight in the AAD or SOS-alone group as compared with the control group. Furthermore, there are no significant changes of body weight in all groups for the periods of administration. In the tumor, the concentration of DOX in the group treated with SOS + DOX was 2.57 ± 0.41 ng/mg protein, a significant increase of 2.1-fold compared with the DOX-alone group (P < 0.01).

In the liver and heart, which were normal, the combined
Fig. 2 Effects of glutamate transporter inhibitors on L-[^14]C]glutamate uptake in M5076 ovarian sarcoma cells. M5076 ovarian sarcoma cells (5 × 10⁶ cells/ml) were incubated with 1.0 μM glutamate in the presence or absence of inhibitors (100 μM). A, L-[^14]C]glutamate uptake is expressed as a percentage of the control level. Significant differences from the level of the control group are indicated by a) (P < 0.001) and b) (P < 0.05). B, each point is the percentage of inhibition compared with the control group. Each point is the mean ± SD of four samples. B, □, SOS; ⋄, AAD.

Table 1 Effects of glutamate transporter inhibitors on DOX concentrations in the tissues of M5076 tumor-bearing mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DOX</th>
<th>DOX + AAD</th>
<th>DOX + SOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3.82 ± 0.38</td>
<td>4.36 ± 0.60</td>
<td>3.44 ± 0.56</td>
</tr>
<tr>
<td>Heart</td>
<td>1.07 ± 0.19</td>
<td>1.22 ± 0.10</td>
<td>0.76 ± 0.02a</td>
</tr>
</tbody>
</table>

a P < 0.05, a significant difference from the DOX group.

The treatment of AAD + DOX tended to bring about an increase in the concentration of DOX as compared with treatment with DOX alone. However, SOS + DOX tended to reduce the DOX concentration in the liver and heart, and the DOX concentration in the heart was particularly reduced by 29.0% (P < 0.05; Table 1).

Effect of SOS on L-[^14]C]Glutamate Uptake by P388 Leukemia Cells. The effects of SOS (500 μM) on glutamate levels in P388 leukemia cells and M5076 ovarian sarcoma cells are shown in Fig. 4.

In the sarcoma cells, SOS remarkably inhibited the glutamate uptake to 37.1% of the control value. On the other hand, the inhibitory effect of the same concentration of SOS against P888 leukemia cells was smaller than that on sarcoma cells. Furthermore, 0.01–500 μM SOS inhibited the uptake of glutamate in M5076 ovarian sarcoma cells in a concentration-dependent manner. In P888 leukemia cells, there was a significant decrease in the glutamate uptake at 1–500 μM SOS. However, 0.01–0.1 μM SOS raised the level of glutamate (Fig. 5). Furthermore, the initial rate of uptake in the P388 leukemia cells treated with SOS (0.1 μM) was 12.54 ± 0.94 pmol/min/10⁷ cells and tended to increase relative to the control (10.10 ± 1.27 pmol/min/10⁷ cells; Fig. 5).

DISCUSSION

Previously, the glutamate transporter inhibitor DHK was shown to enhance the antitumor activity of DOX against Ehrlich ascites carcinoma and to be a potential modulator of DOX (1). To clarify the usefulness of DHK and search for a more appropriate glutamate transporter inhibitor, we investigated the effects of inhibitors with different affinities to glutamate transporter isoforms in combination with DOX.

In the membrane transport of DOX in M5076 ovarian sarcoma cells, the transport was significantly reduced by 37.1% in the presence of SOS (500 μM). However, the combination of SOS and DHK tended to reduce the DOX concentration in the liver and heart, and the DOX concentration in the heart was particularly reduced by 29.0% (P < 0.05; Table 1).
sarcoma cells, DHK tended to increase the uptake of DOX and significantly inhibited the release of theanine-like DOX (Fig. 1). Because theanine had a similar effect in combination with DOX on Ehrlich ascites carcinoma cells and M5076 ovarian sarcoma cells (16, 17), and because the action in common between theanine and DHK is inhibition of the glutamate transporter, it was considered that DHK prevented the efflux of DOX by inhibiting the glutamate transporter, the same mechanism used by theanine. On the other hand, AAD and SOS, which are glutamate transporter inhibitors like DHK, also caused the inhibition of DOX efflux, so we expected then to be modulators of DOX activity.

There are five types of high-affinity glutamate transporters, and the tissue distribution of each one is different (3–8). Among these isoforms, GLAST and GLT-1 are extensively distributed in the brain and nowhere else, whereas EAAC1 is distributed in the liver and heart, which are normal tissues (11–14). Substances known as glutamate transporter inhibitors have different affinities for each isoform (7, 11–14). We examined the effects of inhibitors with different affinities to each isoform on the uptake of glutamate by M5076 ovarian sarcoma cells expressing GLAST and GLT-1 (18). All inhibitors significantly blocked the glutamate uptake, but the actions of SOS and AAD were remarkable (Fig. 2). Although the actions were only against tumor cells in vitro, it is considered that they could affect other tissues expressing glutamate transporters in vivo. Therefore, we examined the effect of SOS and AAD, which have strong inhibition and different affinities to each isoform, on the antitumor activity of DOX against M5076 ovarian sarcoma.

There was little change in tumor weight among M5076 tumor-bearing mice after treatment with DOX alone. Namely, no significant difference in the inhibition of tumor growth was observed. However, the combination of SOS + DOX showed significant antitumor activity (Fig. 3). The effect of AAD + DOX was similar to that of DOX alone in the tumors, but the concentration of DOX in tumors in the SOS + DOX group significantly increased by 2.1-fold \((P < 0.01)\) as compared with the DOX-alone group. Hence, it is thought that this increase in the DOX concentration in tumors contributes to the effect of SOS on the antitumor activity of DOX as well as theanine.

![Graph](image-url)
Consequently, because we gained significant antitumor activity by DOX against M5076 ovarian sarcoma in combination with SOS, which had selective affinity to GLAST, the relation between the glutamate transporter isoforms expressed in tumors and the affinity of inhibitors to the isoforms was important.

On the other hand, there are several risks, including the possibility that the increase in the concentration of DOX in normal tissues can lead to side effects. However, SOS, which increased the concentration in tumors, tended to bring about a decrease in the concentration of DOX in the liver and heart. Therefore, it was expected that there could be a SOS-induced decrease in the side effects of DOX. In contrast, on cotreatment with AAD, the DOX concentration tended to increase in the liver and heart. As mentioned above, EAAC1 was mainly distributed in normal tissues, and AAD has selective affinity to EAAC1 (11–14), so it was thought that the combination would affect normal tissues rather than tumors in vivo. Consequently, it was also shown that the relation between glutamate transporter isoforms and the selective affinity of inhibitors could selectively affect the antitumor activity and side effects of DOX.

One can expect a difference of isoforms according to the effects of SOS in different cells between tumors and normal tissues. Whereas SOS remarkably inhibited the uptake of glutamate in M5076 ovarian sarcoma cells expressing GLAST and GLT-1, it had less effect in P388 leukemia cells expressing GLT-1 and EAAC1 (Fig. 4). In contrast, the rate of glutamate uptake in both cell lines was at a similar level. Namely, the effects of inhibitors were expected to reflect differences and quantities of isoforms because SOS has selective affinity for GLAST. Furthermore, SOS inhibited glutamate uptake in M5076 ovarian sarcoma in a concentration-dependent manner, whereas a low concentration of SOS actually increased the uptake in P388 leukemia cells (Fig. 5). The initial rate of the glutamate uptake in P388 leukemia cells was found to be increased. In almost all cells, two or three isoforms of glutamate transporter are expressed. In contrast, inhibitor of glutamate transporter has specificity to one or two isoforms. Namely, it is possible that the weaker inhibition on a certain isoform by the inhibitor induced increasing activity of other isoform. One can expect the actions of inhibitors to be affected by the transport activity in addition to the isoform, and it was shown to be necessary to select the inhibitors and dose for each tumor. If the induction of glutamate uptake by inhibitors occurred in normal tissues, this effect could contribute to a decrease in the side effects of DOX.

In conclusion, a combination of glutamate transporter inhibitors affected the membrane transport of DOX by a glutamate transporter-mediated mechanism. This action, based on the characteristics of inhibitors combined with DOX, enhanced the antitumor activity of DOX without increasing the side effects of it by the selection of inhibitors with a selective affinity to isoforms expressed in tumor cells. In particular, it was shown that in M5076 ovarian sarcoma cells expressing GLAST and GLT-1, SOS enhanced the antitumor activity of DOX and reduced the concentration of DOX in normal tissues. Namely, SOS appeared to be a useful modulator of the antitumor activity of DOX. On the other hand, the effects of inhibitors differed among cells, showing the importance of selecting the inhibitors and dose for tumor cells.

REFERENCES


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