Estrogen Down-regulator Fulvestrant Potentiates Antitumor Activity of Fluoropyrimidine in Estrogen-responsive MCF-7 Human Breast Cancer Cells

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Abstract. *Background: Endocrine therapy is clinically administered in hormone-responsive breast cancer. Combinations of fluoropyrimidine S-1 and an aromatase inhibitor or anti-estrogen are considered beneficial in Japan. Herein we assessed new combinations of S-1 and fulvestrant. Patients and Methods: Cytotoxicity of fulvestrant and 5-fluorouracil (5-FU) was assessed in hormone-responsive (MCF-7) and non-responsive (MDA-MB-231) breast cancer cell cultures. Fulvestrant and S-1 were evaluated for antitumor activity in mice and their effects on estrogen receptor (ER)- α and progesterone receptor (PgR) levels in MCF-7 xenografts using immunohistochemical methods. Results: Fulvestrant inhibited growth of MCF-7, but not of MDA-MB-231 xenografts. Combinations of 5-FU and fulvestrant were superior to monotherapy in vitro. In vivo antitumor activity of S-1/fulvestrant combination therapy was significantly ($p < 0.05$) enhanced compared to that of both monotherapies. Fulvestrant partially down-regulated expression of ER α and PgR, but in combination with S-1, it almost completely blocked their expression. Conclusion: Chemo-endocrine combination therapy using S-1 and fulvestrant is beneficial in estrogen-responsive breast cancer.*

Breast cancer is the most common cancer in women around the world, including in the United States (1, 2) and Japan,

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where it remains the fifth-leading cause of cancer-related death (3). Endocrine therapy is a well-established first-line treatment for estrogen receptor (ER)- α -positive, metastatic breast cancer (4). Current endocrine therapy treatment options for menopausal patients with hormone-responsive breast cancer include selective ER α modulators (*e.g.* tamoxifen), aromatase inhibitors (*e.g.* anastrozole, letrozole, and exemestane), and, in addition, selective ER α down-regulators (*e.g.* fulvestrant). An evaluation of several clinical trials of combination therapies testing fulvestrant with cyclin-dependent kinase 4 and 6 inhibitors [PALOMA-3 (5), MONALEESA-3 (6), and MONARCH2 (7)] or phosphoinositide 3-kinase inhibitors [FERG1 (8), BELLE-2 (9), BELLE-3 (10)] indicated that combination with the cyclin-dependent kinase 4 and 6 inhibitor palbociclib was associated with a significantly extended progression-free survival as compared with that of fulvestrant monotherapy. According to the guideline from the European Society for Medical Oncology, antagonistic interactions are predicted to occur using concomitant combination therapy of endocrine therapy and chemotherapy, and, therefore, simultaneous chemo-endocrine therapy is not recommended (11).

However, in contrast to other chemotherapeutic agents, clinical trials in Japan showed that only tegafur (a masked 5-FU derivative)/uracil (UFT), an oral fluoropyrimidine, used in combination with tamoxifen, improved overall survival, compared to surgery alone (12, 13). Furthermore, there was no significant difference in relapse-free survival between those treated with UFT therapy and cyclophosphamide/methotrexate/5-fluorouracil (5-FU) therapy (14, 15). We attempted to confirm the conclusions of this clinical trial. *In vitro* studies on ER-positive human breast cancer cell lines KPL-1 and ML-20 showed that 4-OH-tamoxifen exerted synergistic inhibitory effects with 5-FU but not with doxorubicin or paclitaxel (16). Furthermore, *in vitro* growth inhibition by 5-FU was assessed in the presence and absence of 17 β -estradiol (E₂). Growth of KPL-1 and ML-20 cells was inhibited in E₂-depleted fetal

bovine serum (FBS), which mimics the status after treatment with an aromatase inhibitor (17).

Oral fluoropyrimidine S-1 contains tegafur in combination with gimeracil (a potent inhibitor of 5-FU degradation) and potassium oxonate at a molar ratio of 1: 0.4: 1; the combination has lower gastrointestinal toxicity by blocking 5-FU activation in the gastrointestinal tract (18, 19). S-1 is considered beneficial and is approved as a clinical therapy for gastric (20), breast (21) and other solid tumor types in over 40 countries. S-1 potentiates the antitumor activity of anastrozole *in vitro* as observed using an aromatase-transfected, ER-positive human breast cancer cell line, and *in vivo* (22). In addition to aromatase inhibitors, the specific ER down-regulator fulvestrant (23) is approved for menopausal patients with hormone-responsive breast cancer (24-26). However, studies on combination therapies consisting of fulvestrant and other chemotherapeutics are limited. In this study, we aimed to evaluate a new chemo-endocrine therapy using S-1 in combination with fulvestrant.

Patients and Methods

Chemicals. Tegafur, gimeracil, and potassium oxonate were obtained from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Fulvestrant was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). E₂, Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) and FBS were purchased from Sigma-Aldrich Japan (Tokyo, Japan). SE-121 pellet, which contained 0.025 mg E₂/pellet for a 60-day release period, was obtained from Innovative Research of America (Sarasota, FL, USA). Hydroxypropyl methylcellulose was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). 5-FU, peanut oil, dimethyl sulfoxide (DMSO), and crystal violet were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other reagents were commercially available products of the highest grade.

Antibodies. Rabbit monoclonal antibodies to human ER α (ab16660, Clone SP1) and human progesterone receptor (PgR) (ab16661, Clone SP2) were purchased from Abnova Japan KK (Tokyo, Japan). Horseradish peroxidase-conjugated goat anti-rabbit IgG Fab fragment Histostar™ (Rb) for mouse tissue analysis was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan) and used for immunohistochemical (IHC) staining.

In vitro cell culture and growth inhibition testing. ER-positive human breast cancer cell line MCF-7, originally obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA), was kindly provided by the Osaka University Graduate School of Medicine (Osaka, Japan) and ER-negative human breast cancer cell line MDA-MB-231 was purchased from the ATCC. MCF-7 was cultured using DMEM/Ham's F-12 (1:1) supplemented with 0.1 nM E₂, 10% FBS and Glutamax™ (Life Technologies Japan Ltd, Tokyo, Japan). MDA-MB-231 was cultured using RPMI-1640 supplemented with 10% FBS at 37°C with 5% CO₂. Cells were collected using TrypLETM Express (Life Technologies Japan Ltd, Tokyo, Japan) and seeded into 96-well plates at a density of 3,000 cells/well on day 0. The *in vitro* E₂ concentration was equivalent to

the serum level in mice achieved by administering the E₂ pellet based on our previous experiment (data not shown). Fulvestrant and 5-FU were dissolved in DMSO and diluted with culture medium on day 1. The final DMSO concentration was less than 0.5%. Cell growth rates were determined colorimetrically on day 6 by crystal violet staining according to the method reported by Saotome *et al.* (27). The concentration inhibiting cell growth by 50% (IC₅₀) was calculated by regression analysis.

Interactions between 5-FU and fulvestrant against MCF-7 cells were analyzed using the isobologram method (28). The dose-response curves for treatment with 5-FU and fulvestrant alone were used to derive three isoeffect curves (Mode I, IIa, and IIb), which were analyzed in relation to the IC₅₀ values. The areas enclosed by the three isoeffect curves shown in Figure 2 indicate the envelope. The data points located within the envelope represent additive effects, and data points below the envelope represent supra-additive effects.

In vivo antitumor activity. Five-week-old female BALB/cAJcl nu/nu mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and housed under specific pathogen-free conditions; food and water were provided *ad libitum*. After the animals had been in quarantine for 5 days, SE-121 pellet (0.025 mg E₂/pellet) was implanted into the left flank subcutaneously. Because high E₂ doses were found to be associated with high mortality in mice due to renal toxicity in a preliminary experiment, one pellet containing 0.025 mg E₂ per animal was identified as the optimal amount (data not shown).

One week after the procedure, MCF-7 cells suspended in saline were injected in the right axillary subcutaneously at a dose of 4×10⁶ cells per mouse. To evaluate the antitumor activity, the mice were randomized according to tumor volume once the mean tumor volume reached approximately 38-46 mm³ (day 0). Each group consisted of seven mice. One group of mice with no tumor- and SE-121-pellet-free was used as negative control (n=7).

S-1 was prepared by mixing tegafur, gimeracil, and potassium oxonate at a molar ratio of 1:0.4:1 in 0.5% hydroxypropyl methylcellulose. S-1 was administered orally at the reported effective dose (10 mg/kg, at 10 ml/kg) (18) once daily for 18 consecutive days. Fulvestrant was dissolved in peanut oil and administered into the left femor subcutaneously on day 1 at a dose of 5 mg in 50 μ l per animal.

The tumor diameters were measured twice a week until day 18, the last day of administration, and the tumor volume was estimated using the formula 0.5× length × width².

All the animal studies were performed according to the guidelines of and approved by the Institutional Animal Care and Use Committee at Taiho Pharmaceutical Co., Ltd. (Approval Number: 17PB06).

IHC staining of ER α and PgR. To perform the IHC staining on mouse tissue, tumors were excised on day 19, the day after the last S-1 administration, fixed in 10% phosphate-buffered formaldehyde (pH 7.4) for 1 day followed by the preparation of paraffin-embedded tissue sections according to the conventional method. Heat-induced antigen retrieval was applied using a pressure cooker at 121°C for 5 min in Tris-Borate-EDTA buffer (Takara Bio Inc., Kusatsu, Japan) at pH 8.3. Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide in methanol for 10 min at room temperature. ER α and PgR were detected using rabbit monoclonal anti-human ER α (1:200 dilution) and anti-human PgR (1: 100 dilution) as primary antibodies, respectively. Horseradish

Figure 1. Growth inhibition by single-drug treatment. 5-Fluorouracil (5-FU) (A) and fulvestrant (B) were tested for growth inhibition of estrogen receptor (ER)-positive human breast cancer cell line MCF-7 and ER-negative human breast cancer cell line MDA-MB-231 in the presence of 0.1 nM estradiol. The values are the means and SD of cell growth relative to that of the control (T/C) from three independent experiments.

peroxidase-conjugated goat anti-rabbit antibody for mouse tissue was used as secondary antibody, and a 3, 3'-diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide was added as the substrate followed by Mayer's hematoxylin solution added as counterstain.

Negative control samples were processed without applying the primary antibodies. Three fields of each sample were randomly selected for qualitative analysis without using an unblinded procedure; three slides were evaluated for each treatment group. Approximately, 300 cells/field were counted at a magnification of 400 $\times$, except in the necrotic area of the central tumor and the connective tissues or blood vessels.

Statistical analysis. The significance of the differences in the mean tumor volume between the treated and control groups on day 14 was analyzed using the Dunnett's test. The combinational effect of S-1 and fulvestrant was analyzed according to the closed testing procedure using the Aspin-Welch two-tailed *t*-test (29). The significance of the differences in the mean uterine weight on day 1

Figure 2. An isobologram curve for the combination treatment with 5-fluorouracil (5-FU) and fulvestrant. Da and Db indicate the ratio of the concentration inhibiting cell growth by 50% (IC_{50}) for 5-FU and fulvestrant in combination therapy to monotherapy, respectively. An envelope of additivity is enclosed by mode I, IIa, and IIb. Data points within the envelope of interaction between 5-FU and fulvestrant indicate additivity, whereas data points below the envelope indicate synergy.

was analyzed using the Aspin-Welch two-tailed *t*-test. The statistical analyses were performed using EXSAS, Ver. 7.11 (Arm Systex Co., Ltd., Osaka, Japan).

Results

In-vitro growth inhibition. The single-drug treatment with chemotherapeutic agent 5-FU inhibited the growth of both ER-positive MCF-7 and ER-negative MDA-MB-231 cells in a dose-dependent manner (Figure 1A). Fulvestrant alone inhibited the growth of MCF-7 cells but not of MDA-MB-231 cells (Figure 1B). The IC_{50} values of 5-FU for MCF-7 and MDA-MB-231 were 4.6 and 12.0 μ M, respectively, and their ratio was 2.6, whereas the respective IC_{50} values of fulvestrant were 0.8 nM and >1 μ M and their ratio was $>12,500$.

In this study, the efficacy of the combination therapy using 5-FU and fulvestrant was assessed in MCF-7 cells. Interestingly, the isobologram data points for the combination therapy of MCF-7 cells were located either within the envelope of additivity or below the envelope, indicating supra-additive effects (Figure 2). Hence, the combination of 5-FU and fulvestrant appears to have a beneficial effect.

Figure 3. Tumor volume of MCF-7 xenograft *in vivo*. Nude mice bearing MCF-7 xenograft were randomized into four groups ($n=7$) on day 0, which was 7 days after the SE-121 pellet (containing 0.025 mg estradiol for 60 days release) had been subcutaneously implanted. Fulvestrant (5 mg/mouse) was administered subcutaneously on day one, S-1 (10 mg/kg) was administered orally once daily from day 1 to 18. Tumor volume was measured twice a week until day 19. The values are the means and SD of tumor volume ($n=7$). Significantly different on day 15 at *** $p<0.001$ vs. control by Dunnett's test; # $p<0.05$ vs. either monotherapy by Aspin-Welch *t*-test.

***In vivo* antitumor activity.** As shown in Figure 3, tumor volume reached a plateau on day 15; therefore, S-1 administration was terminated on day 18, and antitumor effects were evaluated based on the tumor volume on day 14. Importantly, tumor growth was significantly inhibited by S-1 or fulvestrant ($p<0.001$). The tumor volume in the combination therapy group was significantly ($p<0.05$) lower as compared to that in both monotherapy groups. Body weight loss was not observed during the experiment (data not shown).

Table I shows the uterine weight in mice without and with implanted SE-121 pellet containing 0.025 mg E_2 that were either treated or not treated with fulvestrant. The SE-121 pellet significantly ($p<0.05$) increased the uterine weight, and this increase was completely inhibited by concomitant fulvestrant treatment ($p<0.01$) according to the Aspin-Welch *t*-test.

IHC analysis of ER- α and PgR. ER α and PgR were detected in the MCF-7 xenografts by IHC staining. The intensity and proportion of staining for ER α was partially reduced by fulvestrant, whereas S-1 alone did not appear to affect ER α staining. However, the combination therapy significantly reduced the intensity and proportion of cells staining for ER α as compared to that of both monotherapies. PgR

Table I. Weight of uterus in nude mice (mean $\pm$ SD).

Treatment	Uterine weight (g)	n	p-Value*
SE-121 and tumor-free	0.066 $\pm$ 0.009	8	
SE-121 alone	0.097 $\pm$ 0.031	7	<0.05 ^a
Fulvestrant and SE-121	0.035 $\pm$ 0.010	6	<0.01 ^b

*Aspin-Welch two-tailed *t*-test. ^aVersus SE-121 free; ^bversus SE-121 alone.

expression was barely detectable in the group treated with fulvestrant alone, although ER α expression was only partially reduced, indicating that the ER α transduction signal was reduced (Figure 4).

Discussion

In vitro combination treatment of estrogen-responsive MCF-7 breast cancer cells with 5-FU and fulvestrant was associated with additive or supra-additive effects. Combination therapy reduced tumor growth more significantly than either monotherapy, which had a more limited reduction in tumor volume relative to the starting volume. Tumoral ER α expression, which was targeted by fulvestrant, was partially reduced by the treatment with this drug, whereas the combination therapy more significantly reduced the expression of ER α than did each monotherapy. These results appeared to corroborate the synergistic cytotoxic effects of 5-FU and fulvestrant observed *in vitro*.

In our previous report, 4-hydroxytamoxifen and estrogen-depleted FBS, which mimics the effect of aromatase inhibitor activity *in vitro*, were shown to increase the activity of fluoropyrimidine (12, 13). In addition, combination therapy using the aromatase inhibitor anastrozole combined with S-1 or UFT was reported to be superior to both monotherapies (22). Furthermore, fulvestrant has been reported to reduce signaling *via* ER- α by down-regulating the ER α level. The effects of the S-1/fulvestrant combination on MCF-7, a tumorigenic cell line with ER α expression, were similar to those of other chemo-endocrine therapies using an anti-estrogen (4-hydroxytamoxifen) and an aromatase inhibitor (anastrozole) (12, 13, 22).

An *in vivo* analysis of the combination of fulvestrant and 5-FU in MCF-7-bearing nude mice has been already reported by Ogasawara *et al.* (30); the antitumor activity, 5-FU level, and the effect on tumor-associated mRNA was significantly increased, whereas the tumoral ER α level was reduced as compared to that in the 5-FU monotherapy group (30). Interestingly, these results are corroborated by the data from our study. In a previous study, S-1 treatment significantly reduced tumoral mRNA expression of ER (22). Because fulvestrant down-regulates ER α protein in tumor by

Figure 4. Immunohistochemical staining of estrogen receptor- α (ER α) (upper panel) and progesterone receptor (PgR) (lower panel) in a MCF-7 xenograft subjected to monotherapy with either S-1 (10 mg/kg, days 1-18, orally) or fulvestrant (1 mg/animal, day 1, subcutaneously), or to combination therapy. Tumors were excised on day 19. The bar indicates 100 μ m. The original magnification was 400 $\times$.

accelerated degradation and not by suppression of translation/transcription, the addition of S-1 contributed to the down-regulation of ER α .

In comparison with other standard chemotherapeutic agents, S-1 has some advantages. Specifically, for long-term treatment, this oral drug appears to be more beneficial than intravenous therapy. Furthermore, an open-label randomized phase III trial (SELECT BC) demonstrated that S-1 was non-inferior to taxane, a key drug for breast cancer with respect to overall survival, which is used as first-line therapy in metastatic breast cancer and associated with improved quality of life (21). It has been reported that patients with incurable cancer clearly prefer oral chemotherapy as compared to other administration routes if the efficacy is equivalent without reducing the quality of life, which also contributes to better medication adherence (31). The toxicities of S-1 and fulvestrant do not overlap in patients (19, 20, 23-25). Hair loss is a common adverse effect that severely reduces quality of life, especially for female patients, but the frequency of hair-loss associated with S-1 is low (less than 5%) in contrast with other standard chemotherapies, such as taxanes or anthracyclines (20, 32).

This study used only MCF-7 cells due to the limited availability of established tumorigenic human breast cancer cell lines which are responsive to estrogen. We also attempted to use T-47D, which is another typical ER-positive human breast cancer cell line, but failed to obtain *in vivo* growth of T-47D cells in a female nude or SCID mouse model (data not shown). In our current experiment, the maximum E₂ dose of 0.025 mg, which did not induce renal toxicity, was used. Since this E₂ dose significantly increased the uterine weight ($p < 0.05$), it appeared to be adequate for

inducing the desired biological activity. However, the mechanism for potentiating ER degradation needs to be investigated further. Future studies should use patient-derived xenograft models that are ER-positive to assess the effects of fulvestrant and S-1.

In conclusion, chemo-endocrine therapy using S-1 in combination with fulvestrant may represent a new beneficial candidate therapy for menopausal patients with estrogen-responsive breast cancer that should be evaluated in clinical trials.

Conflicts of Interest

MN, HS, and TT are employees of Taiho Pharmaceutical Co., Ltd. SN has been an adviser for and received the honoraria and the research funding related to this study from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan); SN has also been an adviser for and received the honoraria and the research funding not related to this study from AstraZeneca (Cambridge, UK). This study was funded by Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). The Authors have no conflict of interest to declare regarding this study.

Authors' Contributions

MN and TT planned the experiments. MN is the corresponding Author and wrote the article. HS and MN carried out the experiments. SN contributed to the interpretation of the results. All Authors provided critical feedback and helped shape the research, analysis, and article.

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