Effects of Glutamate Transporter Inhibitors on the Antitumor Activity of Doxorubicin

Yasuyuki Sadzuka,¹ Yasuyo Yamashita, and Takashi Sonobe
School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

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. L-serine-O-sulfate, which has high affinity to glutamate/aspartate transporter, particularly enhanced the antitumor activity of DOX in M5076 tumor-bearing mice. In contrast, L-α-aminoadipate tended to increase the DOX concentration in normal tissues rather than tumors. It was 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. Furthermore, the effects of inhibitors varied among cells expressing different isoforms. Notably, a low concentration of L-serine-O-sulfate actually increased the uptake of glutamate in P388 leukemia cells.

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 that the enhancement of antitumor activity to these tumors is more important. We previously confirmed that DHK, 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). 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.

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 resulting cell suspension (5 × 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 \times 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 \((5 \times 10^6\) cells/ml medium) were suspended in incubation buffer \([125\text{ mM NaCl}, 4.5\text{ mM KCl}, 1.2\text{ mM CaCl}_2, 1.2\text{ mM MgCl}_2,\) and 5.0 mM glucose (pH 7.4)] and then preincubated at 25°C for 5 min (15). The cells were then incubated with 1.0 \mu M glutamate (containing 0.1 \mu M \(\text{L-}^{14}\text{C} \text{glutamate}\)) and inhibitors \((100 \mu M)\) at 25°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 \times g for 5 min, washed twice with PBS, and then dissolved in 400 \mu l of 1.0 N NaOH. Aliquots of this solution \((200 \mu l)\) 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 \((5 \times 10^6\) cells/animal) were transplanted onto the backs of BDF, \(1\) mice. DOX \((2.0 \text{ mg/kg/day for 4 days})\) was administered i.p. at 18, 20, 22, and 24 days after the inoculation. Glutamate transporter inhibitors \((10 \text{ mg/kg/day for 4 days})\) 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 \((1200 \times g, 15\text{ min})\). 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\((100 \mu M)\) 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 \((100 \mu M)\) group \((1.72 \pm 0.05 \mu g/10^7\text{ cells})\) was significantly increased as compared with that in the DOX-alone group \((1.39 \pm 0.13 \mu g/10^7\text{ cells})\; (P < 0.01)."

**Effects of Glutamate Transporter Inhibitors on L-\(^{14}\text{C} \text{Glutamate Uptake by M5076 Ovarian Sarcoma Cells.** The effects of glutamate transporter inhibitors on the intracellular uptake of L-\(^{14}\text{C} \text{glutamate}\) 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 \((1–1000\mu M)\) inhibited the uptake in a concentration-dependent manner (Fig. 2B). Furthermore, the effects of inhibitors \((100 \mu M)\) on glutamate transport in M5076 ovarian sarcoma after DOX treatment were similar to those in Fig. 2, but the inhibition tended to increase as compared with that for inhibitor alone (data not shown).

**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 \pm 0.41 \text{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
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).

**Table 1** Effects of glutamate transporter inhibitors on DOX concentrations in the tissues of M5076 tumor-bearing mice. Each value is the mean SD (n = 4–7).

<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 ± 0.02</td>
</tr>
</tbody>
</table>

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

**Fig. 3** Effects of glutamate transporter inhibitors on antitumor activity against M5076 ovarian sarcoma. M5076 ovarian sarcoma cells (5 × 10⁵ cells/animal) were transplanted onto the backs of BDF₁ mice. DOX (2.0 mg/kg/day × 4 days, i.p.) and glutamate transporter inhibitors (10 mg/kg/day × 4 days, i.p.) were injected. Each column shows the mean ± SD (n = 4–7). A significant difference from the level of the DOX alone group is indicated by a) (P < 0.01).

**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 (5 × 10⁵ cells/ml) were incubated with 1.0 µM glutamate in the presence or absence of inhibitors (100 µM). A, L-[¹⁴C]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.
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.

![Fig. 4](image-url) Effects of SOS (500 μM) on L-[14C]glutamate uptake in P388 leukemia cells and M5076 ovarian sarcoma cells. Glutamate uptake is expressed as a percentage of the control level. Each column is the mean ± SD of four samples. A significant difference from the level of the control group is indicated by a) ($P < 0.001$).

![Fig. 5](image-url) Effects of SOS on L-[14C]glutamate uptake in M5076 ovarian sarcoma cells and P388 leukemia cells. M5076 and P388 cells (5 × 10⁵ cells/ml) were incubated with 1.0 μM glutamate in the presence or absence of SOS (0.01–500 μM). A, each point shows the percentage of inhibition compared with the control group. Each point is the mean ± SD of four samples. □, M5076; ●, P388. B, each column is the initial rate of glutamate uptake (the mean ± SD of three experiments). The initial rate of glutamate uptake was calculated from the glutamate level for the initial 5 min of the incubation. SOS, 0.1 μM.
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 liver and heart. Consequently, because we gained significant antitumor activity 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.

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 DOX 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 each tumor cell.

REFERENCES

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