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ORIGINAL ARTICLE

Preclinical rationale for combined use of endocrine therapy and 5-fluorouracil but neither doxorubicin nor paclitaxel in the treatment of endocrine-responsive breast cancer

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Abstract
Purpose Our previous study indicated that concurrent administration of 4-OH-tamoxifen (TAM) and 5-fluorouracil (5-FU), but not doxorubicin (Dox), resulted in additive antitumor effects on endocrine-responsive breast cancer cells. We further clarified the effects of combined administration of endocrine therapy with chemotherapeutic agents in this study.
Methods Concurrent treatment with 4-OH-TAM and paclitaxel (Ptx) was investigated in estrogen receptor (ER)-positive breast cancer cells. Additionally, the combined effects of estrogen depletion from culture medium mimicking estrogen ablative therapy with 5-FU, Dox, and Ptx were investigated.
Results Concurrent treatment with 4-OH-TAM and Ptx yielded less than additive antitumor effects in ER-positive breast cancer cells, as observed with Dox in our previous study. More interestingly, estrogen depletion with 5-FU, but with neither Dox nor Ptx, yielded additive antitumor effects on these cells. We also performed preliminary experiments to elucidate the mechanisms of action responsible for the combined antitumor effects observed. Ptx up-regulated the level of expression of one of the molecules related to TAM resistance, Eph-A2, as observed with Dox in our previous study. Estrogen depletion down-regulated the level of expression of one of the molecules related to 5-FU resistance, thymidylate synthase, as observed with 4-OH-TAM in our previous study.
Conclusions These findings, together with those of our previous study, suggest that concurrent treatment with endocrine therapy, administration of TAM, or estrogen ablative therapy and 5-FU but neither Dox nor Ptx may yield additive antitumor effects on endocrine-responsive breast cancer.

Keywords Breast cancer · Chemo-endocrine therapy · Tamoxifen · Doxorubicin · Paclitaxel

Abbreviations

IC_{50}	50% Inhibitory concentration
CI	Combination index
DCC-FBS	Dextran-coated charcoal-stripped fetal bovine serum
Dox	Doxorubicin
E2	17β-Estradiol
ER	Estrogen receptor
FU	5-Fluorouracil
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Ptx	Paclitaxel
RT-PCR	Reverse transcription-polymerase chain reaction
TAM	Tamoxifen
TS	Thymidylate synthase

Introduction

Postoperative adjuvant concurrent chemo-endocrine therapy with tamoxifen (TAM) and uracil plus tegafur, a 5-fluorouracil (5-FU) derivative, has been reported to improve

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overall survival (OS) compared with UFT alone, and to improve disease-free survival (DFS) compared with TAM or UFT alone in patients with endocrine-responsive breast cancer [1]. In contrast, concurrent administration of TAM with combined chemotherapy (cyclophosphamide, methotrexate, and 5-FU) failed to yield additional benefit in terms of DFS or OS in patients with endocrine-responsive breast cancer [2]. Additionally, concurrent chemo-endocrine therapy with TAM and anthracycline-based combined chemotherapies have been reported to be inferior to chemotherapy followed by TAM [3, 4]. A large clinical trial, INT-0100, showed a marginally significant advantage of sequential cyclophosphamide-doxirubicin-5-FU chemotherapy plus TAM over the concurrent therapy [3]. The GEICAM 9401 study comparing epirubicin-cyclophosphamide adjuvant chemotherapy plus TAM administered concurrently versus sequentially showed non-significant superiority of the sequential arm to the concurrent arm [4].

Although several basic studies on the interaction between TAM and chemotherapeutic agents have been performed, their conclusions have been inconsistent [5–10]. In our previous study, concurrent administration of 4-OH-TAM additively enhanced the growth-inhibitory activity of 5-FU in ER (estrogen receptor)-positive breast cancer cells in vitro, probably due to down-regulation of thymidylate synthase (TS) activity by 4-OH-TAM. However, in contrast to 5-FU, 4-OH-TAM failed to enhance the growth-inhibitory effect of Dox on these cells [11]. To further clarify the effects of combined administration of endocrine therapy and chemotherapeutic agents, concurrent treatment of 4-OH-TAM with a standard chemotherapeutic agent for breast cancer, paclitaxel (Ptx), was investigated in ER-positive breast cancer cells in this study. In addition, combined effects of estrogen depletion from culture medium (mimicking estrogen ablative agents such as aromatase inhibitors or luteinizing hormone-releasing hormone) with 5-FU, Dox, and Ptx were also investigated.

Materials and methods

Reagents

17β-estradiol (E2), 4-OH-TAM, RPMI-1640 medium, and phenol red-free RPMI-1640 medium were purchased from Sigma-Aldrich Inc. Chemical Co. (St. Louis, MO, USA). 5-FU, Ptx, ethanol, and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dox was purchased from Kyowa Hakko Co., Ltd. (Tokyo, Japan). E2 was dissolved in ethanol, and 4-OH-TAM and Ptx in DMSO. Dox was dissolved in sterile saline. Dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS) was purchased from Tissue Culture Biological, Inc. (Tulanta, CA, USA).

The RNeasy mini kit and Quantitect probe were purchased from Qiagen Inc. (Valencia, CA, USA). High-capacity cDNA reverse transcriptional kits and TaqMan probe and primer were purchased from Applied Biosystems (Foster, CA, USA). Anti-human TS rabbit polyclonal antibody was prepared by our laboratory [12], and anti-human β-actin mouse antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit goat antibody, and HRP-conjugated anti-mouse goat antibody were purchased from Sigma-Aldrich. A substrate of HRP, luminal, was purchased from Thermo Fisher Science (Rockford, IL, USA).

Cell lines

The KPL-1 human breast cancer cell line was established in our laboratory [13]; this line is ER-α-positive and estrogen-responsive [14]. ML-20 and MDA-MB-231 human breast cancer cell lines were kindly provided by the late Dr. Robert B. Dickson (Lombardi Cancer Research Center, Georgetown University Medical Center, Washington DC, USA). The ML-20 cell line is a subclone of the MCF-7 cell line, and is ER-α-positive and estrogen-dependent [15]. MDA-MB-231 cells are ER-α-negative and estrogen-unresponsive. All cell lines were routinely cultured in RPMI-1640 medium supplemented with 5% FBS.

Cell growth assay

To reduce endogenous estrogen-like activity, cells were pre-cultured with phenol red-free RPMI-1640 medium supplemented with 5% DCC-FBS (estrogen-depleted medium) for a week. Approximately 3,000 cells/well were seeded into 96-well plates on day 0. To test the effects of 4-OH-TAM on the growth-inhibitory activity of Ptx, various concentrations of Ptx were added to medium with or without the indicated concentrations of 4-OH-TAM supplemented with 0.1 nM E2 on day 1. The medium was changed to Ptx-free medium on day 2. Six wells were treated with each concentration of Ptx. Growth ratio was determined on day 6 using spectroscopic measurements according to the following formula [16]: growth ratio = (OD_{540} of Ptx-treated well)/(OD_{540} of Ptx-free well) $\times$ 100 (%) or (OD_{540} of well treated with Ptx and 4-OH-TAM)/(OD_{540} of well treated with 4-OH-TAM alone) $\times$ 100 (%). To test the effects of E2 depletion on the growth-inhibitory activities of chemotherapeutic agents, various concentrations of 5-FU, Dox, or Ptx were added to estrogen-depleted medium or medium supplemented with 0.1 nM E2 on day 1. Medium containing Dox or Ptx was changed to drug-free medium on day 2. In the case of 5-FU, cells were continuously to this agent until day 6. The number of viable cells and growth ratio

were measured using the simplified crystal violet method on day 6 as noted above. Reproducibility of results was determined by performing three separate experiments.

To evaluate antitumor effects of combined treatments, a combination index (CI) based on 50% inhibitory concentration (IC_{50}) values was calculated according to the following formula [11]: CI = IC_{50} with combined treatment/IC_{50} with single treatment. CI < 0.5 was considered evidence of additive interaction.

Determination of levels of TS protein expression by western blotting

KPL-1 cells were cultured in a 75 cm^2 flask in the absence of E2, and medium was changed to fresh medium supplemented with 0.1 or 1 nM E2 or E2-free medium for 48 h. Cell pellets were collected by a scraper, and the cytosol was extracted by centrifugation (105,000 × *g* for 1 h). Total protein concentration was spectroscopically measured with the Bradford assay using bovine serum albumin as standard. The cytosol was subjected to western blotting (50 μg protein/lane). Relative levels of expression of TS protein were measured using β-actin as an endogenous control with the LAS 3000 (Fuji Film Co., Ltd., Tokyo, Japan).

Real-time reverse transcription (RT)-polymerase chain reaction (PCR) for Eph-A2

To test the effects of Ptx on level of mRNA expression of Eph-A2, which has been reported to be related to TAM resistance [17], KPL-1 cells were treated with 1, 3, or 5 nM Ptx for 24 h in the presence of 0.1 nM E2. Total RNA was extracted using the RNeasy Mini Kit following the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Archive Kit as described by the manufacturer. PCR reactions were carried out by TaqMan Real-time RT-PCR. Gene expression was measured using the QuantiTect Probe PCR Kit and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. TaqMan gene expression assay primer probe sets for each gene were used.

Briefly, 50 ng cDNA was added to a reaction mixture containing 25 μL of 2 × QuantiTect Probe PCR Master Mix and 2.5 μL of 20 × TaqMan gene expression assay mix in a final volume of 50 μL. The conditions for real-time RT-PCR were 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 15 min; and 40 cycles at 94°C for 15 s and 56°C for 30 s. Gene expression profiling was performed using the comparative CT method for relative quantification. The calibration samples were untreated cells, and GAPDH was used as an endogenous control. The Gene Assay IDs of the TaqMan gene expression assay supplied by Applied Biosystems were Hs00171656_m1 for Eph-A2 and Hs99999905_m1 for GAPDH.

Statistical analysis

All values are the mean ± SD. For statistical analysis, EXAS ver. 7.11 software (Arm Systex Co., Ltd. Osaka, Japan) was used to examine differences between two groups. Two-sided *P* values less than 0.05 were considered significant.

Results

Growth-inhibitory activities of 5-FU, Dox, and Ptx in estrogen-depleted condition

Estrogen depletion from culture medium supplemented with 0.1 nM E2 reduced the growth of estrogen-responsive KPL-1 and ML-20 cells by 11.3 ± 6.8 and 21.3 ± 6.9%, respectively. Concurrent 5-FU treatment and E2 depletion yielded additive growth-inhibitory effects (CIs of 0.35 and 0.37 for ER-positive ML-20 and KPL-1 cells, respectively), though additive effects were observed with neither with Dox and E2 depletion nor with Ptx and E2 depletion (CIs of 0.74 and 0.97 for ML-20 cells, and CIs of 0.76 and 0.84 for KPL-1 cells, respectively), as shown in Table 1 and Figs. 1 and 2.

Down-regulation of level of TS expression in estrogen-depleted condition

Because 4-OH-TAM reduced the level of expression of TS in our previous study [11], level of TS expression was determined in E2-depleted condition. As expected, TS protein level was decreased by E2 depletion in estrogen-responsive KPL-1 cells (Fig. 3).

Table 1 Interaction between chemotherapeutic agents and E2 depletion in ER-positive breast cancer cells

Cell line	Agent	50% IC with E2	E2-depleted	Combination index
ML-20	Dox	62.1 nM	45.9 nM	0.74
	5-FU	2.01 μM	0.70 μM	0.35
	Ptx	3.2 nM	3.1 nM	0.97
KPL-1	Dox	82.2 nM	62.6 nM	0.76
	5-FU	2.06 μM	0.76 μM	0.37
	Ptx	3.1 nM	2.6 nM	0.84

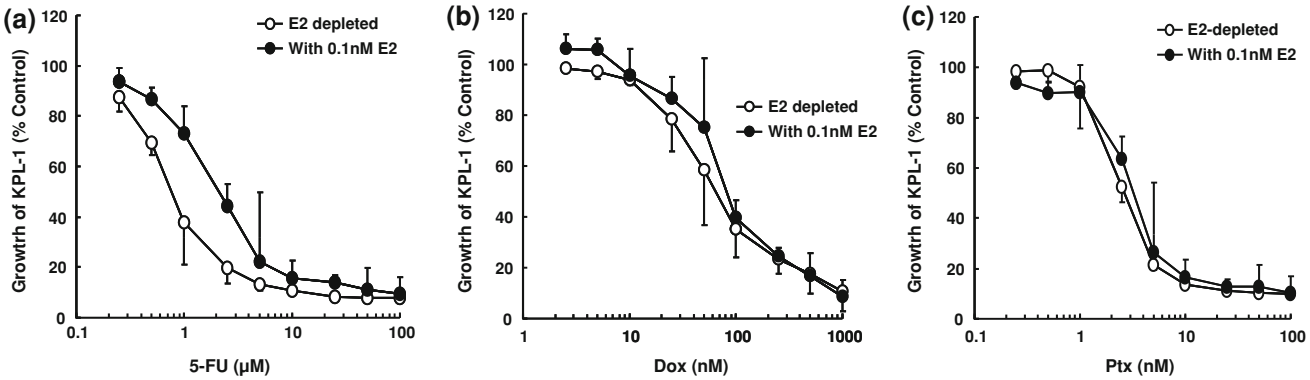

Fig. 1 Growth-inhibitory activities of 5-FU (**a**), Dox (**b**), and Ptx (**c**) in estrogen-responsive KPL-1 breast cancer cells. KPL-1 cells were treated with these agents in E2-depleted (*open circle*) or E2 (0.1 nM)-supplemented conditions (*closed circle*) as described in "Materials and Methods". Values and *bars* represent means and SDs, respectively ($n = 6$)

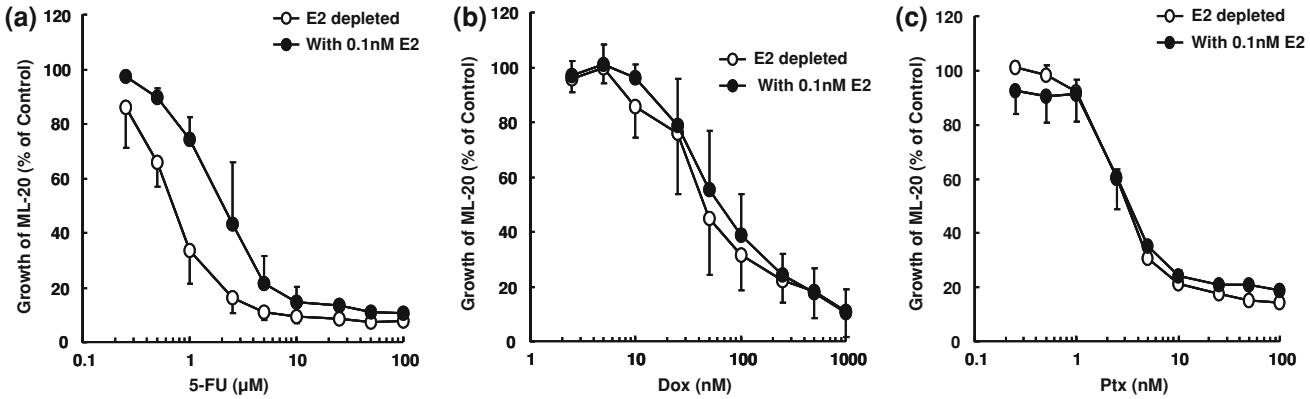

Fig. 2 Growth-inhibitory activities of 5-FU (**a**), Dox (**b**), 4-OH-TAM (**c**), and Ptx (**d**) in estrogen-responsive ML-20 cells. ML-20 cells were treated with these agents in E2-depleted (*open circle*) or E2 (0.1 nM)-supplemented conditions (*closed circle*) as described in "Materials and Methods". Values and *bars* represent means and SDs, respectively ($n = 6$)

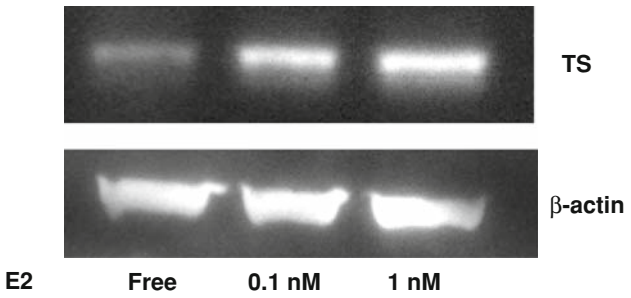

Fig. 3 Levels of TS protein expression in estrogen-responsive KPL-1 breast cancer cells as determined by western blotting. KPL-1 cells were treated with 0, 0.1, or 1 nM E2 for 48 h. *β*-actin was used as an endogenous control

Growth-inhibitory activity of concurrent treatment with 4-OH-TAM and Ptx

Concurrent treatment with 5-FU and 4-OH-TAM yielded additive antitumor effects in estrogen-responsive KPL-1 and ML-20 cells in our previous study [11]. In contrast, in the present study concurrent treatment with 4-OH-TAM and Ptx yielded less than additive antitumor effects in each of these ER-positive cell lines (Fig. 4a, b). No additive antitumor effects of 4-OH-TAM and Ptx were observed on ER-negative MDA-MB-231 cells (Fig. 4c).

Levels of mRNA expression of Eph-A2 after treatment with Ptx

Since the level of expression of one of the genes related to TAM-resistance, Eph-A2, was increased by Dox in our previous study [11], changes in levels of Eph-A2 mRNA expression were examined after treatment with Ptx in the present study. Levels of expression of Eph-A2 were increased 1.5- to 2.2-fold after exposure to 1–5 nM Ptx for 24 hours in ER-positive KPL-1 cells (Fig. 5).

Discussion

Chemotherapeutic and endocrine agents are widely used for the treatment of breast cancer patients. However, the

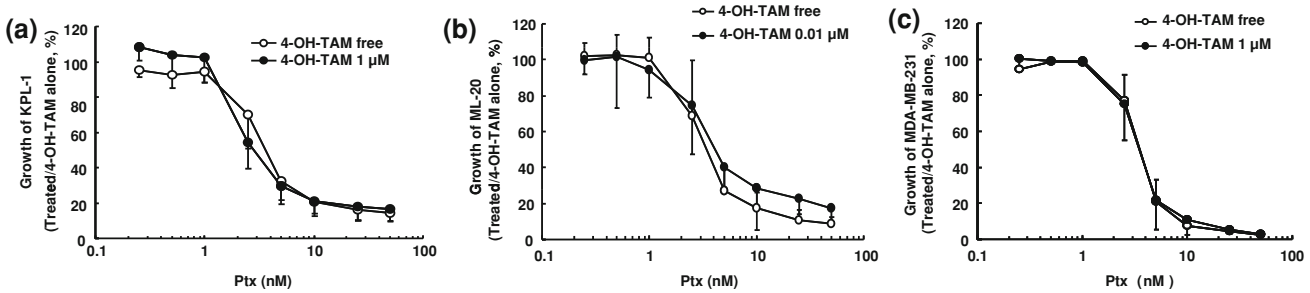

Fig. 4 Growth-inhibitory activities of concurrent treatment with Ptx and 4-OH-TAM in ER-positive KPL-1 cells (**a**), ER-positive ML-20 cells (**b**), and ER-negative MDA-MB-231 cells (**c**) in the presence of 0.1 nM E2. These cells were treated with (*closed circles*) or without (*open circles*) 4-OH-TAM and the indicated concentrations of Ptx as described in "Materials and Methods". Values and *bars* represent means and SDs, respectively ($n = 6$)

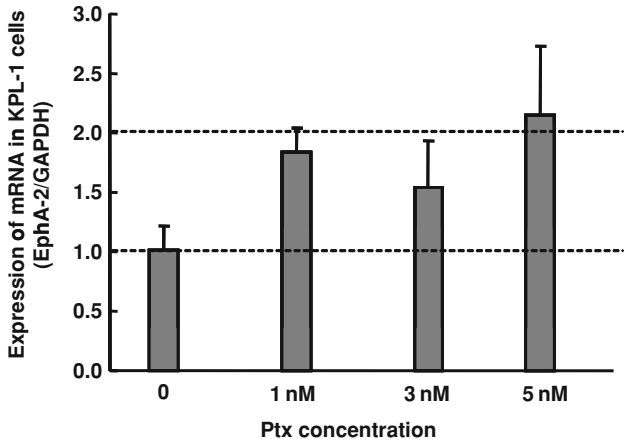

Fig. 5 mRNA expression of Eph-A2 as determined by quantitative RT-PCR. KPL-1 cells were treated with the indicated concentrations of Ptx in the presence of 0.1 nM E2 for 24 h. Values and *bars* represent means and SDs, respectively. Relative expression ratios were calculated using GAPDH as an endogenous control ($n = 4$)

optimal combinations of chemotherapy and endocrine therapy remain to be determined [18]. Although there have been a number of preclinical studies on the efficacy of chemo-endocrine therapy, their conclusions have been inconsistent, with findings of synergistic [6, 10] or antagonistic antitumor effects [5, 7–9] in the case of concurrent treatment with chemotherapeutic and endocrine agents.

To investigate the interaction of chemotherapeutic agents (5-FU, Dox, and Ptx) with endocrine therapies including TAM or estrogen-ablative therapies, we determined the growth-inhibitory activities of concurrent treatment with them in estrogen-responsive breast cancer cells in the present study. Since it is difficult to test estrogen-ablative effects of LH-RH agonists or aromatase inhibitors in vitro, we instead used estrogen-depleted culture conditions, which mimic the tumor microenvironments obtained with LH-RH agonists in premenopausal patients or with aromatase inhibitors in postmenopausal patients. As shown in Table 1 and Figs. 1 and 2, 5-FU exhibited additive growth-inhibitory effects with estrogen depletion while neither Dox nor Ptx exhibited additive effects with estrogen depletion in estrogen-responsive breast cancer cells.

Our previous study suggested that 4-OH-TAM significantly decreased TS activity, and might thus enhance the antitumor activity of 5-FU in ER-positive breast cancer cells. TS is one of the key enzymes responsible for the antitumor activity of 5-FU. In addition, the antitumor activity of 5-FU has been reported to be inversely correlated with the level of TS expression in human tumors [19]. To clarify whether estrogen depletion also down-regulates TS expression in estrogen-responsive breast cancer cells, protein expression of TS was investigated in the present study. As shown in Fig. 3, the level of expression of TS protein was significantly decreased by estrogen depletion in ER-positive KPL-1 cells. Coincidentally, it has been reported that the level of TS mRNA expression was increased by E2 at the transcriptional level in ER-positive breast cancer cells [20].

In an attempt to elucidate the interaction of TAM with chemotherapeutic agents in terms of antitumor activity, we previously found that 4-OH-TAM additively enhanced the antitumor activity of 5-FU but not that of Dox in estrogen-responsive breast cancer cells [11]. In the past decade, taxanes such as Ptx have been frequently used for breast cancer patients in the setting of adjuvant treatment and for patients with metastatic disease. In the present study, we therefore examined the interaction of 4-OH-TAM with Ptx. Interestingly, as shown in Fig. 5, 4-OH-TAM failed to enhance the growth-inhibitory activity of Ptx, as observed for Dox in ER-positive breast cancer cells. These findings, together with those of our previous study, suggest that the two major chemotherapeutic agents Dox and Ptx may yield less than additive effects when used concurrently with TAM in the treatment of patients with ER-positive breast cancer.

To determine the mechanisms of action responsible for the negative interaction between 4-OH-TAM and Dox, the profiles of mRNA expression of selected genes related to ER signaling, TAM resistance, and Dox sensitivity were examined in our previous study. It was found that one of the molecules responsible for TAM resistance, the tyrosine

kinase receptor Eph-A2 [17], was significantly up-regulated by Dox at either the mRNA or protein level in ER-positive breast cancer cells. To clarify whether Ptx also increases the level of expression of Eph-A2 in ER-positive breast cancer cells, the level of mRNA expression of Eph-A2 was examined in the present study. As expected, the level of expression of Eph-A2 was significantly increased by Ptx in ER-positive KPL-1 breast cancer cells (Fig. 5). These findings, together with those of our previous study, suggest that the up-regulation of Eph-A2 level induced by Dox or Ptx might be, at least in part, responsible for the reduction of antitumor activity of TAM in ER-positive breast cancer.

The present study has some limitations. First, it is unclear whether estrogen depletion from culture medium in vitro yields conditions representative of those of the breast tumor microenvironment upon treatment with LH-RH agonists in premenopausal patients or aromatase inhibitors in postmenopausal patients. It has been pointed out that the results of estrogen ablative therapy in vivo are poorly reproducible in vitro. Second, we tested changes in the levels of expression of TS and Eph-A2 after treatment with 4-OH-TAM and Ptx, respectively, in the present study, based on findings of our previous study. A number of molecules are related to sensitivity and resistance to TAM, Dox, or Ptx. A large-scale cDNA microarray analysis may provide more comprehensive findings on the mechanisms of action responsible for the interaction between chemotherapeutic and endocrine agents. Furthermore, clinical and correlative studies using neoadjuvant chemo-endocrine therapy may be more straightforward means of testing the suitable combination regimens.

In conclusion, the findings of this as well as our previous study suggest that concurrent treatment with TAM or estrogen ablative therapy and 5-FU may yield additive antitumor effects on ER-positive breast cancer. In contrast, concurrent treatments with TAM or estrogen ablative therapy and Dox or Ptx may yield less than additive effects on ER-positive breast cancer. In addition, down-regulation of TS expression by 4-OH-TAM may enhance the antitumor activity of 5-FU while up-regulation of Eph-A2 expression may inhibit the antitumor activity of 4-OH-TAM in ER-positive breast cancer cells.

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References

1. Noguchi S, Koyama H, Uchino J, Abe R, Miura S, Sugimachi K, Akazawa K, Abe O (2005) Postoperative adjuvant therapy with tamoxifen, tegafur plus uracil, or both in women with node-negative breast cancer: a pooled analysis of six randomized controlled trials. J Clin Oncol 23:2172–2184
2. Boccardo F, Rubagotti A, Bruzzi P, Cappellini M, Isola G, Nenci I, Piffanelli A, Scanni A, Sismondi P, Santi L (1990) Chemotherapy versus tamoxifen versus chemotherapy plus tamoxifen in node-positive, estrogen receptor-positive breast cancer patients: results of a multicentric Italian study. Breast Cancer Adjuvant Chemo-Hormone Therapy Cooperative Group. J Clin Oncol 8:1310–1320
3. Albain KS, Green SJ, Ravdin PM, Cobau CD, Levine EG, Ingle JN (2002) Adjuvant chemohormonal therapy for primary breast cancer should be sequential instead of concurrent: initial results from intergroup trial 0100 (SWOG-8814). Proc ASCO 21:37a
4. Pico C, Martin M, Jara C, Barnadas A, Pelegri A, Balil A, Camps C, Frau A, Rodriguez-Lescure A, Lopez-Vega JM, De La Haba J, Tres A, Alvarez I, Alba E, Arcusa A, Oltra A, Batista N, Checa T, Perez-Carrion R, Curto J (2004) Epirubicin-cyclophosphamide adjuvant chemotherapy plus tamoxifen administered concurrently versus sequentially: randomized phase III trial in postmenopausal node-positive breast cancer patients. A GEICAM 9401 study. Ann Oncol 15:79–87
5. Osborne CK (1983) Combined chemo-hormonal therapy in breast cancer: a hypothesis. Breast Cancer Res Treat 1:121–123
6. Benz C, Cadman E, Gwin J, Wu T, Amara J, Eisenfeld A, Dannies P (1983) Tamoxifen and 5-fluorouracil in breast cancer: cytotoxic synergism in vitro. Cancer Res 43:5298–5303
7. Hug V, Hortobagyi GN, Drewinko B, Finders M (1985) Tamoxifen-citrate counteracts the antitumor effects of cytotoxic drugs in vitro. J Clin Oncol 3:1672–1677
8. Osborne CK, Kitten L, Arteaga CL (1989) Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens. J Clin Oncol 7:710–717
9. Woods KE, Randolph JK, Gewirtz DA (1994) Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line. Biochem Pharmacol 47:1449–1452
10. Leonessa F, Jacobson M, Boyle B, Lippman J, McGarvey M, Clarke R (1994) Effect of tamoxifen on the multidrug-resistant phenotype in human breast cancer cells: isobologram, drug accumulation, and M(r) 170,000 glycoprotein (gp170) binding studies. Cancer Res 54:441–447
11. Kurebayashi J, Nukatsuka M, Nagase H, Nomura T, Hirono M, Yamamoto Y, Sugimoto Y, Oka T, Sonoo H (2007) Additive antitumor effect of concurrent treatment of 4-hydroxy tamoxifen with 5-fluorouracil but not with doxorubicin in estrogen receptor-positive breast cancer cells. Cancer Chemother Pharmacol 59:515–525
12. Okabe H, Tsujimoto H, Fukushima M (1997) Preparation of the antibodies against recombinant human thymidylate synthase for the detection of its intratumoral levels and the application to sensitivity-study of 5-fluorouracil. Oncol Rep 4:385–690
13. Kurebayashi J, Kurosumi M, Sonoo H (1995) A new human breast cancer cell line, KPL-1 secretes tumour-associated antigens and grows rapidly in female athymic nude mice. Br J Cancer 71:845–853
14. Kurebayashi J, Otsuki T, Yamamoto S, Kurosumi M, Nakata T, Akinaga S, Sonoo H (1998) A pure antiestrogen, ICI 182,780, stimulates the growth of tamoxifen-resistant KPL-1 human breast cancer cells in vivo but not in vitro. Oncology 55:23–34
15. Kunisue H, Kurebayashi J, Otsuki T, Kunisue H, Kurebayashi J, Otsuki T, Tang CK, Kurosumi M, Yamamoto S, Tanaka K, Doihara H, Shimizu N, Sonoo H (2000) Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2. Br J Cancer 82:46–51
16. Saotome K, Morita H, Umeda M (1989) Cytotoxicity test with simplified crystal violet staining method using microtitre plates

and its application to injection drugs. Toxicol In Vitro 3:317–321
17. Lu M, Miller KD, Gokmen-Polar Y, Jeng MH, Kinch MS (2003) EphA2 overexpression decreases estrogen dependence and tamoxifen sensitivity. Cancer Res 63:3425–3429
18. Goldhirsch A, Glick JH, Gelber RD, Coates AS (2005) Meeting highlights: international expert consensus on the primary therapy of early breast cancer. Ann Oncol 16:1569–1583
19. Beck A, Etienne MC, Cheradame S, Fischel JL, Formento P, Renee N, Milano G (1994) A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumour sensitivity to fluorouracil. Eur J Cancer 30A:1517–1522
20. Xie W, Duan R, Chen I, Samudio I, Safe S (2000) Transcriptional activation of thymidylate synthase by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology 141:2439–2449