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Antitumor effect of combination of S-1 and docetaxel on the human breast cancer xenograft transplanted into SCID mice

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Abstract. *In vivo* experiments were performed on breast cancer xenografts to examine whether the combination therapy with S-1, an oral dihydrouracil dehydrogenase (DPD) inhibitory fluoropyrimidine, plus docetaxel functions as an additive/synergistic modulator in tumor growth. The human breast cancer xenograft, MDA-MB-435SHM, was inoculated into SCID female mice. The tumor growth and thymidylate synthase (TS)/DPD activity of tumors treated with the agents were investigated. The T/C value (relative mean tumor weight of the treated group/relative tumor weight of the control group) of the group treated with docetaxel, S-1 and combination therapy were 45.3, 63.1 and 29.8%, respectively; suggesting the positive antitumor effects of the combination therapy in particular. In addition, significant down-regulation of DPD activity was also observed in the tumors treated with S-1, docetaxel and their combination. Down-regulation of the DPD activity of the tumors is also considered to be correlated with the antitumor effect of the treated groups, suggesting its influence on the synergistic effect of the combination therapy.

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Abbreviations: MBC, metastatic breast cancer; SCID, severe combined immune-deficient; DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase; RR, response rate; 5-FU, 5-fluorouracil

Key words: breast cancer, combination therapy, S-1, docetaxel, DPD-inhibitory fluoropyrimidine

Introduction

Adjuvant chemotherapy and/or hormone therapy clearly improve the survival of breast cancer patients (1,2). Metastatic breast cancer (MBC), however, demonstrates resistance against the present therapeutic procedure. Thus, new protocols with chemotherapy play a very important role in the treatment or control of advanced breast cancer. Clinical reports have demonstrated the clinical role of new agents, such as taxanes and/or oral fluoropyrimidines, especially for metastatic sites (3-7). Docetaxel, a semi-synthetic taxane and convincing agent against breast cancer (8,9), acts as a potent anti-mitotic agent by the promotion of abnormal microtubule stabilization (10). Clinical studies of single-agent docetaxel treatment in MBC patients with prior anthracycline treatment have been reported demonstrating higher response rates (RR) (11-13). The results of these studies also revealed that patients who received single-agent docetaxel achieved significantly RR superior to other combination regimens, including mitomycin C plus vinblastine, methotrexate plus 5-fluorouracil (5-FU) and 5-FU plus vinorelbine. In addition, the administration of oral fluoropyrimidines, such as capecitabine, also revealed subtle clinical responses for MBC (14,15).

S-1, a newly developed oral dihydropyrimidine dehydrogenase (DPD) inhibitory fluoropyrimidine drug, consisting of tegafur (FT), 5-chloro-2,4-dihydroxypyrimidine (gimeracil) and potassium oxonate (oteracil) at a molar ratio of 1:0.4:1 was reported to be a promising agent for gastric and breast cancers in phase II registration studies (4,6). A recent clinical study also suggested the effectiveness of the combination regimen with S-1 and docetaxel for advanced/recurrent gastric cancer (7). The combination with S-1 and docetaxel is, therefore, expected to be an effective regimen for anthracycline and/or taxane pre-treated MBC.

The mechanism of action of the combination therapy with fluoropyrimidine and taxanes, however, has not been fully explored. *In vivo* experiments were designed to measure the extent of tumor growth of MDA-MB-435SHM, a human breast cancer xenograft, treated with S-1, docetaxel and their combination. The experiments also measured the modulation of the thymidylate synthase (TS) and DPD activities of the xenograft treated with chemotherapy, in order to investigate

the relationship between 5-FU-related biochemical parameters and tumor suppression.

Materials and methods

Mice. CB17/Icr SCID mice (female, 4-week-old) were purchased from CLEA Japan, Inc., Tokyo. The mice were maintained under specific pathogen-free conditions using an Isorack system and were fed sterile food and water *ad libitum* in the Keio University Animal Center. Six- to eight-week-old mice weighing >20 g were used for the experiments.

Xenograft. MDA-MB-435SHM was established in the Cancer Research Laboratory, Taiho Pharmaceutical Co. Ltd. (16) and was serially transplanted at the Keio University Animal Center.

Briefly, the xenograft was established from MDA-MB-435, a human breast cancer cell line purchased from American Tissue Culture Collection (ATCC). Orthothopic transplantation of the cells was performed and lung metastasis deposits obtained were excised and transplanted into the mammary fat pad of the other 6- to 8-week female mice. This was repeated 8 times, and the new xenograft, with a high potential for tumor growth and/or lung metastasis, was established and named MDA-MB-435SHM (17). This xenograft was serially transplanted into SCID mice and used for the subsequent experiments.

Chemicals. S-1 was prepared in our laboratory by mixing tegafur, gimeracil and oteracil in a molar ratio of 1:0.4:1 in 0.5% hydroxypropyl methylcellulose. Docetaxel (Taxotere®) was purchased from Aventis Pharma Ltd, Tokyo, Japan. [6-^{14}C]-5-FU(56 mCi/mmol) and [6-^{3}H]-FdUMP(625 GBq/mmol) were obtained from American Radiolabeled Chemicals Inc. (MO, USA) and Moravek Biochemicals Inc. (CA, USA), respectively.

Tumor inoculation. The tumor tissue was minced and one or two tissue fragments of the xenograft (~3x3 mm) were aseptically prepared for the inoculation. The tissue fragment was subcutaneously transplanted into the dorsum of ether-anesthetized SCID mice using a trocar needle. Two fragments per mouse were inoculated separately into the dorsum to form two tumors. The transplanted tumors were measured (length and width) with sliding calipers 3 times weekly by the same observer and the tumor weight was calculated from the measurements obtained, using the formula (18):

$$\text{Tumor weight (mg)} = \text{length (mm)} \times [\text{width (mm)}]^2 / 2.$$

The mice were randomized into 4 groups (control and test groups) when the estimated tumor weight reached ~100-300 mg. The growth curves were generated by plotting the mean tumor weight (mg) against the treatment duration (days).

Treatment. The mice were stratified into 4 groups of 5 mice each, including: 1) control (no treatment), 2) docetaxel monotherapy, 3) S-1 monotherapy, 4) combination of S-1 and docetaxel. Docetaxel was prepared using appended solvent and the maximum tolerated dose of docetaxel (25 mg/kg) was administered intravenously on days 0, 7, 14 and 21. The administration of S-1 was completed with the effective dose (8.3 mg/kg) orally once daily on day 0-4, 7-11, 14-18 and 21-25. The schedule showed above was performed according to previously used regimens (16), and the administration schedule is derived from a clinical schedule previously reported (19,20).

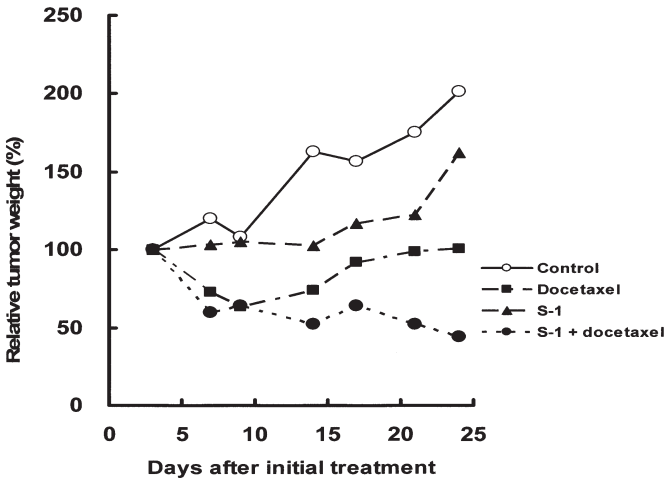

Figure 1. Antitumor activity of the agents against the growth of the xenograft. Treatment with docetaxel alone and with in S-1 showed substantial growth inhibition, while treatment with S-1 demonstrated modest change in tumor growth. Values are the mean of relative tumor weights. ○, control; ■, docetaxel alone; ▲, S-1 alone; ●, combination with docetaxel plus S-1.

Evaluation. The effects of the agents on the tumor-growth curve were evaluated in terms of the lowest T/C value (%) during the experiment, where T is the relative mean tumor weight of the treated group and C the relative tumor weight of the control group at any given time. The growth kinetic antitumor activity was evaluated as positive when the lowest T/C was <42%, which was calculated from $(0.75)^3$, corresponding to a 25% reduction of each diameter.

The mice were sacrificed on day 28 after the initial treatment. The spleen was excised and weighed to investigate any adverse effect of the agent. The inoculated tumors were also excised and the effect of the agents on their tumor growth was evaluated in terms of the T/C value (%), where T is the actual tumor weight of the treated group and C the actual tumor weight of the control.

Assays of enzyme activity. The TS and DPD activities of the tumors were also investigated using radio-assay, as described (21,22).

Briefly, the tumors resected from the mouse dorsum were thawed at 4˚C and placed in a 4-fold excess of 0.2 M Tris-HCl buffer, pH 7.4, containing 20 mM 2-mercaptoethanol and the phosphatase inhibitor cytidylate (15 mM) and NaF (100 mM). The tissues were disrupted by use of a ground-glass hand homogenizer. Aliquots of the crude sonicates were removed for 105,000 x g centrifugation and for the TS assay, the cytosol supernatant (175 μl), 1 M acetic acid extraction of nucleotides (300 μl) were added. The TS levels were assayed by the addition of 6 pmol of [6-^{3}H]FdUMP (18 Ci/mmol) in 50 μl of 5 mM potassium phosphate buffer, pH 7.4, plus 25 μl of

Table I. Growth kinetics with the NCI protocol and actual tumor weight.

	T/C (%)[a]	On day	Mean ± SD (mg)[c]	T/C (%)[c]	p-value[c]
Control			2908±537	100	
Docetaxel	45.3	14	1268±724	43.6	<0.001
S-1	63.1	14	2938±833	101	NS
Docetaxel + S-1	29.8[b]	21	407±624	13.9	<0.001

[a]NCI protocol with tumor growth; T/C = minimum, T relative weight/C relative weight. [b]Positive effect for antitumor activity, [c]data are derived from actual tumor weights.

Table II. Enzyme activities of the xenografts.

	DPD	p-value[c]	TS	p-value[c]
Control	462.03±83.8[a]		26.6±8.0[b]	
Docetaxel	349.33±95.5	<0.05	25.3±5.0	NS
S-1	256.44±97.9	<0.05	37.1±12.0	NS
Docetaxel + S-1	198.41±172.8	<0.05	20.5±9.0	NS

[a]pmol/min/mg protein; [b]fmol/min/mg protein; [c]p-value vs. control. DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase. Statistical analysis of DPD activities between docetaxel and combination, S-1, and combination revealed no significant change, NS.

cofactor solution. The results are shown as bound contents of [^{3}H]FdUMP to TS via reduced folate to TS. The DPD activity was determined as the sum of the degeneration products from [6-^{14}C]-5-FU by a modification of the method of Naguib *et al* (22).

Statistical analysis. The statistical analysis was performed using the Student's t-test and the Chi-square test. $p<0.05$ was considered to be significant.

Results

The growth kinetics of the MDA-MB-435SHM xenotransplants are presented in Fig. 1. The treatment with docetaxel alone and the combination with docetaxel plus S-1 resulted in substantial growth inhibition, while the treatment with S-1 demonstrated only a modest change in the growth of the MDA-MB-435SHM xenografts. The *in vivo* growth modulation of the tumors was also assessed by determining the % T/C values of growth kinetics after treatment with S-1, docetaxel and their combination (Table I). In the recipients treated with docetaxel, S-1 and their combination, the T/C values decreased by 45.3, 63.1 and 29.8%, respectively. In the recipients treated with a combination of the two agents, a significant suppression of tumor growth with a T/C value <42% was observed. A comparison of the T/C ratio derived from the actual tumor weight, assessed by resection of the tumor xenograft also indicated the advantage of combination therapy (Table I). In the recipients receiving docetaxel alone or a combination of the two agents, the T/C values decreased by 43.6% ($p<0.001$) and 13.9% ($p<0.001$), respectively; while in the recipients receiving S-1 monotherapy, the T/C value was not significantly different from those of the control. Furthermore, the product of T/C values of the two monotherapy groups equals 44% (0.436x1.01), which is greater than that of combination-treated group (13.9%). Since S-1 showed no inhibitory effect in terms of the weight of the xenograft, the combination therapy can be considered to synergistically affect the tumor growth property.

The DPD activities in the control, docetaxel-treated, TS-1-treated and combination-treated tumors were 462.03, 349.33, 256.44 and 198.41 pmol/min/mg/protein, respectively (Table II). These results indicate a significant down-regulation of tumor DPD activity by these agents. In particular, the tumors treated with the combination demonstrated an inhibition of DPD activity. However, the TS activities of the xenografts were not significantly changed from control after any of the treatments (Table II).

Adverse effects of the agents were shown only in the combination group (Table III). The total body and excised spleen weight gain of the recipients from the combination-treated group differed by >5% of that of control, indicating moderate treatment-related toxicity. In addition, one mouse

Table III. Adverse effects on body weight and actual spleen weight.

	Day 3 weight in g	Day 7 weight in g	Day 14 weight in g	Day 21 weight in g	Mean ± SD[b]	p-value[c]
Control	22.8	24.0 (△ 5.2%)[a]	25.8 (△ 13.1%)	26.4 (△ 15.7%)	186±28.8	
Docetaxel	21.2	21.2	22.2 (△ 4.7%)	22.5 (△ 6.1%)	150±46.9	NS
S-1	22.2	23.2 (△ 4.5%)	24.2 (△ 9.0%)	24.2 (△ 9.0%)	186±45.0	NS
Docetaxel + S-1	21.5	20.2 (▼ 6.0%)[a]	18.5 (▼ 13.9%)	19.7 (▼ 8.3%)	140±34.6	<0.05

[a]△, percent volume of weight increase; ▼, percent volume of weight gain. [b]Actual spleen weight (mg), [c]spleen weight p-value vs. control.

treated with combination died on day 24 after the initial treatment.

Discussion

This study was aimed at clarifying whether the combination therapy with S-1 and docetaxel is effective for the treatment of breast cancer, using the *in vivo* SCID mouse system as a model. Docetaxel, a potent antitumor agent, has been widely used, and the clinical advantage for breast cancer patients has also been fully confirmed (8-10). In addition, recent clinical reports focused on the efficacy of the docetaxel and oral fluoropyrimidine combination (3,7). Of the selected test compounds, S-1, a new oral fluoropyrimidine, demonstrates both potent anti-metabolite and DPD-inhibiting properties due to the effects of tegafur and gimeracil, respectively. DPD, a catabolizing enzyme of 5-FU has been demonstrated to play an important role in the antitumor effect against several solid tumor types (23). The results of our previous study also suggest that the combination with S-1 and paclitaxel has potently higher antitumor, and antimetastatic properties *in vivo* (16). A clinical study of S-1 plus docetaxel combination therapy with breast cancer patients has not been assessed to date.

For gastric cancer in Japan, the clinical investigation of the combination of S-1 and docetaxel was reported for a Phase I registration study, showing a significant effect of the metastatic site, with a response rate (RR) of 71.4% (7). This RR is significantly higher than that of S-1 and docetaxel-monotherapy (40 and 20%, respectively) (24-29). The data shown above suggest that the combination of S-1 and docetaxel has a promising property for the treatment of pre-treated metastases. In addition, the RR of docetaxel-monotherapy in MBC is reported to be 55.3-67.7% (30), indicating higher RR than for gastric cancer.

The kinetic curve for the combination with S-1 and docetaxel (Fig. 1) clearly demonstrates that the combination of the 2 tumor-suppressing agents is effective in suppressing the tumor growth. The T/C ratio derived from tumor growth kinetics also decreased especially in the combination group. These *in vivo* observations corroborate those reported by Nukatsuka *et al* (16), and, therefore, demonstrate significant antitumor advantage of treatment with S-1 and taxanes. It is also clear from the data presented in Table I that the combination of 2 tumor-suppressing agents strongly affects the actual tumor weight, compared to that of monotherapy groups.

The administration of S-1, with the maximum tolerated dose, demonstrated no inhibitory effect for tumor growth. Although the reason for this lack of tumor suppression is unclear, it is well known that the distribution volume related to the 5-FU metabolic pathway is very small in the nude mouse (athymic mouse) system (31); thus, a similar distribution may be observed in the degeneration of S-1 in SCID mice. Takahashi *et al* reported that 5-FU plasma levels in rats did not change by the simultaneous intravenous administration of docetaxel with oral S-1 (32). Since the tumoral drug concentration of 5-FU after administration of S-1 is closely related to plasma drug concentration, the 5-FU levels in breast tumors used in this study were considered to be almost similar between S-1 alone and S-1 combined with docetaxel.

The induction of the suppression of tumor weight in this experiment, however, was derived from the combination with moderately effective and ineffective agents. The effects of the individual agents on tumor growth, taken together with the moderate and ineffective agents, suggest that the observed anti-proliferative activity may be synergistic. The synergistic effect of the 5-FU and docetaxel combination has been already reported using mouse mammary epithelial cells both *in vitro* and *in vivo* (33). However, the mechanism of synergism of these compounds is not fully understood. 5-FU affects the 'arrest' of cell proliferation by blocking the G2 and S phases of the cell cycle. In addition, the G2/M phase is affected by taxanes. With the manifestation of different mechanisms of action of the individual agents, an additive (not synergistic) modulation might be expected when combination therapy is implemented.

The adverse effects of the experimental therapies were observed and considered to be similar with those of clinical studies (7). The spleen and body weight loss of mice during the experiments may indicate hematological and non-hematological toxicities.

The DPD activity of primary breast cancers has been reported to be higher than in other cancers (34). In addition, the DPD activity in metastatic tumors has been reported to be higher than that of primary tumors or normal tissue in colorectal and breast cancer patients (35,36). In the *in vivo* mouse system, however, the tumor xenograft DPD activity is generally lower than that of clinical tumors (37). The MDA-MB-435SHM xenograft used for our experiment, has been confirmed to have higher DPD activity than other human tumor xenografts (16). The results of the enzyme activity assay (Table II) clearly demonstrate that the tumor-suppressing agents are effective for down-regulation of DPD, while no significant enhancement in terms of TS activity was observed. It is interesting to note that the modulation of tumor growth *in vivo* strongly correlated with a similar modulation (down-regulation) of DPD activity. It is also noteworthy that the combination treatment caused the greatest down-regulation of DPD activity. Unlike S-1, docetaxel is not a DPD-inhibitory compound, and thus, would not be expected to affect a significant influence on DPD activity. DPD alteration in malignant tumors induced by docetaxel has not been previously reported, with the exception of one report of the investigation of the induction of DPD expression by docetaxel in gastric cancer. However, the data resulted in no induction of DPD expression (38). Our results regarding DPD activity, however, demonstrate a significant down-regulation upon the administration of docetaxel. Thus, the modulation seen in the combination of S-1 and docetaxel on DPD activity is largely dependent upon the additive reaction led by two individual DPD-inhibitory agents. Therefore, it is conceivable that synergistic growth regulation of xenotransplanted breast cancer by the combination of S-1 and docetaxel may be a manifestation of the combined strong down-regulation of DPD activity. The synergistic effect of combination therapy on tumor growth may partly be due to the additive DPD inhibition.

In conclusion, the present study on breast cancer xenografts has shown that tumor growth kinetics and DPD activity of the tumor can be down-regulated by agents that are known to suppress tumor growth in clinical studies. The down-regulation of DPD activity of tumor cells might be a key parameter in tumor suppression. Although adverse effects are present, the combination of S-1 and docetaxel is a promising candidate in the treatment of MBC.

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