Theanine induced inhibition of glutamate transporter enhances the activity of an antitumor agent.

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Summary

We showed that theanine, a specific amino acid in green tea and glutamate derivative, increased doxorubicin (DOX) induced antitumor activity, and confirmed that this action contributed to the increase in the DOX concentration in a tumor with inhibition of the DOX efflux from tumor cells by theanine. To clarify the mechanism of theanine, we studied tthe correlation with the glutamate transporter and glutathione (GSH) S-conjugate export (GS-X) pump on theanine induced inhibition of DOX efflux. Theanine inhibited GLAST and GLT-1 as glutamate transporter subtypes in the Na^+-coupled cotransport system. The decrease in the glutamate concentration in tumor cells caused by theanine decreased in the intracellular GSH level. Furthermore, the intracellular generation of the GS-DOX conjugate decreased with the theanine induced reduction of the GSH level. In M5076 cells, the expression of MRP5/GS-X pump, has a function in DOX efflux, was confirmed. MRP5/GS-X pump is expected to be closely concerned with the extracellular transport of the GSH conjugate in tumor cells. We thought that the GS-DOX conjugate was transported extracellularly via the MRP5/GS-X pump in M5076 cells and that theanine affected this route. In conclusion, it is clear that theanine increases the DOX concentration in a tumor in vivo through inhibition of glutamate transporter via the GS-X pump. The glutamate transporter mediated increase in antitumor activity caused by theanine is a novel mechanism, and it is hoped that this action will lead to a useful and novel cancer chemotherapy.

Keywords

theanine, doxorubicin, glutamate transporter, glutathione, MRP5/GS-X pump

Introduction

Chemotherapy using antitumor agents plays an important role in clinical cancer therapy. Among the treatments involving antitumor agents, biochemical modulation leads to effective therapy. The enhancement of antitumor activity was observed on biochemical modulation, however, the side toxicities of antitumor agents increased at the same time. Thus, improvement of the therapeutic index may not necessarily be achieved. Therefore, the development of more effective modulators without elevation of the side effects has been expected. We have investigated the antitumor effects of doxorubicin (DOX), an anthracycline antibiotic, and some modulators. We have also looked for modulators in foodstuffs, as opposed to medicine.

Green tea is shown to have some antioxidative components, preventing life-style related diseases such as cancer. Thus, green tea has been noted to have healthy effects by regulation of biological functions. EGCG is an antioxidative component of tea leaves and has been reported to have the effect of anticarcinogenesis. Although the effects of antioxidative components have been studied in regard to catechins, other effective components in green tea have not been clarified. On the other hand, theanine is γ-glutamylethylamide and an amino acid found specifically in green tea, is contained at about 2 % of the dry weight of tea leaves and is one of the tastier components. We have investigated the effects of theanine on the antitumor activity of DOX and report the effects of theanine as a modulator and its usefulness.[1-5)]

Materials and Methods

Doxorubicin hydrochloride was purchased from Kyowa Fermentation. L-Theanine was purchased from Tokyo Kasei Co. Ltd.

M5076 ovarian sarcoma cells were s.c. transplanted onto the backs of mice, and then DOX 2 mg/kg/day was intraperitoneally administered at 14, 16, 18 and 20 days after tumor inoculation.

Theanine 10 mg/kg/day was intraperitoneally injected at 15, 17, 19 and 21 days after tumor inoculation. The animals were killed by cervical dislocation on the 22nd day after inoculation. The solid tumor was removed and weighted.

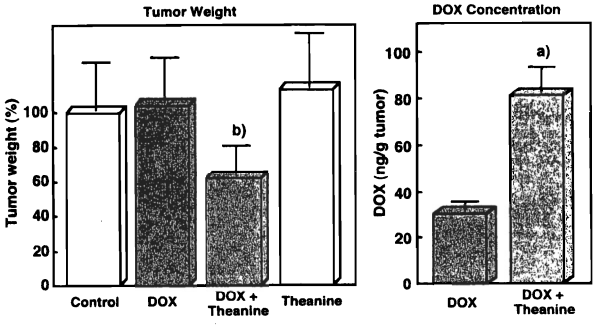
The examination of DOX uptake and release in M5076 ovarian sarcoma cells and determinations of glutamate and glutahione levels were performed according to our previous paper.[1,4,5]

Results and Discussion

Since M5076 ovarian sarcoma has low sensitivity to DOX, this dose of DOX did not inhibit tumor growth compared to the control level. Whereas, DOX combined with theanine significantly reduced the tumor weight to 62 % of the control level (Fig. 1). Since theanine is a glutamate derivative, it has no antitumor activity. Therefore, it appeared that the reduction in the tumor weight with the combination of theanine and DOX was the result of enhancement of the antitumor effect of DOX by theanine. In the tumor, as the target site of antitumor agents, the administration of theanine increased the DOX concentration by about 3 fold compared to the DOX alone level (Fig. 1). Thus, we confirmed that the increase in the DOX level in a tumor is the direct cause of the enhancement of the antitumor activity induced by theanine. On biochemical modulation, examination of both the antitumor activity and the side effects of antitumor agents is essential. Cardiac toxicity is an especially typical side toxicity of DOX. In the heart, no increase in the DOX concentration was observed in combination with theanine and DOX. These results suggested that theanine increases the DOX concentrations selectively in the tumor, but not in normal tissues. Thus, theanine induced antitumor activity of DOX without the increase in side toxicity of DOX.

Fig. 1

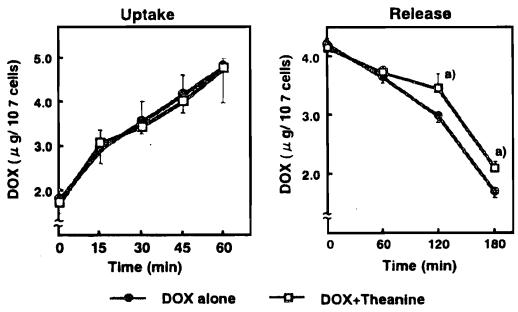
Effects of Theanine on the Antitumor Activity of Doxorubicin against M5076 Ovarian Sarcoma



Tumor weights are expressed as a percentage of control level. Each column is the mean ± SD (n=8). Significant differences from the level of the DOX alone group are indicated by a) $P < 0.001$ and b) $P < 0.01$.

Fig. 2

Effect of Theanine on the Membrane Transport of Doxorubicin in M5076 Ovarian Sarcoma Cells



M5076 cells were incubated with 9.0nmol/ml DOX in the presence or absence of theanine (1.0 μM). Each point is the mean ± SD (n=4). Significant difference from the DOX alone group is indicated by a) $P<0.05$.

We have attempted to clarify a part of the mechanism underlying the action of theanine. Since theanine increases the DOX concentration in the tumor in vivo, we became interested in the transport of DOX across the tumor cell membrane. Therefore, we investigated the effects of theanine on the DOX influx and efflux in M5076 ovarian sarcoma cells in vitro. Theanine did not affect the DOX influx into M5076 cells, whereas inhibition of the DOX efflux by theanine was observed (Fig. 2). The reduction of the DOX efflux across the tumor cell membrane caused by theanine may induce an increase in the DOX concentration in solid tumors in vivo.

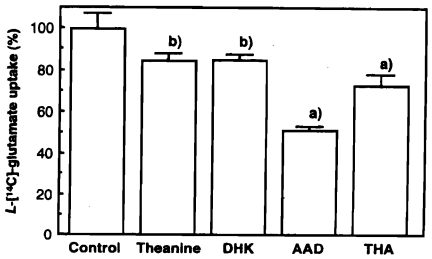
Recently, many investigators have reported the functions of drug transporters related to multidrug resistance, such as P-glycoprotein. Concurrently, some inhibitors of these drug pumps have been utilized to overcome drug resistance. However, on M5076 ovarian sarcoma cells, the overexpression of P-glycoprotein is never observed on the cell membrane. Therefore, as the functional site for theanine, these drug efflux pumps could not be considered. Since theanine is a derivative of glutamate, we hypothesized that theanine-mediated inhibition of DOX efflux may be due to its effect on glutamate transporters. As well as theanine, glutamate transporter inhibitors reduced the efflux of DOX in M5076 ovarian sarcoma. The fact that these glutamate analogues changed DOX efflux suggested that glutamate transporters should be related to the membrane transport of DOX in M5076 ovarian

sarcoma. Furthermore, as shown in Fig. 3, theanine as well as glutamate transporter inhibitors significantly reduced the glutamate uptake. Theanine was probably transported via glutamate transporters into M5076 cells and also competitively inhibited the glutamate uptake. The inhibition of doxorubicin efflux by glutamate transport inhibitors suggested that theanine inhibits the DOX efflux with a reduction of glutamate uptake. In addition, the inhibition of glutamate uptake by these glutamate analogues accounted for the expression of glutamate transporters in M5076 cells.

Some types of glutamate transporter as to four subtypes, GLT-1, GLAST, EAAC1 and EAAT4, were cloned, and their tissue distributions and physiological functions were clarified. We confirmed that GLAST and GLT-1 were detected in M5076 cells.[4] We speculated that these two types bring glutamate into M5076 cells. GLAST and GLT-1 are astrocytic glutamate transporters. The inhibition of glutamate transporters increases the extracellular glutamate concentration and excessive accumulation of glutamate causes neuronal death. Inhibiting glutamate uptake, theanine may possibly enhance the toxicity of glutamate. Although theanine is distributed in brain, there have been no reports on its toxicity to date. Furthermore, theanine was reported to be an antagonist of the NMDA receptor, one of the glutamate receptors related to the neuronal death induced by glutamate. Accordingly, theanine could protect brain from glutamate neurotoxicity. On the other hand, DOX can not permeate through the blood-brain barrier. Therefore, the combination of DOX and theanine are not likely to cause neurotoxicity.

Fig. 3

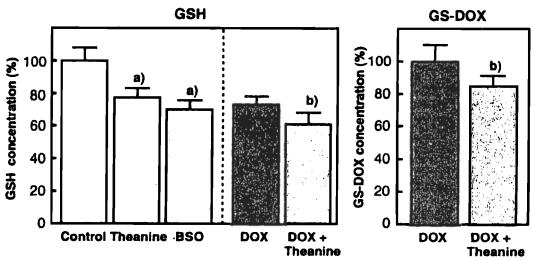
Effects of Theanine and Glutamate Transport Inhibitors on the *L*-[^{14}C]-Glutamate Uptake in M5076 Ovarian Sarcoma Cells



M5076 cells incubated with 1.0 μM glutamate in the presence or absence of theanine, DHK, AAD and THA (100 μM). *L*-[^{14}C]-glutamate uptakes are expressed as % of the of control level. Each column is the mean ± SD (n=4). Significant differences from the level of control are indicated by a) $P < 0.001$ and b) $P < 0.01$.

Fig. 4

Effects of Theanine on the Glutathione and Doxorubicin-Glutathione Conjugate Concentrations in M5076 Ovarian Sarcoma Cells



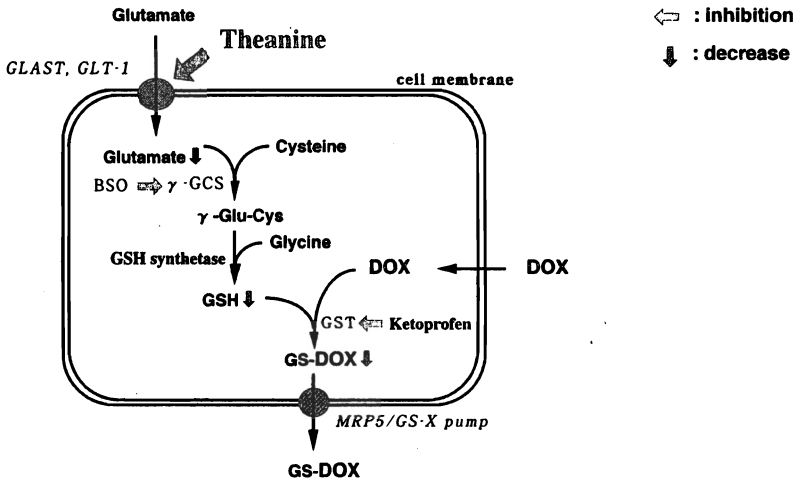
M5076 cells are incubated with theanine or BSO (100 μM) or DOX (9.0nmol/ml). GSH concentrations are expressed as % of control level (mean±SD, n=4). GS-DOX concentrations are expressed as % of the level of DOX alone group (mean±SD, n=4). Significant difference from the level of control is indicated by a) $P < 0.001$. Significant difference from the level of DOX alone group is indicated by b) $P < 0.05$.

It is expected that the inhibitory effect on DOX efflux by theanine is connected with inhibition of the glutamate transporter. However, it is difficult to believe that DOX is directly transported by the glutamate transporter and that this transport is inhibited by theanine. In contrast, glutathione (GSH) is synthesized from glutamate. In the body, GSH is a substrate of a conjugative reaction in drug metabolism. Recently, it has been reported that the ATP-dependent GSH S-conjugate export (GS-X) pump has something to do with the transport of antitumor agents in cell membranes. It is suggested that the GS-X pump exhibits a high affinity to GSH conjugated DOX. It is expected that the intracellular synthesis of GSH and GSH conjugate reaction may affect the transport of DOX. As well as BSO, a specific inhibitor of the rate-limiting enzyme for GSH, theanine significantly decreases the intracellular concentration of GSH in M5076 cells (Fig. 4). Thus, the inhibition of glutamate uptake by theanine was considered to induce a decrease in the synthesis of GSH from glutamate as a substrate. The decrease in GSH evoked with the combination of theanine and DOX was shown to be greater than that caused by DOX alone. Furthermore, the amount of GS-DOX conjugate in the presence of theanine decreased to 85 % of that in the DOX alone group. Thus, the intracellular generation of the GS-DOX conjugate was confirmed to decrease with the theanine induced reduction in the level of GSH. In M5076 cells, we confirmed the expression of MRP5/GS-X pump, which functions in the efflux of DOX[5]. Therefore, it was shown that the GS-DOX conjugate was transported extracellularly via the MRP5/GS-X pump in M5076 cells.

From these results, we speculated on the mechanism for the theanine induced effect, as shown in Fig. 5. Some of the DOX taken up by M5076 cells binds to GSH and thereby generates the GS-DOX conjugate. The conjugate is released extracellularly by the M5076/GS-X pump. This pathway is closely connected with the intracellular synthesis of GSH and the GSH conjugated reaction. On the other hand, theanine suppressed the uptake of glutamate and thereafter the biosynthesis of GSH through inhibition of GLAST and GLT-1 as glutamate transporters in M5076 cells. Then, generation of the conjugate of intracellular DOX and GSH is affected and the release of the GS-DOX conjugate by tumor cells via the MRP5/GS-X pump decreases. It is considered that these phenomena suppress the decrease in the concentration of DOX in a tumor and induce the increase in the DOX induced antitumor activity caused by theanine. As GLAST and GLT-1 are not expressed in the heart and liver, theanine did not have an effect in these tissues. Thus, it appears that theanine does not have an enhancing effect on the DOX induced side toxicity. In the brain, where GLAST and GLT-1 are expressed, DOX is not present. Thus, we concluded that the combination of theanine with DOX is an ideal chemotherapy for increasing antitumor activity without intensifying side effects.

Fig. 5

Mechanism for Theanine to inhibit of Doxorubicin Release from M5076 Ovarian Sarcoma Cells



In conclusion, it is clear that theanine increases the concentration of DOX in a tumor in vivo through inhibition of the glutamate transporter via the GS-X pump. The glutamate transporter mediated increase in antitumor activity caused by theanine is a novel mechanism, and it is hoped that this action will lead to the discovery of a useful cancer chemotherapy for drug sensitive, drug resistant and metastatic tumors.

References

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