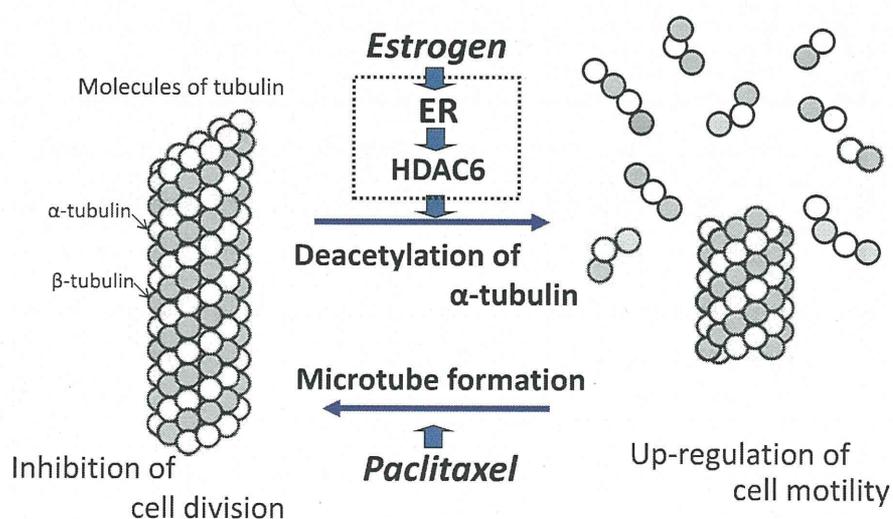**Fig. 5** Paclitaxel and estrogen show the opposite effects on tubulin assembly. Paclitaxel inhibits the proliferation of tumor cells via induction of microtubule formation, whereas estrogen induces deacetylation of α-tubulin via HDAC6. Our previous study has showed that deacetylation of α-tubulin causes up-regulation of cell motility [27]

tubulin involving HDAC6. These findings provide a new understanding of the mechanisms underlying the roles of the ER and chemotherapeutic agents such as paclitaxel, as well as insights into developing new molecular targets for breast cancer treatment.

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Antitumor activity of chemoendocrine therapy in premenopausal and postmenopausal models with human breast cancer xenografts

MOTOYUKI KATAOKA[1], YURI YAMAGUCHI[2], YOICHIRO MORIYA[1], NORIAKI SAWADA[1], HIDEYUKI YASUNO[1], KUMIKO KONDOH[1], DEAN B. EVANS[3], KAZUSHIGE MORI[1] and SHIN-ICHI HAYASHI[4]

[1]Product Research Department, Kamakura Research Laboratories, Chugai Pharmaceutical Co., Ltd., Kamakura 247-8530; [2]Research Institute for Clinical Oncology, Saitama Cancer Center, Saitama 362-0806, Japan; [3]Oncology Research, Novartis Institutes for BioMedical Research, Basel, Switzerland; [4]Center for Regulatory Epigenome and Diseases, Molecular and Functional Dynamics, Graduate School of Medicine, Tohoku University, Sendai 980-8575, Japan

Abstract. We examined the efficacy of chemoendocrine therapy using capecitabine as a chemotherapeutic agent in premenopausal and postmenopausal models with estrogen receptor (ER)-positive human breast cancer xenografts. Tamoxifen and letrozole were used as endocrine therapeutic agents for premenopausal and postmenopausal models, respectively. The antitumor activity of capecitabine in combination was significantly superior to either monotherapy treatment in both premenopausal ($p<0.01$) and postmenopausal ($p<0.05$) models. No increase in toxicity in terms of body weight loss was observed during treatment in either of the xenograft models. In the premenopausal model, the level of thymidine phosphorylase (TP), a key enzyme generating 5-FU from capecitabine, was upregulated ($p<0.05$) in tumors by tamoxifen but not by letrozole treatment in the postmenopausal model. The combination of 5'-deoxy-5-fluorouridine (5'-DFUR; an intermediate of capecitabine) with 4-hydroxytamoxifen (4-OHT; an active form of tamoxifen) or letrozole was also evaluated *in vitro* by using estrogen-responsive element (ERE) reporter gene assays aimed to model premenopausal and postmenopausal breast cancer. Both combinations decreased the number of estrogen-responding cells in a concentration-dependent manner and further analysis by isobolograms revealed a synergistic effect of the combination of 5'-DFUR with 4-OHT, and at least an additive effect of the combination of 5'-DFUR with letrozole. These results suggest that chemoendocrine therapy using capecitabine may be a useful treatment modality for patients with hormone-receptor-positive breast cancer, regardless of the menopausal status and should be explored in clinical trials.

Correspondence to: Dr Yoichiro Moriya, Product Research Department, Kamakura Research Laboratories, Chugai Pharmaceutical Co. Ltd., 200 Kajiwara, Kamakura 247-8530, Japan
E-mail: moriyayui@chugai-pharm.co.jp

Introduction

Two decades ago, *in vitro* antagonism in cytotoxicity was reported between chemotherapeutic agents and antiestrogens in breast cancer cells (1). This observation translated to the clinic as results with chemoendocrine therapies in patients have been disappointing (2,3). However, not all reports of addition of chemotherapies, such as CMF [a chemotherapy regimen consisting of cyclophosphamide, methotrexate and 5-fluorouracil (5-FU)], to endocrine therapies showed negative results. For example, clinical results from NSABP B-20 trial (4) and the International Breast Cancer Study Group (IBCSG) trial VII (5) indicated that the addition of CMF to tamoxifen yielded superior five-year disease-free survival rate. Therefore, it is likely that some chemotherapeutic agents may not be antagonistic to antiestrogens.

Capecitabine is an oral 5-FU derivative widely used for breast cancers which generates the active substance 5-FU in tumors by a three-step cascade of enzymes located in the liver and tumors. The final step is the conversion of 5'-DFUR, an intermediate metabolite, to 5-FU by TP, which is highly expressed in tumors. Therefore, a higher level of 5-FU would be expected in tumor tissues which have higher expression levels of TP, when treated with capecitabine. Indeed, it has been reported that the antitumor activity of capecitabine did correlate with tumor levels of TP activity in xenograft models, whereas that of 5-FU did not (6).

Estrogen is known to play a crucial role in estrogen receptor (ER)-positive breast cancer development and growth through binding to ER. It is reported that 71% of invasive breast cancer is ER-positive (7). Antiestrogen therapies are an indispensable treatment modality for almost all ER-positive breast cancer patients. Tamoxifen, an estrogen antagonist, was widely used for the treatment of ER-positive breast cancer patients with premenopausal, as well as postmenopausal, status. It was reported in a P-1 study that tamoxifen reduced the cumulative rate of invasive breast cancer (8), and that it reduced the incidence of contralateral breast cancer in premenopausal patients (9).

In postmenopausal women, the blood estrogen levels decrease because the ovaries cease to produce estrogen. However, estrogen level in breast cancer is still 10 times higher

than that in normal breast tissue (10,11) because estrogen is supplied through the conversion of androgen by aromatase, a key enzyme of estrogen synthesis. In addition, aromatase is known to be highly expressed in the adipose stromal cells adjacent to breast cancer cells (12). Thus, the locally produced estrogen promotes the growth of ER-positive tumor cells in postmenopausal breast cancer patients. Letrozole, an aromatase inhibitor, improved disease-free survival and time to distant recurrence compared to tamoxifen in postmenopausal ER-positive breast cancer patients (BIG 1-98 study) (13,14).

In the present study, we examined the antitumor activity of capecitabine in combination with endocrine therapies in both premenopausal and postmenopausal breast cancer xenograft models. We also examined the effect of the combination capecitabine plus antiestrogens in *in vitro* ERE reporter gene assays (15,16).

Materials and methods

Chemicals. Capecitabine and 5'-DFUR were synthesized at Hoffmann-La Roche (Basel, Switzerland). Tamoxifen citrate, 4-hydroxytamoxifen (4-OHT) and androstenedione were purchased from Sigma-Aldrich Co. (St. Louis, MO). Letrozole was obtained from Novartis Pharmaceutical Co., Ltd.

For *in vivo* study, capecitabine was dissolved in 40 mmol/l citrate buffer, pH 6.0 containing 5% gum arabic. Tamoxifen was dissolved in the same buffer as capecitabine solution. Letrozole was dissolved in dimethyl sulfoxide, and further diluted with 0.5% aqueous solution of sodium carboxymethylcellulose containing 20% propylene glycol. For *in vitro* study, 5'-DFUR was dissolved in phosphate-buffered saline (10 mmol/l). 4-OHT was dissolved in ethanol (1 mmol/l), and further diluted with phosphate-buffered saline, pH 7.4. Letrozole was dissolved in the same manner as in the *in vivo* study.

Animals. Female 4-5-week-old BALB/c-nu/nu mice [CAnN. Cg-Foxn1(nu)/CrlCrlj nu/nu] were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All mice were housed in a pathogen-free environment under controlled conditions (temperature 20-26°C, humidity 40-70%, light/dark cycle 12 h/12 h). Chlorinated water and irradiated food (CE-2; Clea Japan, Inc., Tokyo, Japan) were provided *ad libitum*. All mice were allowed to acclimatize and recover from shipping-related stress for at least 1 week prior to the study. The health of the mice was monitored by daily observation. The protocol was reviewed by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd. and all mouse experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals promulgated in Chugai Pharmaceutical Co., Ltd.

Cell lines and culture conditions. Estrogen receptor-positive MCF-7 human breast cancer cell line was kindly provided by Dr Y. Iino (Gunma University, Maebashi, Japan) and was maintained in Eagle's minimum essential medium (MEM; Sigma-Aldrich Co.) supplemented with 0.1 mmol/l MEM non-essential amino acids (Invitrogen Corp., Carlsbad, CA), 1 mmol/l sodium pyruvate (Invitrogen Corp.) and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermo Trace Ltd., Victoria, Australia).

Preparation of aromatase transfectant. The aromatase gene transfectant cell line MCF-7A25F3 was established according to the method of Zhou *et al* (17). Briefly, the first-strand cDNA synthesis was carried out with 5 μg of total RNA from human placenta (Toyobo Co., Ltd., Osaka, Japan), 100 pmoles of oligo (dT) and 200 units of SuperScript II (Invitrogen Corp.). The target cDNA for aromatase gene was obtained by the polymerase chain reaction. The sequences of the primer were as follows: forward; 5'-GCT CTA GAG GAA CAC AAG ATG GTT TTG GAA-3' containing *Xba*I restriction site for cloning, reverse; 5'-GCT CTA GAT GGG TAC TGA CCA GCC TTC T-3'. The PCR product was digested with *Xba*I and then was extracted from the gel. The cDNA fragment was inserted into the *Xba*I and *Sma*I site of the mammalian expression vector pCI-Neo (Promega Co., Ltd., WI). The plasmid DNA was purified using DNA miniprep column (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction and then sequenced.

This sequence-confirmed construct was used for the transfection to generate the MCF-7 cell line expressing aromatase gene. To establish a stably transfected cell line, 1 μg of plasmid DNA was incubated with 0.5 ml of Lipofectamine (Clontech Laboratories Inc., CA) for 30 min and overlaid onto 1×10^6 MCF-7 cells for 1 h in serum-free medium. Cells were then selected in the presence of 350 μg/ml Geneticin (G-418; Calbiochem Co., Ltd., San Diego, CA). After selection for 2 weeks, the cells were screened for aromatase cDNA in genomic DNA by PCR. The ratio of copy numbers of aromatase cDNA in the parent MCF-7 cells and in the transfected cells were estimated. Primers for quantification were as follows: forward; 5'-GGA AAT GCT GAA CCC GAT AC-3', reverse; 5'-GAG AAA AAG GCC AGT GAG GA-3' for aromatase, and forward; 5'-GGT TGG CCA ATC TAC TCC CAG G-3', reverse; 5'-TGG TCT CCT TAA ACC TGT CTT G-3' for β-globin. The copy number of the aromatase gene of the transfected cells as a whole was 2-fold higher than that of the parent MCF-7 cells. The cloning of transfected cells was performed and clones revealing a high proliferation response to androstenedione were selected. Next, the clones were inoculated into ovariectomized BALB/c-nu/nu mice which had been implanted with a slow-release androstenedione pellet to determine the tumor growth in response to the hormone *in vivo*.

Measurement of aromatase activity. Aromatase activity was determined by using a tritiated water (^3H_2O) method in which the ^3H_2O released from 1β-^{3}H-androstenedione was measured. The measurement was conducted by Teikoku Hormone Medical Co., Ltd. (Kawasaki, Japan). Briefly, the collected MCF-7A25F3 cells were homogenized and centrifuged at 200 x g for 3 min. The supernatant (0.5 ml) was incubated with 1β-^{3}H-androstenedione and NADPH at 37°C for 60 min. The incubation was terminated by cooling, and the reaction mixture was extracted with 2 ml of ice-chilled chloroform. The aqueous layer was removed and mixed with 100 mg of activated charcoal. The mixture was vortexed well and centrifuged at 1,500 x g for 10 min at 4°C to remove the charcoal. An aliquot (0.5 ml) of the supernatant was added to 8 ml of Scintisol fluid (Dojin Chemical Co., Kumamoto, Japan) in scintillation vials, vortexed, and measured for radioactivity. Blank values were subtracted, and the aromatase activity was expressed as fmol

^3H_2O released/min/mg protein. Protein concentration of the supernatant of the cell homogenate was determined by BCA Protein Assay Kit (Takara Bio Inc., Otsu, Japan).

Human breast cancer xenograft models. The premenopausal breast cancer xenograft model was established as follows. A suspension of MCF-7 cells ($5x10^6$ cells/mouse) was subcutaneously inoculated into the right flank of the BALB/c-nu/nu mice which had been subcutaneously implanted with slow-release estrogen pellets (0.25 mg/pellet 17β-estradiol; Innovative Research of America, Sarasota, FL) the day before tumor cell inoculation. Several weeks after tumor inoculation, mice bearing a tumor of ~200-400 mm^3 in volume were selected and were randomly allocated to control and treatment groups. Each group consisted of 8 mice. The mice received 6-week oral therapy with capecitabine (days 1-14, every 3 weeks) at maximum tolerated dose (MTD; 539 mg/kg/day) or two thirds MTD (359 mg/kg/day) and/or tamoxifen daily for 6 weeks at doses of 30 or 100 mg/kg/day.

The postmenopausal breast cancer xenograft model was established as follows. BALB/c-nu/nu mice were ovariectomized and subcutaneously implanted with slow-release androstenedione pellets (1.5 mg/pellet; Innovative Research of America) the day before tumor cell inoculation. MCF-7A25F3 cells ($5x10^6$ cells/mouse) were subcutaneously inoculated into the right flank of the mice. Mice bearing a tumor of ~200-400 mm^3 in volume were selected and were randomly allocated to control and treatment groups of 5 mice each. The mice received 6-week oral administration of capecitabine (days 1-14, every 3 weeks) at two thirds MTD and/or letrozole, at 0.1 mg/kg/day. In each model, control mice received vehicle alone.

Tumor volumes (V) were estimated from the equation $V = ab^2/2$, where a and b are the length and width of the tumor, respectively. Tumor volumes and body weights were monitored two or three times a week starting from the first day of the treatment.

Measurement of thymidine phosphorylase (TP). The tumor tissues were homogenized with a glass homogenizer in 10 mmol/l Tris buffer (pH 7.4) containing 15 mmol/l NaCl, 1.5 mmol/l $MgCl_2$ and 50 μmol/l potassium phosphate. The homogenates were then centrifuged at 10,000 x g for 20 min at 4°C, and the supernatants were stored at -80°C until use. The TP level was measured by ELISA using monoclonal antibodies specific to human TP as described previously by Nishida *et al* (18). One unit corresponds to the TP level of the standard enzyme solution (extracts of human colon cancer HCT116 xenograft) which converts 5'-DFUR to 5-FU at a rate of 1 μg 5-FU/h (18). The protein concentration was determined using DC protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Preparation of estrogen-responsive element-green fluorescence protein transfected cell line, MCF-7E10. The procedure for preparation of estrogen-responsive element-green fluorescence protein (ERE-GFP)-transfected cell line was described previously (16). Briefly, MCF-7 cells were transfected with the d2E-green fluorescent protein (GFP) vector alone (Clontech Lab., Inc., Palo Alto, CA) or carrying the ptk-estrogen-responsive element (ERE) insert using Trans IT LT-1 reagent (Takara Shuzo Co., Ltd., Tokyo, Japan) according to the manufacturer's

instructions. After 24 h, the cells were subjected to selection in growth medium containing geneticin (1 mg/ml). The ERE-GFP-MCF-7 clone that expressed high levels of GFP in the presence of estrogen, but not in the absence of estrogen, was selected (MCF-7E10).

Isolation of primary stromal cells from tumors and preparation of breast cancer extract. To obtain breast cancer extract, tissue specimens obtained from three premenopausal patients were minced in phenol red-free RPMI-1640 medium (PRF-RPMI; Sigma-Aldrich Co.) and suspended. The suspension was centrifuged (600 x g, 10 min, 4°C) and the supernatant was further subjected to centrifugation (12,000 x g, 10 min, 4°C) to obtain breast cancer extract. The isolation procedure of cancer stromal cells is similar to that described by Ackerman *et al* (19). The breast cancer tissue specimen obtained from a postmenopausal patient was minced in PRF-RPMI and suspended. The suspension was centrifuged (600 x g, 10 min, 4°C) and the pellet was washed with Hanks' balanced salt solution (HBSS: Sigma-Aldrich Co.) and treated with the mixture of collagenase (1 mg/ml: Nitta Gelatin Co. Ltd., Osaka Japan), bovine serum albumin (40 mg/ml: Sigma-Aldrich Co.), glucose (2 mg/ml: Wako Pure Chemical Industries, Osaka, Japan), antibiotic-antimycotic (Invitrogen, Corp.) and gentamicin (50 μg/ml: Schering-Plough, NJ) for 2-3 h at 37°C followed by filtration through nylon mesh. The cells, including stromal cells, were recovered by centrifugation (600 x g, 10 min, 4°C) and washed several times with HBSS. The cells were suspended in MEM-α medium (Invitrogen, Corp.) containing 10% FBS (Tissue Culture Biologicals, Turale, CA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

The study was approved by the Saitama Cancer Center (Saitama, Japan) Ethics Committee. Human breast cancer tissues were obtained from surgical specimens at the Saitama Cancer Center Hospital after obtaining informed consent from the patients.

Breast cancer extract ERE reporter gene assay and coculture ERE reporter gene assay. The breast cancer extract ERE reporter gene assays were carried out according to the method of Yamaguchi *et al* (20). The MCF-7E10 cells were cultured with 5% (v/v) breast cancer tissue extract for 4 days in the presence of 5'-DFUR and/or 4-OHT. The cells were collected by mild trypsinization, and the number of cells expressing GFP was counted under fluorescence microscopy (16).

Coculture ERE reporter gene assays were performed as previously described (16). Briefly, MCF-7E10 cells and the stromal cells were precultured in PRF-RPMI containing 10% dextran-coated, charcoal-treated FBS (DCC-FBS; Hyclone Lab., UT) for 72 h. Harvested stromal cells were seeded at $5x10^4$/ml in a 24-well multidish, and the next day, $5x10^4$ MCF-7E10 cells were seeded on top of the adipose stromal cells with testosterone at 10^{-7} mol/l as a substrate for aromatase. After being cocultured for 4 days in the presence of 5'-DFUR, an intermediate of capecitabine, and/or letrozole, the cells were collected by mild trypsinization, and the number of cells expressing GFP was counted under fluorescence microscopy. Data are expressed as a percentage of cells expressing GFP. The isobole method was performed in each experiment to analyze the mode of interaction between capecitabine and
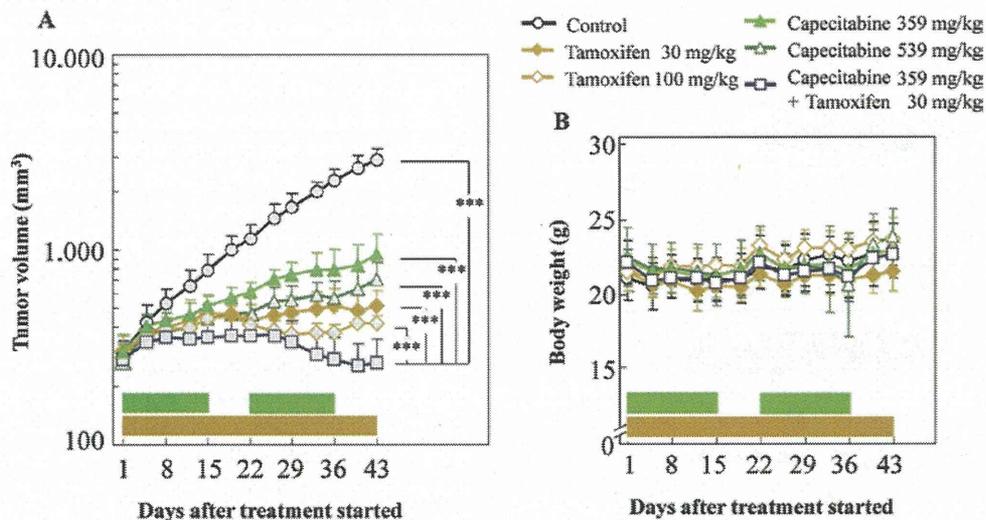
Figure 1. Antitumor activity of combination therapy of capecitabine with tamoxifen in a premenopausal breast cancer xenograft model. Mice were randomized into 6 groups of 8 mice each. Capecitabine (359 or 539 mg/kg) was orally administered once a day from day 1 to day 14 and day 22 to day 35 (green horizontal bars). Tamoxifen (30 or 100 mg/kg) was orally administered once a day for 6 weeks (brown horizontal bar). Data points indicate mean ± SD of tumor volume (A) and mean ± SD of body weight (B). Statistically significant differences versus capecitabine 359 mg/kg + tamoxifen 30 mg/kg group are shown with asterisks: ***p<0.001 by Wilcoxon test.
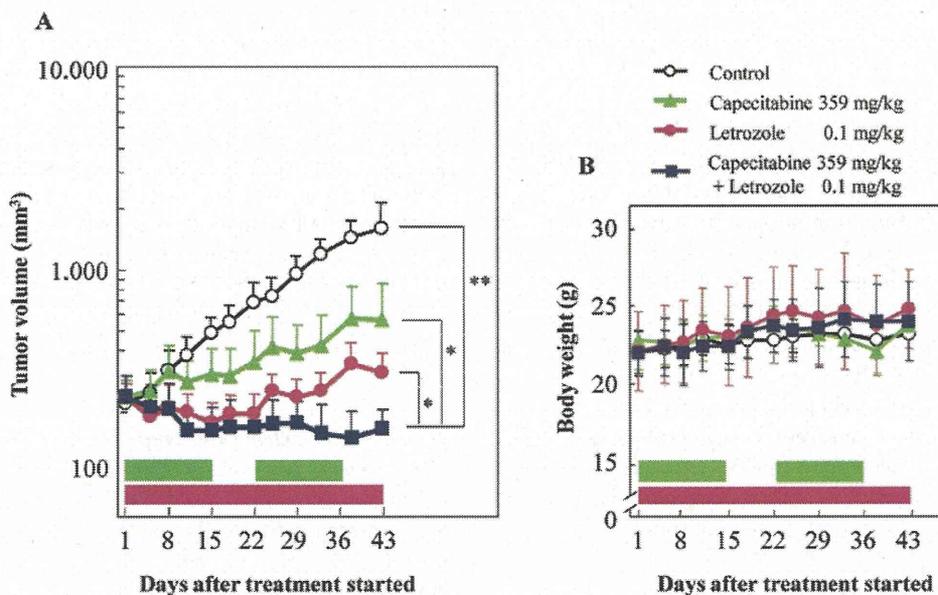

Figure 2. Antitumor activity of combination therapy of capecitabine with letrozole in a postmenopausal breast cancer xenograft model. Mice were randomized into 4 groups of 5 mice each. Capecitabine (359 mg/kg) was orally administered once a day from day 1 to day 14 and day 22 to day 35 (green horizontal bars). Letrozole (0.1 mg/kg) was orally administered once a day for 6 weeks (purple horizontal bar). Data points indicate mean ± SD of tumor volume (A) and mean ± SD of body weight (B). Statistically significant differences versus capecitabine 359 mg/kg + letrozole 0.1 mg/kg group are shown with asterisks: *p<0.05, **p<0.01 by Wilcoxon test.

4-OHT/letrozole according to the method of Berenbaum MC (21).

Statistical analysis. The Wilcoxon test was used to detect the statistical differences in tumor volume, body weight and TP level. Statistical analyses were carried out using the SAS preclinical package (version 5.0; SAS Institute Inc., Tokyo, Japan). Differences were considered to be significant at p<0.05.

Results

Establishment of MCF-7 aromatase-transfectant clone. Three aromatase-transfectant clones were selected by *in vitro* screening. The 3 clones were inoculated into ovariectomized BALB/c-nu/nu mice which had been implanted with slow-release estradiol or androstenedione pellets to determine the tumor growth in response to each hormone *in vivo*. The clone, MCF-7A25F3,
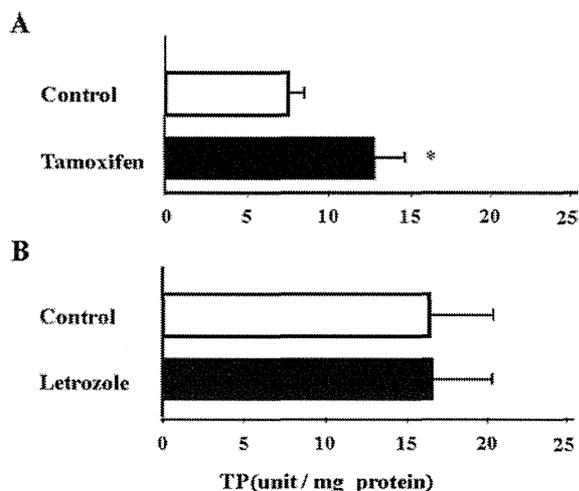
A

B

TP(unit / mg protein)

Figure 3. TP activity in tumor tissue after tamoxifen or letrozole treatment. Mice inoculated with MCF-7 or MCF-7A25F3 cells were randomly assorted to groups of 4 or 5 mice each. Tamoxifen (A) or letrozole (B) was orally administered once a day for 3 weeks and tumor tissues were excised on day 22. TP levels in tumor tissues were measured by ELISA. Data bars indicate mean ± SD. Statistically significant differences versus control group are shown with asterisks: *p<0.05 by Wilcoxon test.

which showed the highest tumor growth was selected (data not shown). The MCF-7A25F3 cells were maintained in the same medium as MCF-7 cells supplemented with 10% DCC-FBS and 350 µg/ml geneticin. The tumor of parent MCF-7 cells grew in BALB/c-nu/nu mice with estrogen pellet but not in BALB/c nu/nu mice with androstenedione pellet (data not shown). The aromatase activity in MCF-7A25F3 cell homogenate was 420-fold higher (50.4 fmol/min/mg protein) than that of parent MCF-7 (0.12 fmol/minute/mg protein) measured by the ^3H_2O release from 1β-^3H-androstenedione. To confirm the androstenedione-dependent growth of MCF-7A25F3 cells, tumor growth inhibition by letrozole was examined *in vivo*. MCF-7A25F3 cells were inoculated into BALB/c nu/nu mice which had been ovariectomized and subcutaneously implanted with androstenedione pellets. BALB/c nu/nu mice received daily oral administration of 0.03-3 mg/kg of letrozole for 6 weeks. Letrozole significantly inhibited the tumor growth in a dose-dependent manner with the maximum effect at 1-3 mg/kg (data not shown).

Antitumor activity of capecitabine in combination with tamoxifen in a premenopausal xenograft model. The antitumor activity of capecitabine in combination with tamoxifen was evaluated using MCF-7 premenopausal breast cancer xenograft model. The dosages of these agents were determined by a preliminary dose-finding experiment (data not shown). To detect an interaction between the two agents distinctly, suboptimal doses of each drug were used in combination therapy. The MTD for capecitabine administered daily for 2 weeks in 3-week cycles in this model was determined as 539 mg/kg/day in a previous experiment (22,23). The daily oral administration of tamoxifen for 6 weeks showed maximum antitumor activity at a dose of 100 mg/kg. Therefore, in the present combination therapy experiments, 359 mg/kg (2/3 MTD) for capecitabine, and the dose of 30 mg/kg (1/3 of maximum effective dose) for tamoxifen were selected.

As shown in Fig. 1A, both capecitabine and tamoxifen showed only moderate antitumor activities at selected doses for each agent. In contrast, the antitumor activity of combination therapy was significantly superior to the antitumor activities of monotherapy of each agent (p<0.001). In addition, the combination therapy produced statistically significant inhibition of tumor growth compared with tamoxifen at 100 mg/kg (p<0.001) or capecitabine at 539 mg/kg in monotherapy. None of the treatment groups in this experiment showed significant weight loss during the treatments (Fig. 1B).

Antitumor activity of capecitabine in combination with letrozole in a postmenopausal xenograft model. The anti-tumor activity of capecitabine in combination with letrozole was evaluated in the postmenopausal breast cancer xenograft model using MCF-7A25F3 cells. To detect the interaction between the two agents distinctly, the dose of 0.1 mg/kg for letrozole, which was 1/30 of the maximum effective dose (data not shown) and the dose of 359 mg/kg (2/3 MTD) for capecitabine, were used for combination therapy as a suboptimal dose in each monotherapy. The combination therapy of capecitabine with letrozole showed significantly higher antitumor activity than each monotherapy (Fig. 2A). None of the treatment groups in this experiment showed significant weight loss during the treatments (Fig. 2B).

TP levels in the tumor tissues. Mice inoculated with MCF-7 or MCF-7A25F3 cells were randomly assorted into groups of 4 and 5 mice each, respectively. The TP levels in tumor tissues were measured 22 days after treatment started. The TP level of MCF-7 tumor was significantly increased (12.8±2.0 U/mg protein) by tamoxifen (30 mg/kg/day) treatment for 3 weeks compared with vehicle-treated group (7.6±1.1 U/mg protein) (Fig. 3A). On the other hand, the TP level of MCF-7A25F3 tumor was higher (16.4±3.8 U/mg protein) than that of MCF-7 parent tumor and no further upregulation of TP was observed by 0.1 mg/kg of letrozole treatment (16.6±3.8 U/mg protein) (Fig. 3B).

Breast cancer extract ERE reporter gene assay and coculture ERE reporter gene assay. The inhibitory effect of 5'-DFUR in combination with 4-OHT, an active form of tamoxifen, on estrogen-responding cells was evaluated using breast cancer extract ERE reporter gene assay (Fig. 4A). The percentage of GFP-positive cells was decreased by the addition of 5'-DFUR or 4-OHT in a concentration-dependent manner. The analysis by isobole method showed a synergistic effect of 5'-DFUR and 4-OHT (Fig. 4B).

The inhibitory effect of 5'-DFUR in combination with letrozole on estrogen-responding cells was evaluated using coculture ERE reporter gene assay (Fig. 4C). The percentage of GFP-positive cells was decreased by the addition of 5'-DFUR or letrozole in a concentration-dependent manner. The isobole analysis showed an additive effect of 5'-DFUR and letrozole (Fig. 4D).

Discussion

The combination effects of antiestrogens and chemotherapeutic agents have been controversial for many years. Specifically,
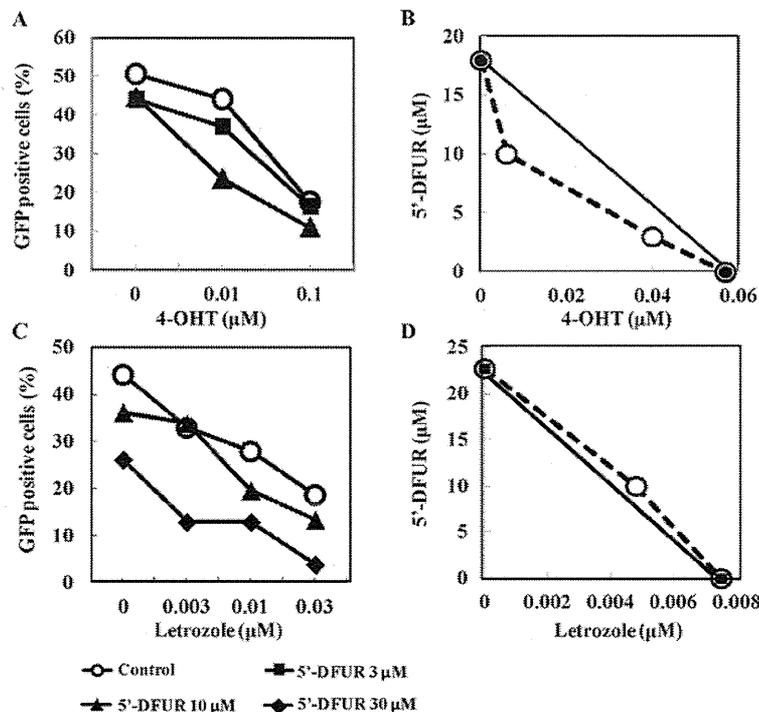
Figure 4. Combination effect of 5'-DFUR with 4-OHT, or 5'-DFUR with letrozole in ERE reporter gene assay. The MCF-7E10 cells were cultured with various concentrations of 5'-DFUR and 4-OHT in the presence of breast cancer extract for 4 days, and the number of cells expressing GFP was counted. Data were expressed as a percentage of GFP-expressing cells against the observed number of MCF-7E10 cells (A). Isobolograms of the interactions between 5'-DFUR and 4-OHT were taken at the point of 30% GFP-positive cells (B). The MCF-7E10 cells were cultured with various concentrations of 5'-DFUR and letrozole in the presence of breast cancer stromal cells for 4 days, and the number of cells expressing GFP was counted. Data were expressed as a percentage of GFP-expressing cells against the observed number of MCF-7E10 cells (C). Isobolograms of the interactions between 5'-DFUR and letrozole were taken at the point of 30% GFP-positive cells (D).

chemoendocrine combinations of tamoxifen with chemotherapy containing 5-FU revealed both positive and negative results. Fisher *et al* reported the prolongation of the disease-free interval in the therapy of tamoxifen with L-phenylalanine mustard and 5-FU in patients with primary operable breast cancer (4). The IBCSG Trial VII reported that early CMF added to tamoxifen significantly improved 5-year disease-free survival in post-menopausal breast cancer patients (5). On the other hand, Rivkin *et al* reported that combination treatment of CMFVP (cyclophosphamide, methotrexate, fluorouracil, vincristine, predonisone) chemotherapy with tamoxifen has not been shown to be superior to tamoxifen alone in the treatment of postmenopausal women with node-positive, ER-positive, operable breast cancer (2). Pritchard *et al* reported that there were no significant differences in overall survival, recurrence-free survival, locoregional recurrence-free survival and distant recurrence-free survival between the tamoxifen treatment group and tamoxifen plus CMF group in postmenopausal breast cancer patients (3). Perry *et al* reported that no difference was evident among postmenopausal patients in overall response rate and duration of responses between CAF (cyclophosphamide, adriamycin, 5-fluorouracil) and CAF plus tamoxifen, and that no difference of response rates was shown among premenopausal patients between CAF and CAF plus tamoxifen (24). However, in *in vitro*, a study on mammary carcinoma cell lines showed that the cytotoxic effects of cotreatment of 5'-DFUR with tamoxifen were dependent on

tamoxifen concentration and treatment duration (25). Since 5'-DFUR is known to be converted to 5-FU by TP and it was also reported that tamoxifen upregulated TP in breast cancer cells (26), data suggest that there is a mechanism for a potentially favorable outcome of chemoendocrine therapy with tamoxifen and such chemotherapeutic agents as were activated by TP.

In this study, we showed the antitumor efficacy of chemo-endocrine therapy using capecitabine as a chemotherapeutic agent, in premenopausal as well as postmenopausal breast cancer xenograft models. Capecitabine has been approved for breast cancer in many countries due to proven efficacy and tolerability in patients with metastatic breast cancer (27,28). Tamoxifen has been the most widely used anti-hormonal agent for many years in the first line treatment of ER-positive breast cancers, and has a profound impact on breast cancer mortality (8,9,29). Letrozole has been used for the breast cancers of postmenopausal women, and Mouridsen *et al* demonstrated the superiority of letrozole to tamoxifen in first-line endocrine therapy in postmenopausal breast cancer patients (30).

In the present study, we examined the antitumor efficacy of combination therapy of capecitabine with tamoxifen or with letrozole in premenopausal and postmenopausal status, respectively. For a premenopausal model, ER-positive MCF-7 human breast cancer cells were used. For a postmenopausal model, we prepared the MCF-7 aromatase transfectant clone, MCF-7A25F3. The feature of MCF-7A25F3 cells as aromatase

transfectants was confirmed by the estradiol- and androstene-dione-dependent growth *in vivo* and the increased (420-fold) aromatase activity in MCF-7A25F3 cells compared with parent MCF-7 cells.

Using the established premenopausal and postmenopausal human breast cancer xenograft models, we evaluated the antitumor efficacy of combination therapy of capecitabine with tamoxifen or letrozole. In each model, dose and schedule of capecitabine was determined according to the study of Sawada *et al* (22,23). To make the combination effect clear, MTD or sub-MTD of capecitabine and suboptimal dose of tamoxifen or letrozole were used according to the preliminary experiments (data not shown). In both models, combination therapy clearly showed the supra-additive antitumor activity to either monotherapy. In the premenopausal model, the combination therapy of capecitabine at 359 mg/kg (2/3 MTD) with tamoxifen at 30 mg/kg showed a significantly higher antitumor activity than each monotherapy (Fig. 1A). The combination therapy showed higher antitumor activity than MTD (539 mg/kg) of capecitabine or 100 mg/kg of tamoxifen. In the postmenopausal model, the combination therapy of capecitabine at 2/3 MTD with letrozole at 0.1 mg/kg showed significantly higher antitumor activity than either monotherapy (Fig. 2A). Concerning the therapeutic experiment of breast cancer aromatase transfectant with letrozole, Long *et al* reported that suppression of tumor growth was observed for at least 44 weeks in MCF-7Ca aromatase transfectant xenograft model in which letrozole was subcutaneously injected daily (10 μg/day) (31). However, we showed the antitumor effect of letrozole by oral administration, which is consistent with the clinical administration route. No augmentation of toxicity in terms of body weight loss was observed in the combination therapy in either model (Figs. 1B and 2B). The results indicate that both chemo-endocrine therapy regimens may be applicable to breast cancer patients.

Capecitabine generates the active substance 5-FU selectively in tumors by using three enzymes located in the liver and in tumors (22,23,32). The final step is the conversion of the intermediate metabolite 5'-DFUR to 5-FU by TP in tumors and this conversion appeared to be a rate-limiting step for the efficacy of capecitabine. Ishikawa *et al* reported that the efficacy of capecitabine correlated with TP activity in tumor tissues using human cancer xenograft models (6). TP is upregulated in tumors by receiving stress such as hypoxia, radiation and chemotherapeutic damage. Several types of cytokines such as interleukin-1, tumor necrosis factor-α, interferon-γ also upregulate the expression of TP in malignant cells (33). Endo *et al* have previously reported that several types of antitumor drugs, such as taxanes, cyclophosphamide and oxaliplatin, upregulated TP in tumor tissues and that capecitabine in combination with these TP upregulators showed synergistic antitumor activity in xenograft models (32). Ustunomiya *et al* reported the upregulation of TP in T47D breast cancer cells treated with tamoxifen *in vitro* (26) and also Evans *et al* reported the induction of hypoxia by tamoxifen in MCF-7 xenografts (34). These findings prompted us to examine the TP activity in the tumor tissue of mice treated with tamoxifen and letrozole. Consequently, we found that the TP activity in MCF-7 tumor tissue of mice treated with tamoxifen was significantly increased compared to that of control mice in the premenopausal model (Fig. 3A). Thus,

the increased efficacy of combination therapy of capecitabine with tamoxifen may, at least partly, be explained by the upregulation of TP by tamoxifen in tumor tissue facilitating the conversion of capecitabine to 5-FU. On the other hand, in the postmenopausal model, the increased efficacy of combination therapy of capecitabine with letrozole could not be explained by TP upregulation because no TP increase was observed by letrozole treatment, suggesting that mechanisms other than TP upregulation are involved in the efficacy of this combination therapy (Fig. 3B).

Next, we tried to analyze the effect of the combinations capecitabine with tamoxifen or capecitabine with letrozole *in vitro*. For this purpose, we established ERE reporter gene assay, to mimic premenopausal or postmenopausal breast cancer (16,20). For the combination effect of capecitabine with tamoxifen, MCF-7E10 cells were cultured with breast cancer extract in the presence of various concentrations of 5'-DFUR and 4-OHT, an active metabolite of tamoxifen (35). To make the assay system closer to the clinical conditions of premenopausal breast cancer, human breast cancer extracts were used as a source of estrogen and other unknown factors which may affect the ER signals and cell proliferation. Both 5'-DFUR and 4-OHT decreased the percentage of GFP-positive cells in a dose-dependent manner. The analysis by isobole method showed the mode of the combination effect of the two drugs to be synergistic. The mechanism of the synergy was considered to come from the TP upregulation in MCF-7E10 cells by tamoxifen facilitating the generation of 5-FU from 5'-DFUR. The combination effect of capecitabine with letrozole was examined using coculture ERE reporter gene assay which corresponds to postmenopausal breast cancer. In this assay, exogenously added testosterone was converted into estrogen by aromatase in stromal cells isolated from breast cancer tissues. Both 5'-DFUR and letrozole decreased the percentage of GFP-positive cells in a dose-dependent manner. The analysis by isobole method showed the mode of the combination effect of the two drugs to be additive. The results indicated that 5'-DFUR and letrozole work independently and do not interfere with each other. Unexpectedly, the results of the *in vitro* letrozole experiment did not coincide with the *in vivo* results. The reason for this discrepancy could not be delineated in the present study.

In conclusion, several types of therapeutic agents including chemotherapeutic agents, endocrine therapy agents and molecular targeted medicines are now available for the treatment of breast cancer. To conduct clinical studies, research demonstrating the scientific rationale is required. In the present study, we showed the efficacy of combination therapy of capecitabine with tamoxifen or letrozole for a premenopausal and postmenopausal breast cancer model, respectively. The usefulness of these combinations in patients needs to be confirmed in clinical trials.

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