Jpn. J. Cancer Res. **90**, 775–780, July 1999

Membrane Transport and Antitumor Activity of Pirarubicin, and Comparison with Those of Doxorubicin

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We have compared the membrane transport and antitumor activity of pirarubicin with those of doxorubicin in M5076 ovarian sarcoma, which exhibits low sensitivity to doxorubicin. Pirarubicin was rapidly taken up by M5076 cells and the intracellular concentration of pirarubicin reached more than 2.5-fold that of doxorubicin. In terms of the 50% cell growth-inhibitory concentration *in vitro*, pirarubicin was more effective than doxorubicin. Thus, the intracellular concentration influenced the cytotoxicity of these anthracycline agents. On comparison of the nuclear uptake of pirarubicin and doxorubicin, the nucleus/cell ratio of pirarubicin was found to be about 40%, whereas that of doxorubicin reached more than 80%. As the intranuclear concentration of pirarubicin is dependent on nuclear transport, the increases in not only cell membrane transport, but also nuclear membrane transport contributed to the enhancement of the efficacy of pirarubicin. In M5076 solid tumor-bearing mice, pirarubicin reduced the tumor weight to 60% of the control level, although doxorubicin had no effect. These results were supported by the intracellular uptake of pirarubicin. Moreover, theanine, which inhibited the pirarubicin efflux from M5076 cells, increased by 1.3-fold the pirarubicin concentration in the tumor and enhanced the therapeutic efficacy of pirarubicin 1.7-fold. In conclusion, our results suggest that an increase in the concentration of an anthracycline derivative in tumor cells due to alteration of cell membrane transport results in enhancement of the antitumor activity.

Key words: Pirarubicin — Doxorubicin — Theanine — Cancer chemotherapy — Nuclear uptake

The therapeutic efficacy of antitumor agents is dependent on their distribution in a tumor. Many studies involving drug delivery systems, such as liposomes, have been performed in order to transfer antitumor drugs selectively to tumors.[1–3] As regards the transport mechanisms of antitumor agents, the functions of P-glycoprotein and multidrug resistance-associated protein were reported to be related to reduction of the drug concentration in tumors and resistance to antitumor agents.[4,5] Although some inhibitors of these drug efflux pumps have been utilized to reverse multidrug resistance, the adverse effects of these inhibitors prevent their clinical use. Moreover, the transport mechanisms of antitumor agents in drug-sensitive tumor cells have never been clarified. In sensitive tumors, no methods for increasing the intracellular concentrations of chemotherapeutic agents to enhance the antitumor activity have been established to date.

We have investigated the transport mechanisms of anthracycline derivatives in tumor cell membrane, and reported some differences in the uptake of anthracyclines by human leukemia HL60 cells.[6–9] However, it was not established how the membrane transport of an antitumor agent *in vitro* would influence its therapeutic activity *in vivo*.

M5076 ovarian sarcoma (M5076) is a transplantable murine reticulum sarcoma originating in the ovary of C57BL/6 mice, and is highly invasive and metastatic.[10–12] We previously reported that clinical doses of doxorubicin (DOX) did not reduce the M5076 tumor weight.[13] Pirarubicin (4′-O-tetrahydropyranyldoxorubicin; THP) (Fig. 1) is an anthracycline antibiotic,[14] developed as a result of studies to mitigate the severe cardiotoxicity of DOX. THP is more lipophilic than DOX, and is used for chemotherapy for ovary cancer. In the present study, we have compared the membrane transport and antitumor activity of THP with those of DOX in order to discover a more effective chemotherapy against M5076 sarcoma.

We previously demonstrated that theanine, a component of Japanese green tea, enhanced the antitumor activity of DOX by biochemical modulation.[13,15,16] Theanine comprises 1–2% of the dry weight of green tea leaves.[17] We found that theanine increased the DOX concentration in tumors by inhibiting the efflux of DOX from the tumor cells.[13,15] However, the action of theanine with other antitumor drugs has never been clarified. We have investi-

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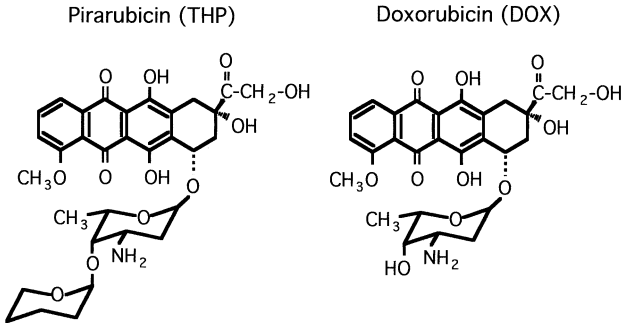

Fig. 1. Chemical structures of THP and DOX.

gated the enhancing effect of theanine on the antitumor activity of THP in M5076 tumor-bearing mice. Furthermore, the relationship of the drug concentration in tumor cells to antitumor activity was clarified.

MATERIALS AND METHODS

Reagents THP injection, 10 mg/vial (Pinorubin), was purchased from Nippon Kayaku Co., Ltd., Tokyo, DOX injection, 10 mg/vial (Adriacin), from Kyowa Fermentation Inc., Tokyo, theanine from Tokyo Kasei Co., Ltd., Tokyo, and RPMI 1640 medium from Nissui Pharmaceutical Co., Ltd., Tokyo. The drugs were dissolved in sterile isotonic saline. The other chemicals used in this study were of the highest purity available.

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

Tumor M5076 ovarian sarcoma was kindly provided by Dr. T. Tashiro (Japanese Foundation for Cancer Research, Tokyo).

***In vitro* experiments** The M5076 cells were washed twice and resuspended in RPMI 1640 medium containing 10% fetal bovine serum.

To examine the drug uptake by M5076 cells, cells (5×10^6 cells/ml medium) were incubated with 5.0 μg/ml THP or DOX at 37°C for 60 min.

To examine the effect of theanine on the THP efflux from M5076 cells, cells were preincubated with 5.0 μg/ml THP for 30 min. After preincubation, the cells were washed and resuspended in fresh medium, and then incubated at 37°C for 120 min in the presence or absence of theanine (1.0 μM).

For determination of the time course of the intracellular drug concentration, aliquots of the cell suspension were removed at definite times. Each aliquot was cooled on ice and then centrifuged at 150g for 3 min. The cells were washed and resuspended in 1.0 ml of ice-cold 10 mM phosphate buffer (pH 7.8), and then mixed for 30 s with 5.0 ml of chloroform-methanol (4:1, v/v) and centrifuged at 1,200g for 15 min. The concentration of drug in the organic phase was determined with a fluorescence spectrophotometer, Hitachi F2000 (Hitachi Ltd., Tokyo) (excitation, 470 nm; emission, 585 nm).

For separation of nuclei from the cell fraction, incubated cells were washed twice with cold Hanks' balanced salt solution (pH 7.2), resuspended in 1 ml of Hanks' solution containing 0.1% Nonidet P-40 (Iwai Co., Ltd., Tokyo), and then allowed to stand at 0°C for 5 min.[18] The nuclear fraction in the cytoplasmic fraction was separated by centrifugation at 100g for 15 min. The nuclear fraction was resuspended in 1.0 ml of ice-cold 10 mM phosphate buffer (pH 7.8), and then the concentration of drug was determined as described above.

Cell growth-inhibition assay *In vitro* drug sensitivity was assessed by means of the tetrazolium dye (MTT) assay.[7, 19] Briefly, 1.0 ml aliquots of cells were placed in the wells of 12-well microculture plates with six graded concentrations of THP and DOX, in triplicate. The concentration range was 0.002–20 μg/ml. Untreated control cells were placed in six wells. After incubation under a humidified atmosphere containing 5% CO_2 for 3 days at 37°C, 100 μl of MTT solution was added and the plates were incubated for 4 h under the same conditions. The tetrazolium salt MTT is reduced to a colored formazan by living but not by dead cells. The formazan crystals were dissolved in 1.5 ml of acid isopropanol, and then the optical density of the wells, which is linearly correlated with the number of cells, was measured with a microplate reader at 540 nm. Cell survival was calculated as (the optical density of the treated wells divided by the mean optical density of control wells)×100%. The 50% cell growth-inhibitory concentration, IC_{50}, represents the drug concentration lethal to 50% of the cells.

Animal experiment M5076 cells (1×10^6 cells/animal) were transplanted onto the backs of BDF_1 mice. After 20 days, the mice were divided into several groups, each consisting of 8 mice. THP or DOX (2.0 mg/kg/day×4 days) was intraperitoneally injected at 20, 22, 24 and 26 days after the transplantation. Theanine (10 mg/kg/day×4 days) was intraperitoneally administered at 21, 23, 25 and 27 days. Control mice were injected with the same volume of sterile isotonic saline. The mice were killed on the 28th day after inoculation by cervical dislocation, and then the solid tumors and tissues were immediately removed and weighed. The concentrations of drugs in the tissues were determined as described above.

Statistical analysis Statistical analysis was performed by means of Student's t test and ANOVA.

RESULTS

Comparison of the uptake of THP and DOX by M5076 ovarian sarcoma cells The uptake of THP and DOX by M5076 cells is shown in Fig. 2. THP was rapidly taken up by cells, and the THP concentration reached the plateau level within 15 min. In contrast, the intracellular concentration of DOX slowly increased. After 60 min, the THP concentration was 2.5-fold ($P<0.001$) greater than the DOX level.

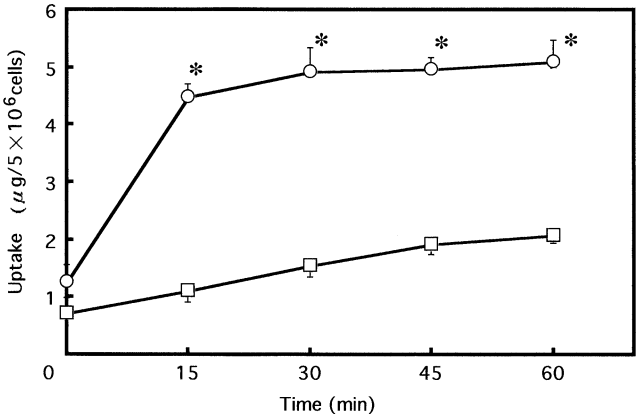

Fig. 2. Comparison of the intracellular uptakes of THP and DOX by M5076 ovarian sarcoma cells. M5076 cells were incubated with 5.0 μg/ml of THP or DOX at 37°C. Each point represents the mean of four samples, the bar indicating the SD. ○ THP, □ DOX. A significant difference from the DOX level is indicated by * $P<0.01$.

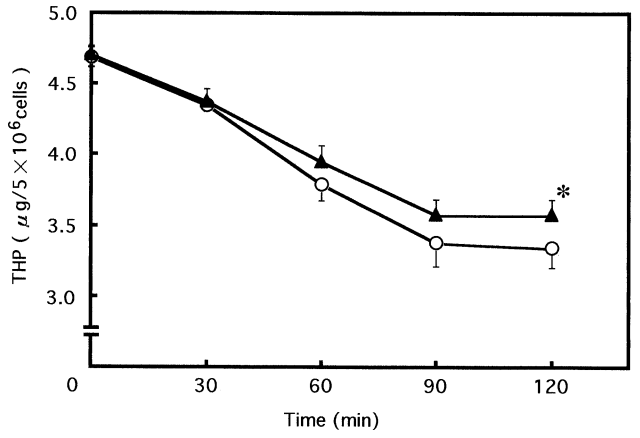

Fig. 3. Effect of theanine on the efflux of THP from M5076 ovarian sarcoma cells. M5076 cells were incubated with 5.0 μg/ml of THP at 37°C for 30 min, washed and then incubated with or without theanine 1.0 μM. Each point represents the mean of four samples, the bar indicating the SD. ○ THP-alone, ▲ THP+theanine. A significant difference from the level of the THP-alone group is indicated by * $P<0.01$.

Comparison of cytotoxicities of THP and DOX in M5076 ovarian sarcoma cells The IC_{50} values of THP and DOX in M5076 cells were 0.366 μM and 1.30 μM, respectively. THP was more effective, by 3.6-fold, than DOX.

Effect of theanine on the efflux of THP from M5076 ovarian sarcoma cells The effect of theanine on the efflux of THP from M5076 cells is shown in Fig. 3. Theanine reduced the efflux of THP by 16% ($P<0.01$) after 120 min incubation. It did not change the THP uptake by M5076 cells (not shown).

Comparison of nuclear uptake of THP and DOX by M5076 ovarian sarcoma cells The intracellular and intranuclear uptakes of THP and DOX by M5076 cells are shown in Fig. 4. The intranuclear concentration of THP

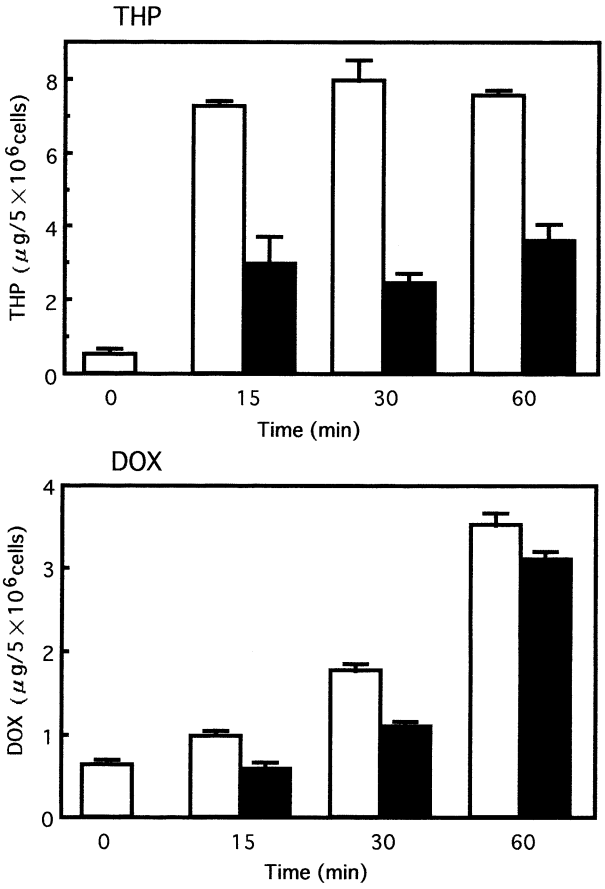

Fig. 4. Comparison of the nuclear uptakes of THP and DOX by M5076 ovarian sarcoma cells. M5076 cells were incubated with 10 μg/ml of THP or DOX at 37°C. Each column represents the mean of four samples, the bar indicating the SD. Open and closed columns express the intracellular and intranuclear concentrations, respectively.

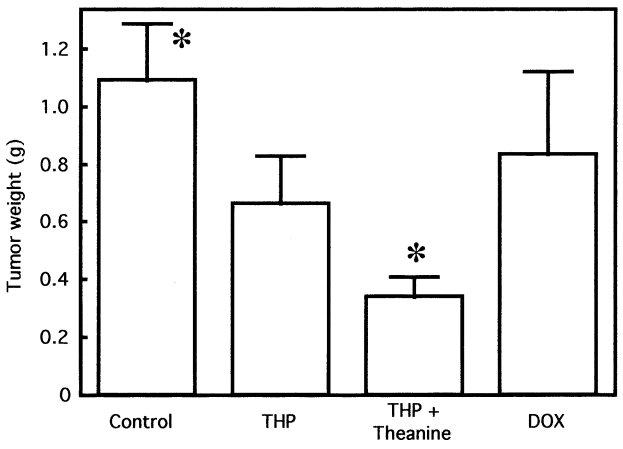

Fig. 5. Effect of theanine on the antitumor activity of THP against M5076 ovarian sarcoma. Each column represents the mean for eight mice, the bar indicating the SD. A significant difference from the level of the THP-alone group is indicated by $* P<0.001$.

Table I. Effects of Theanine on THP Concentrations in Tumors, Hearts and Livers of Mice, and Comparison with DOX Concentrations

Group	THP	THP+theanine	DOX
Tumor	2.00±0.26	2.62±0.47[a)]	1.07±0.11[a)]
Heart	1.49±0.17	1.73±0.29	2.57±0.35[a)]
Liver	3.82±0.28	1.86±0.38[a)]	2.48±0.48[a)]

The THP or DOX concentration is expressed as ng/mg protein in the tissue. Each value represents the mean±SD for eight mice. A significant difference from the level of THP-alone group is indicated by *a*); $P<0.001$.

increased rapidly and reached the maximal level within 15 min, like the intracellular concentration. The nucleus/cell ratio of THP was about 40% at 60 min. The intranuclear uptake of DOX depended on the increase in the intracellular DOX concentration, and the nucleus/cell ratio of DOX was more than 80% at 60 min. In addition, theanine had no effect on the nucleus/cell ratio of the drug concentrations (figure not shown).

Effect of theanine on the antitumor activity of THP against M5076 ovarian sarcoma The tumor weights after the treatment are shown in Fig. 5. THP reduced the tumor weight to 60% ($P<0.001$) of the control level (1.089 g). The combination of theanine with THP reduced the tumor weight to 31%, and theanine enhanced the antitumor activity of THP by 1.7-fold ($P<0.001$). DOX slightly reduced the tumor weight.

Table I shows the concentrations of THP or DOX in the tumors, hearts and livers of mice after the treatment. Theanine significantly increased, by 1.3-fold ($P<0.001$), the THP concentration in the tumor, compared with the THP-alone group. In contrast, the DOX concentration was about half that of THP in the tumor ($P<0.001$). In the heart, the DOX concentration was significantly greater than that of THP ($P<0.001$). Theanine decreased the THP concentration in the liver ($P<0.001$).

DISCUSSION

We have compared the antitumor activity of THP with that of DOX toward M5076 ovarian sarcoma, which exhibits low sensitivity to DOX, and we have further investigated the enhancing effect of theanine.

To examine the membrane transport of antitumor agents in tumor cells, we determined the uptakes of THP and DOX by M5076 cells *in vitro*. The THP concentration in M5076 cells increased faster than that of DOX, and reached the maximal level within 15 min. The intracellular concentration of THP was four times greater than that of DOX at 15 min, and remained at a higher level than that of DOX till 60 min. THP is more lipophilic than DOX, and the octanol/PBS partition coefficient of THP is 37 times that of DOX.[8)] This property suggests that the passive diffusion of THP across the cell membrane is greater than that of DOX. However, it could not explain the greater uptake rate of THP by M5076 cells, so there is a possibility that active or facilitative transport mechanisms contribute. In previous papers, we indicated that THP and DOX were taken up by HL60 cells partly via a common carrier-mediated transport system.[6–8)] Moreover, we suggested that THP is, at least in part, incorporated into HL60 cells via nucleoside transport systems.[9)] Thus, nucleoside transport systems may contribute to the membrane transport of anthracyclines in M5076 cells, as in the case of HL60 cells. We are now investigating the transport mechanisms of the anthracyclines in M5076 cells in more detail.

The therapeutic efficacy of anthracycline antibiotics is dependent on the area under the concentration-time curve after administration. It is expected that THP will be more effective against M5076 than DOX because of its higher accumulation in tumor cells. For comparison of the cytotoxicities of THP and DOX *in vitro*, their IC_{50} values were determined. THP was 3.6-fold more potent, in accordance with the greater intracellular uptake of THP.

Theanine, an amino acid component of green tea, was reported to enhance the antitumor activity of DOX in Ehrlich ascites carcinoma and M5076 ovarian sarcoma.[13, 15, 16)] It was indicated that theanine caused the retention of DOX in tumor cells due to inhibition of the efflux of DOX from the tumor cells.[13, 15)] If theanine increases the concentration of THP in M5076 cells, the cytotoxicity of THP should be enhanced. Whereas theanine did not affect the uptake of THP, it did inhibit the efflux of THP and prolonged the accumulation of THP in M5076 cells. Thus, theanine may

increase the THP concentration in tumors and enhance the therapeutic efficacy of THP against M5076. In addition, because theanine inhibited not only DOX but also THP efflux, it may act on a common transport mechanism for DOX and THP.

Anthracycline antibiotics act by inhibiting the activities of DNA polymerase and topoisomerase II via intercalation into DNA. Thus, the intranuclear concentration is an important determinant of the therapeutic efficacy. We examined the nuclear uptake of THP and DOX by M5076 cells. The intranuclear concentration of THP rapidly increased, similarly to its intracellular concentration, and the nucleus/cell ratio of the THP concentration reached about 40%. In contrast, the nucleus/cell ratio of DOX reached more than 80% at 60 min. It was deduced that the DOX taken up by the cells was rapidly incorporated into the nuclei and did not remain in the cytoplasm. Briefly, the intranuclear concentration of THP was dependent on the transport across the nuclear membrane, while the nuclear uptake of DOX was dependent on the transport across the plasma cell membrane. For enhancement of DOX activity, therefore, it is necessary to increase the cell membrane transport of DOX. In the case of THP, an increase in not only the cell membrane transport, but also that in the nuclear membrane transport would be effective for enhancing the cytotoxicity of THP. Thus, a novel modulator which increases the nuclear uptake of THP may result in an increase in the antitumor activity of THP.

Theanine did not alter the nucleus/cell ratio of DOX concentration in M5076 cells. This suggested that theanine inhibited DOX efflux without influencing the nuclear transport of DOX. However, intracellular THP existing outside the nucleus amounted to more than 50%, and this would be an important factor influencing the antitumor activity of THP *in vivo*. Although theanine prolonged the THP retention in M5076 cells, it was not clear whether or not theanine could increase the nuclear uptake and cytotoxicity of THP. Thus, we investigated the combined effect of theanine on the antitumor activity of THP in M5076 tumor-bearing mice.

The injection of THP alone reduced the tumor weight to 60% of the control level. The combination of theanine with THP enhanced by 1.7-fold the inhibitory effect of THP on tumor growth. Also, theanine specifically increased the THP concentration in the tumor by 1.3-fold. This increase in the THP concentration was probably a consequence of the inhibition of THP efflux by theanine, i.e., the increase in the intracellular retention of THP probably caused the enhancement of antitumor activity. Furthermore, theanine can alter the membrane transport and increase the concentration of an anthracycline in tumor cells without affecting the nuclear uptake ratio, enhancing the antitumor activity.

Injection of DOX slightly reduced the tumor weight. In the tumor, the THP concentration was twice that of DOX, in accordance with the difference in uptake between THP and DOX by M5076 cells *in vitro*. This is also in accord with the antitumor activities *in vivo* and the cytotoxicities *in vitro* of THP and DOX. Briefly, it is suggested that the membrane transport of an antitumor agent in tumor cells is related to its therapeutic efficacy *in vivo*. In normal tissues, the THP concentration was less than that of DOX in the heart. As cardiac toxicity is one of the most severe side effects of anthracyclines, THP is more suitable for chemotherapy, at least against M5076 from the viewpoint of side effects.

In conclusion, a comparison of the membrane transport and antitumor activity of THP with those of DOX indicated that the antitumor efficacy could be estimated from the anthracycline concentration in tumor cells. Furthermore, the results obtained with theanine suggest that a drug which alters the cell membrane transport of antitumor agents would modulate the chemotherapeutic efficacy. If the study of membrane transport mechanisms of antitumor agents in tumor cells reveals specific properties of tumors, such mechanisms could be utilized for the development of novel antitumor agents.

ACKNOWLEDGMENTS

This research was supported in part by the Frontier Research Program of the Ministry of Education, Science, Sports and Culture of Japan.

(Received February 25, 1999/Revised April 30, 1999/Accepted May 7, 1999)

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