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ORIGINAL ARTICLE

Additive antitumor effect of concurrent treatment of 4-hydroxy tamoxifen with 5-fluorouracil but not with doxorubicin in estrogen receptor-positive breast cancer cells

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Abstract
Purpose The sequential addition of tamoxifen (TAM) to chemotherapy seems superior to its concurrent addition in patients with breast cancer. This study was conducted to clarify the hypothesis that there are differential interactions among TAM and chemotherapeutic agents.
Methods Estrogen receptor (ER)-α-positive or -negative breast cancer cells were treated with 4-hydroxy TAM (4OHT), 5-fluorouracil (FU) and/or doxorubicin (Dox). Changes in the expression levels of genes related to sensitivity and resistance to TAM, 5-FU or Dox were tested.
Results Concurrent treatment of 4OHT with 5-FU but not with Dox additively inhibited the growth of ER-α-positive cells. 5-FU did not change the expression levels of any tested genes related to either sensitivity or resistance to TAM. Although Dox did not change the expression levels of any genes related to the sensitivity to TAM, Dox significantly increased the expression levels of some genes related to TAM resistance, Eph A-2, ER-β, Fos and vascular endothelial growth factor. 4OHT significantly decreased thymidilate synthase (TS) activity.
Conclusions Although the antitumor effect of concurrent 4OHT and 5-FU was additive, that of concurrent 4OHT and Dox was less than additive in ER-α-positive cells. The increased expression of genes related to TAM resistance by Dox might be responsible for the interaction. Decreased TS activity by 4OHT might increase the antitumor activity of 5-FU. These findings may provide a preclinical rationale for concurrent use with 5-FU and TAM.

Keywords Breast Cancer · Concurrent · Doxorubicin · 5-Fluorouracil · Tamoxifen

Abbreviations

ANOVA	Analysis of variance
CT	Cycle threshold
DCC	Dextran-coated charcoal-stripped
Dox	Doxorubicin
DPD	Dihydropyrimidine dehydrogenase
E2	Estradiol
ER	Estrogen receptor
FBS	Fetal bovine serum
FdUMP	5-Fluoro-2′-deoxyuridine
FGF	Fibroblast growth factor
FU	5-Fluorouracil
HER	Human epidermal growth factor receptor
HIF	Hypoxia inducible factor
IC_{50}	50% inhibitory concentration
4OHT	4-OH-Tamoxifen
OPRT	Orotate phosphoribosyl transferase
PBS	Phosphate-buffered saline
PgR	Progesterone receptor
pS2	Trefoil factor 1
RT-PCR	Reverse transcriptional polymerase chain reaction

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SDF	Stromal cell-derived factor
TAM	Tamoxifen
TOP	Topoisomerase
TS	Thymidylate synthase
VEGF	Vascular endothelial factor

Introduction

Concurrent anthracycline-based chemotherapy and tamoxifen (TAM) has been suggested to be inferior to chemotherapy followed by TAM in the adjuvant setting for patients with hormone-responsive breast cancer [1, 2]. Recommendations provided by the St. Gallen Consensus Conference 2005 indicated that patients receiving chemotherapy should not start TAM until the completion of chemotherapy [3]. On the other hand, it has been reported that concurrent oral 5-fluorouracil (5-FU) derivative, UFT and TAM provide a better outcome for patients with estrogen receptor (ER)-positive breast cancer than either UFT or TAM alone [4]. This indicates that concurrent administration of 5-FU and TAM may lead to an additive antitumor effect in patients with ER-positive tumors. It could be hypothesized that there are differential interactions among TAM and chemotherapeutic agents, such as anthracycline and 5-FU, in terms of antitumor activity.

A number of basic investigations on the interaction between TAM and chemotherapeutic agents have been conducted in many laboratories and their findings have been inconsistent. In the early 1980s, it was reported that alterations in tumor cell kinetics, such as G_1-S blockade, induced by TAM may diminish the antitumor effect of chemotherapy [5]. In contrast, some researchers have reported synergistic interaction between TAM and 5-FU or an anthracycline, doxorubicin (Dox) in hormone-responsive breast cancer cells [6, 7]. Otherwise, other researchers have reported antagonistic interaction between TAM and 5-FU or Dox in similar model systems [8–10]. These conflicting results prompted us to further investigate the interaction among TAM and chemotherapeutic agents at the molecular level.

Previous basic studies on the interaction among TAM and chemotherapeutic agents have focused their attention on the influences of TAM on the cytotoxic effect of the agents. Therefore, the effects of chemotherapeutic agents, 5-FU and Dox, on various molecules related to the sensitivity or resistance to TAM were investigated in this study. The influences of TAM on the expression levels of molecules affecting sensitivity or resistance to 5-FU and Dox were also investigated.

Materials and methods

Reagents

17-β estradiol (E2) and 4-hydroxy tamoxifen (4OHT) were purchased from Sigma Chemical Co. (St. Louis, MI, USA). E2 and 4OHT were dissolved with 100% ethanol and added to the medium at a final ethanol concentration of 0.1%. 5-FU and Dox were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Kyowa Hakko Co., Ltd. (Tokyo, Japan), respectively. [6−^{14}C]-5-FU (56 mCi/mmol) and [6-^{3}H]-5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP, 625 GBq/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA, USA).

Cell lines

The KPL-1 human breast cancer cell line was established in our laboratory [11]; this cell line is ER-α-positive and estrogen-responsive [12]. ML-20 and MDA-MB-231 human breast cancer cell lines were kindly provided by 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 its biological characteristics are identical [13]; this cell line is ER-α-positive and estrogen-dependent. MDA-MB-231 cells are ER-α-negative and estrogen-unresponsive. All cell lines were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).

Cell growth assay

To reduce endogenous estrogen-like activity, phenol red-free RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% dextran-coated charcoal (DCC)-stripped FBS (Tissue Culture Biological, Tulanta, CA, USA) (estrogen-deprived medium) was used. Approximately 2×10^3 cells/well were inoculated

Table 1 Genes tested by real-time RT-PCR

Category	Tested genes
ER signaling	ER-α, CBP300, NCOA1, NCOA3, NCOR1, NCOR2
TAM resistance	BCAR-1, Cyclin D1, EphA-2, ER-β, Fas ligand, FGF-1, FGF-2, FGF-4, Fos, HER1, HER2, HIF-1α, Jun, p53, VEGF
5-FU metabolism	DPD, OPRT, TS
Dox sensitivity	TOPIIα
Dox resistance	MDR1, MRP
Estrogen responsive	PgR, pS2, Myc, SDF-1α

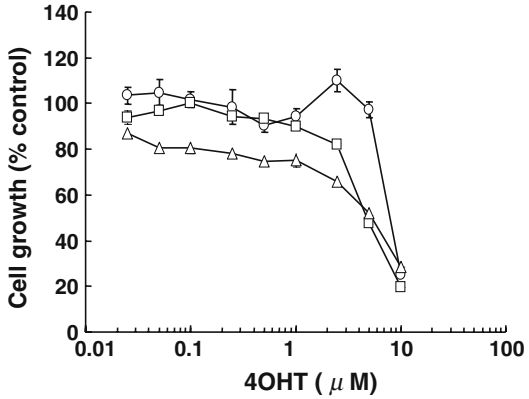

Fig. 1 Antitumor activity of 4OHT in ML-20 cells (*triangle*), KPL-1 cells (*square*) and MDA-MB-231 cells (*circle*). These cells were treated with 0.025–10 μM 4OHT for 4 days. The number of viable cells was evaluated with the crystal violet method. Values represent the means ± SE

into 96-well plates (Costar Corning Inc., Corning, NY, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS for a day. The cells were then washed once with phosphate-buffered saline (PBS) and cultured for 4 days in estrogen-deprived medium with vehicle (control), 0.1 nM E2, 1 μM 4OHT and/or indicated concentrations of 5-FU or Dox. In the case of Dox treatment, the Dox-containing medium was switched to Dox-free medium 16 h after the start of treatment. The number of viable cells was measured using the simplified crystal violet method [14]. Triplicate wells were treated in each experiment. Reproducibility was clarified in at least two separate experiments.

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

Semi-confluent KPL-1 cells in 6-well plates (Costar Corning Inc.) were incubated with phenol red-free RPMI-1640 medium supplemented with 10% DCC-stripped FBS plus vehicle (control), 0.1 nM E2, 1 μM 4OHT and/or indicated concentrations of 5-FU or Dox for 16 h. After incubation, the cells were collected and stored at −80°C. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The quality of total RNA was assessed with the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

TaqMan Low Density Arrays (Applied Biosystems, Foster City, CA, USA) were used in a two-step RT-PCR process. First-strand cDNA was synthesized from 2.5 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) as described by the manufacturer. PCR reactions were then carried out in Low Density Arrays using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. One hundred nanograms of cDNA combined with 1× TaqMan Universal PCR Master Mix (Applied Biosystems) were loaded into each port of the array (one

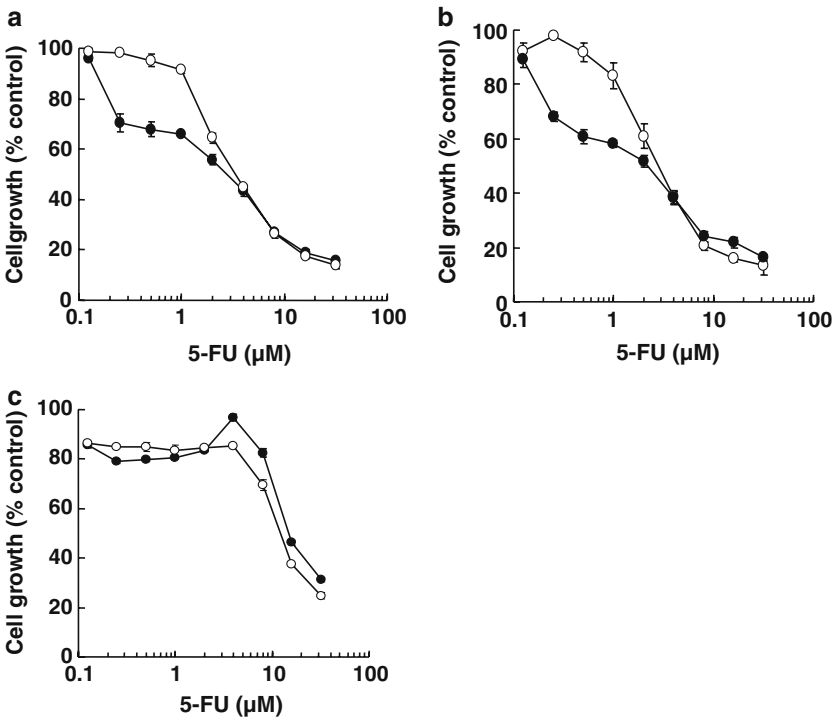

Fig. 2 Antitumor activity of concurrent 4OHT and 5-FU in ML-20 cells (**a**), KPL-1 cells (**b**) and MDA-MB-231 cells (**c**). These cells were treated with vehicle (*control*), 1 μM 4OHT and/or indicated concentrations of 5-FU for 4 days. The number of viable cells was evaluated with the crystal violet method. (*filled circle*) Treated with 4OHT and 5-FU; and (*open circle*), treated with 5-FU alone. Values represent the means ± SE

Table 2 Combination indexes with 5-FU and 4OHT

Cell line	5-FU	4OHT		Combination index (With versus Without)
		With	Without	
ML-20	IC_{50}	2.21[a]	3.73	0.59
	IC_{30}	0.60	1.85	0.33
	IC_{25}	0.41	1.50	0.27
KPL-1	IC_{50}	1.57	2.89	0.54
	IC_{30}	0.37	1.57	0.24
	IC_{25}	0.24	1.31	0.18
MDA-MB-231	IC_{50}	17.38	16.47	1.06
	IC_{30}	10.68	10.36	1.03
	IC_{25}	9.24	9.03	1.02

[a] 5-FU concentration

port contains 48 wells). The Low Density Arrays were thermal cycled at 50°C for 2 min and 94.5°C for 10 min, followed by 40 cycles at 97°C for 30 s, and 59.7°C for 1 min. The TaqMan strategies for each gene have been developed as TaqMan Gene Expression Assays by Applied Biosystems. Gene expression profiling was achieved using the comparative cycle threshold (CT) method of relative quantification (the calibrator samples were untreated cells, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the endogenous control). Relative expression ratios compared between treated and control cells were evaluated following the manufacturer's recommendations: over 2.0 for a significant increase and less than 0.5 for a significant decrease. Reproducibility was clarified in at least two separate experiments.

Six genes were tested as genes related to ER-α signaling, in other words, TAM sensitivity, ER-α, CBP300, NCOA1, NCOA3, NCOR1 and NCOR2. Fifteen genes were tested as genes related to TAM resistance, BCAR-1 [15], cyclin D1 [16], EphA-2 [17], ER-β [18], Fas ligand [19], fibroblast growth factor (FGF)-1 [20], FGF-2 [21], FGF-4 [22], Fos [23], human epidermal growth factor receptor 1 (HER1) [24], HER2 [25], hypoxia inducible factor (HIF)-1α [26], Jun [27], p53 [28] and vascular endothelial growth factor (VEGF) [29]. Three genes were tested as genes related to 5-FU metabolism, dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT) and thymidylate synthase (TS). Two genes were tested as genes related to Dox resistance, MDR-1 and MRP. Topoisomerase (TOP) IIα gene was tested as a gene related to Dox sensitivity. In addition, to clarify the hormone responsiveness of KPL-1 cells, four genes (progesterone receptor [PgR], trefoil factor 1 [pS2], Myc, and stromal cell-derived factor [SDF]-1α) were tested as target genes of ER signaling. All tested genes in this study were selected by the authors according to the survey results from a PubMed search (Table 1).

To clarify the results produced by Low Density Arrays, the TaqMan Real-time RT-PCR system using triplicate samples per gene was applied to four genes related to TAM resistance, EphA-2, ER-β, Fos and

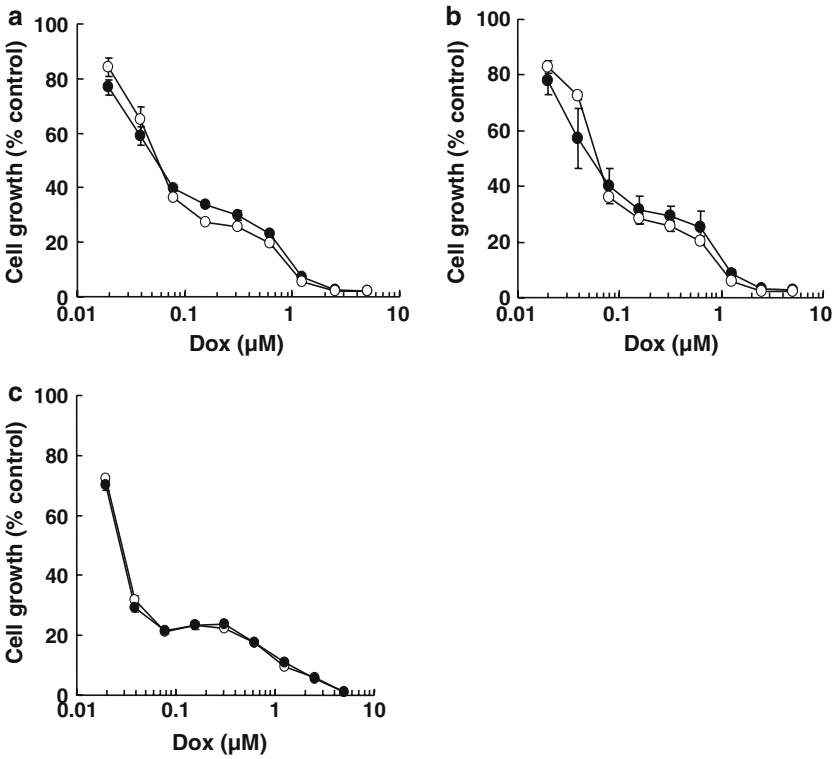

Fig. 3 Antitumor activity of concurrent 4OHT and Dox in ML-20 cells (**a**), KPL-1 cells (**b**) and MDA-MB-231 cells (**c**). These cells were treated with vehicle (*control*), 1 μM 4OHT and/or indicated concentrations of Dox. The number of viable cells was evaluated with the crystal violet method. (*filled circle*), Treated with 4OHT and 5-FU; and (*open circle*), treated with 5-FU alone. Values represent the means ± SE

Table 3 Combination indexes with Dox and 4OHT

Cell line	Dox	4OHT		Combination index (With versus Without)
		With	Without	
ML-20	IC_{50}	0.054[a]	0.056	0.97
	IC_{30}	0.026	0.033	0.78
	IC_{25}	0.021	0.029	0.73
KPL-1	IC_{50}	0.059	0.066	0.90
	IC_{30}	0.025	0.035	0.70
	IC_{25}	<0.02	0.029	NA
MDA-MB-231	IC_{50}	0.029	0.030	0.95
	IC_{30}	<0.02	<0.02	NA
	IC_{25}	<0.02	<0.02	NA

NA Not assessable

[a] Dox concentration

Table 4 Changes in the expression levels of estrogen-responsive genes after exposure to E2 plus-minus 4OHT

Genes	Relative expression ratio (vs. control)		
	Control	E2 with	
		None	4OHT
PgR	ND	633.2[a]	507.0[a]
SDF-1α	1	72.5	6.8
Myc	1	17.9	13.0
pS2	1	7.5	1.4

ND Not detectable

[a] Calculated by assuming that the CT values of control samples were 40

VEGF, which were significantly increased by Dox treatment. First-strand cDNA was synthesized from 2.5 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) as described by the manufacturer. Real-time RT-PCR was performed using the QuantiTect Probe PCR Kit (Qiagen) 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 (Applied Biosystems) were used. Briefly, 50 ng of 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 assays mix in a final volume of 50 μl. The conditions for real-time RT-PCR were 1 cycle of 50°C for 2 min; 1 cycle of 95°C for 15 min; 40 cycles of 94°C for 15 s and 60°C for 1 min. Gene expression profiling was achieved using the comparative CT method of relative quantification (the calibrator samples were untreated cells, with GAPDH used as the endogenous control). Gene Assay IDs of TaqMan gene expression assay by Applied Biosystems were Hs00171656_m1 for Eph-A2, Hs00230957_m1 for ER-β, Hs00170630_m1 for Fos, Hs00173626_m1 for VEGF, and Hs99999905_m1 for GAPDH, respectively.

Enzyme activity

Semi-confluent KPL-1 cells in 75 cm^2 culture flasks (Costar Corning Inc.) were incubated with phenol red-free RPMI-1640 medium supplemented with 10% DCC-stripped FBS and 0.1 nM E2 plus–minus 1 μM 4OHT for 16 h. After incubation, the cells were collected and stored at −80°C. TS activity in crude extract from the cells was measured according to the method reported by Spears et al. using [6-^{3}H]-FdUMP as a substrate [30]. DPD activity was measured using [6-^{14}C]-5-FU as a substrate based on the method previously described by Takechi et al. [31]. OPRT activity was measured using [6-^{14}C]-5-FU as a substrate based on the method previously described by Shirasaka et al. [32]. Intra- and inter-assay coefficients of variations were less than 10% in the respective assays (data not shown). Reproducibility was clarified in at least two separate experiments.

Western blotting

Semi-confluent KPL-1 cells in 75 cm^2 culture flasks were incubated with phenol red-free RPMI-1640 medium supplemented with 10% DCC-stripped FBS and 0.1 nM E2 plus–minus vehicle (control) or 1.0 or 2.0 μM Dox for 16 h. The cells were collected immediately or 24 h after treatment. Cell pellets were washed twice with PBS, lysed in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA), and boiled for 5 min under reducing conditions. Cell lysates were incubated with 10U Benzonase (Merck, Frankfurter, Germany), total protein concentrations were measured using RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the protocol of the manufacturer. The protein extract was subjected to electrophoresis in Bis–Tris gel (NuPAGE 10% Bis–Tris Gels, MOPS running buffer, Invitrogen Japan KK, Kyoto, Japan) for 45 min at a fixed voltage of 200 V under reducing conditions, and the electrophoretically separated protein was transferred to a PVDF membrane at 4°C for 3 h. After blocking with Block Ace (Dainippon Pharm. Co., Ltd., Osaka, Japan), the PVDF membrane was allowed to react with the anti-human EphA2 polyclonal antibody (C-20, 1:200, Santa Cruz, CA, USA), anti-human ER-β polyclonal antibody (H-150, 1:200, Santa Cruz), anti-human c-FOS polyclonal antibody (H-125, 1:200, Santa Cruz) or anti-human VEGF polyclonal antibody (147, 1:200, Santa Cruz) as the primary antibody for 1 h at room temperature, and incubated with alkaline

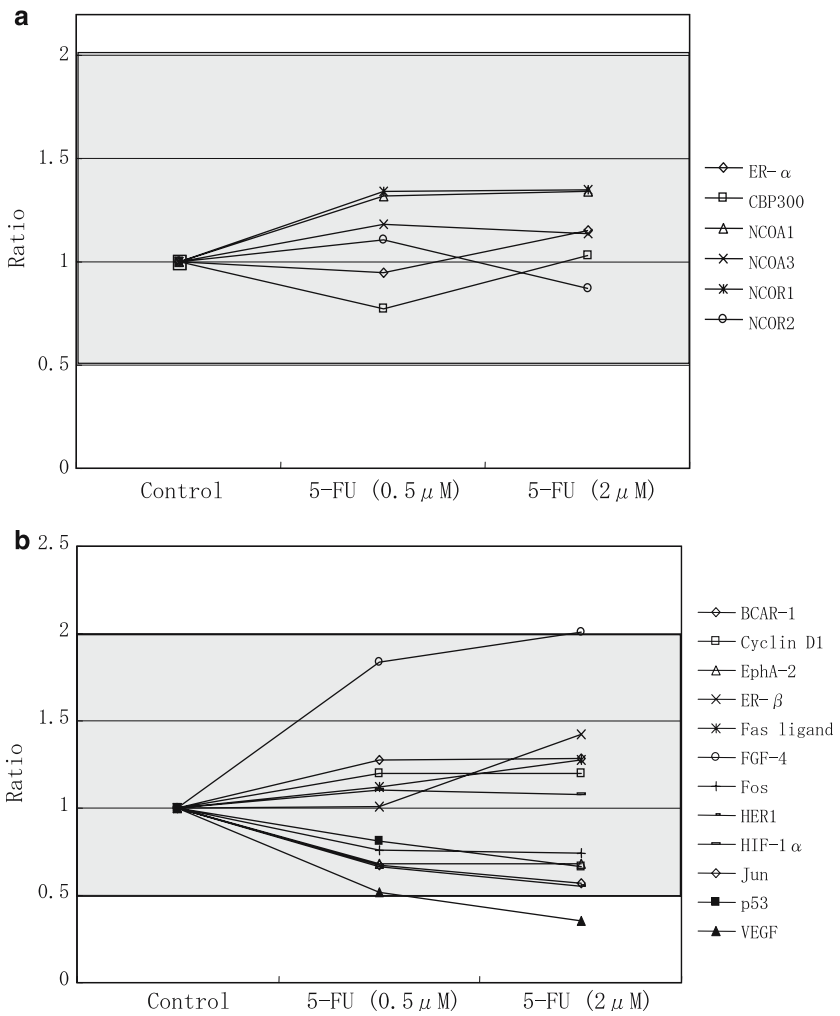

Fig. 4 Changes in the mRNA expression levels of genes related to ER signaling (**a**) and those related to TAM resistance (**b**) after exposure to 5-FU in KPL-1 cells. The cells were treated with vehicle (control) or 0.5 or 2.0 μM 5-FU for 16 h. Total RNA was extracted from collected cells and applied to real-time RT-PCR using TaqMan Low Density Arrays as described in Materials and methods. Relative expression ratios compared between treated and control cells were evaluated as follows: over 2.0 for a significant increase and less than 0.5 for a significant decrease. No detectable expression of FGF-1, FGF-2 and HER2 was observed

phosphatase-conjugated anti-rabbit IgG (1:5,000, Applied Biosystems) as the secondary antibody for 30 min at room temperature. Detection was performed with chemiluminescence substrate (CDP-Star, Applied Biosystems). Chemiluminescent signals were detected using a luminescent image analyzer (LAS-3000 UVmini, Fuji Film Corp., Tokyo, Japan). Membranes were tested for β-actin to confirm equal loading.

VEGF production

Semi-confluent KPL-1 cells in 75 cm^2 culture flasks were incubated with phenol red-free RPMI-1640 medium supplemented with 10% DCC-stripped FBS and 0.1 nM E2 plus–minus 1 μM 4OHT for 16 h. The culture medium was collected and filtered through a 0.45-μm filter to remove cell contamination. VEGF content in the culture medium was determined by enzyme-linked immunosorbent assay using the Quantikine assay kit (R&D Systems, Inc., MN, USA).

Statistical analysis

All values are expressed as the mean ± SE. ANOVA analysis with StatView computer software (ATMS Co., Tokyo, Japan) was used to compare the differences between two groups. A two-sided *P* value less than 0.05 was considered significant.

Results

Antitumor effects of 4OHT, 5-FU and Dox

A single treatment with 4OHT (1–10 μM) dose-dependently inhibited the growth of ER-α-positive ML-20 and KPL-1 cells but not that of ER-α-negative MDA-MB-231 cells. Only a pharmacologic dose of 10 μM 4OHT inhibited the growth of MDA-MB-231 cells (Fig. 1).

To investigate the interaction between 4OHT and 5-FU or Dox, three breast cancer cell lines were

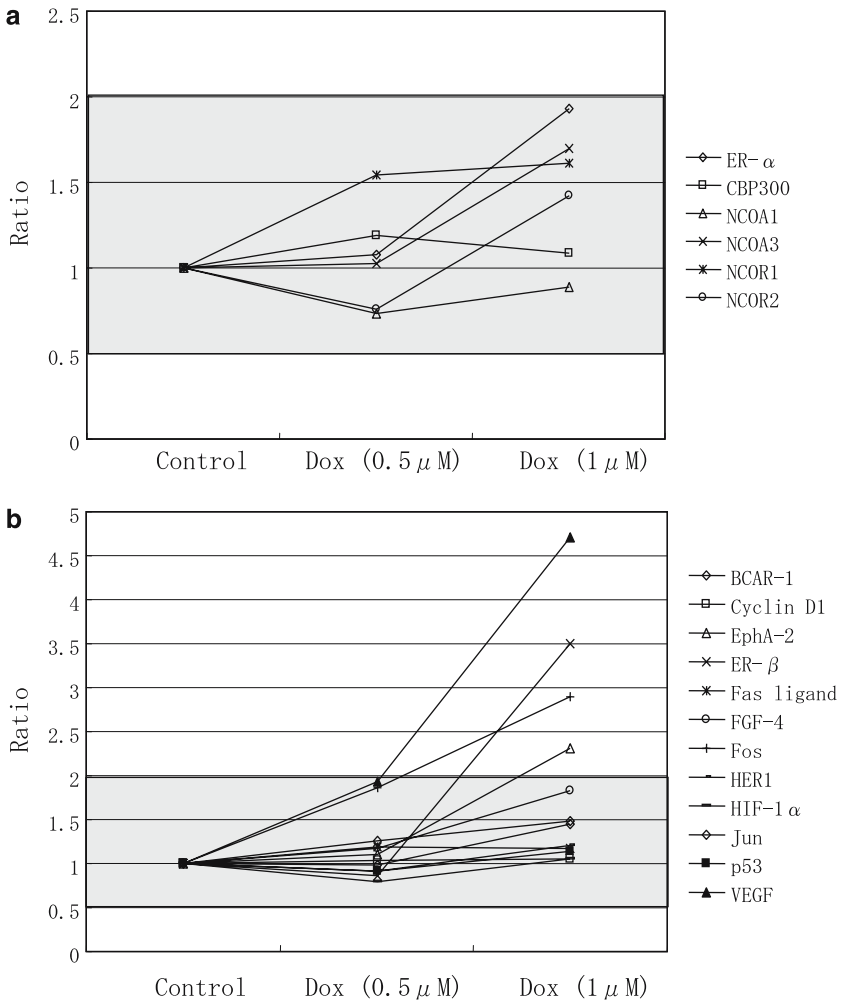

Fig. 5 Changes in the mRNA expression levels of genes related to ER signaling (**a**) and those related to TAM resistance (**b**) after exposure to Dox in KPL-1 cells. The cells were treated with vehicle (*control*) or 0.5 or 1.0 μM Dox for 16 h. Total RNA was extracted from collected cells and applied to real-time RT-PCR using TaqMan Low Density Arrays as described in Materials and methods. Relative expression ratios compared between treated and control cells were evaluated as follows: over 2.0 for a significant increase and less than 0.5 for a significant decrease. No detectable expression of FGF-1, FGF-2 and HER2 was observed

concurrently treated with 1 μM 4OHT and various concentrations of 5-FU or Dox. Concurrent treatments with 4OHT and 5-FU (0.25–1.0 μM) additively inhibited the growth of either ML-20 cells or KPL-1 cells (Fig. 2a, b). No such interaction was observed in MDA-MB-231 cells (Fig. 2c). Although the combination index of 5-FU (with versus without 4OHT) was 0.59 and 0.54 at 50% inhibitory concentration (IC_{50}) in ML-20 and KPL-1 cells, respectively, the index was 1.06 in MDA-MB-231 cells (Table 2). The antitumor effect of concurrent treatment with 4OHT and Dox (0.02–5 μM) was identical to that of a single treatment

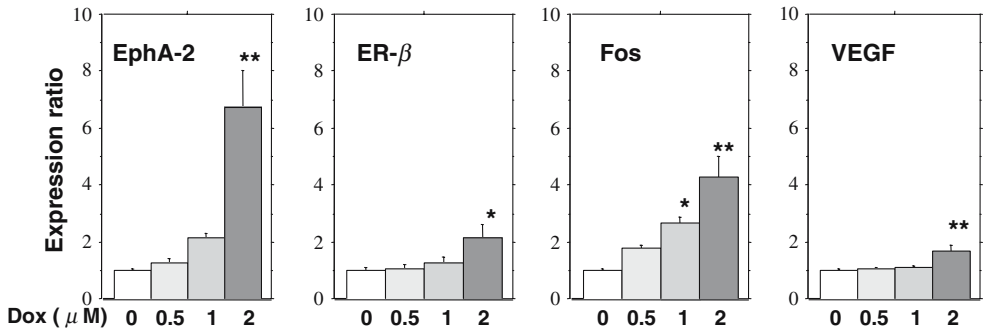

Fig. 6 Changes in the mRNA expression levels of four genes (EphA-2, ER-β, Fos and VEGF) related to TAM resistance after exposure to Dox in KPL-1 cells. Triplicate cells were treated with vehicle (*control*) or 0.5, 1.0 or 2.0 μM Dox for 16 h. Total RNA was extracted from collected cells and applied to real-time RT-PCR using the TaqMan Real-Time RT-PCR system as described in Materials and methods. Values represent the means ± SE. $^{*}P < 0.05$; and $^{**}P < 0.01$

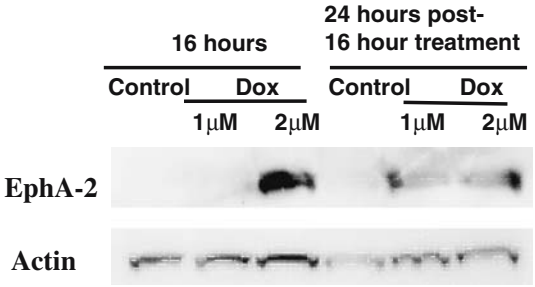

Fig. 7 Western blot analysis for EphA-2 in cell lysates extracted from KPL-1 cells. The KPL-1 cells were treated with vehicle (*control*) or 1.0 or 2.0 μM Dox for 16 h. The cells were collected immediately or 24 h after treatment

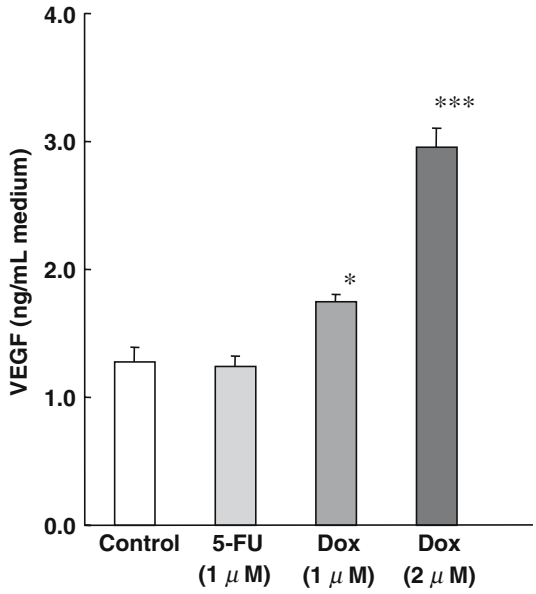

Fig. 8 VEGF secretion from KPL-1 cells into culture medium. The cells were treated with vehicle (*control*), 1.0 μM 5-FU, or 1.0 or 2.0 μM Dox for 16 h. VEGF content in the medium was determined by the enzyme-linked immunosorbent assay. $*P < 0.05$; and $***P < 0.001$

with Dox in all three cell lines (Fig. 3a–c). The combination index of Dox (with versus without 4OHT) was equal to or more than 0.90 in all three-cell lines at the IC_{50} (Table 3).

Estrogen responsiveness of KPL-1 cells

To clarify the estrogen responsiveness of ER-α-positive KPL-1 breast cancer cells, changes in the expression levels of four estrogen responsive genes were tested by real-time RT-PCR using TaqMan Low Density Arrays after exposure to 0.1 nM E2. The effects of concurrent E2 and 4OHT on their expression levels were also investigated. The expression levels of all four genes were significantly increased by E2 and decreased by 4OHT as expected (Table 4).

Table 5 Changes in the expression levels of genes related to 5-FU metabolism and Dox resistance/sensitivity after exposure to 4OHT

Category	Gene	Relative expression ratio	
		Control	4OHT
5-FU metabolism	DPD	1	1.10
	OPRT	1	0.80
	TS	1	0.63
Dox resistance	MDR1	ND	ND
	MRP	1	0.68
Dox sensitivity	TOP IIα	1	0.93

ND Not detectable

Effects of 5-FU and Dox on the expression levels of genes related to TAM sensitivity or TAM resistance

To test the hypothesis that 5-FU and Dox differentially influence the antitumor activity of 4OHT in ER-α-positive breast cancer cells, changes in the expression levels of genes related to TAM sensitivity or TAM resistance were tested by real-time RT-PCR using TaqMan Low Density Arrays after exposure to 5-FU or Dox in KPL-1 cells.

Treatment with 0.5 μM 5-FU (the concentration at which additive antitumor activity was observed in Fig. 2b) for 16 h did not significantly change the expression levels of six genes related to TAM sensitivity and 15 genes related to TAM resistance (Fig. 4a, b). Although neither 0.5 μM nor 1.0 μM Dox significantly changed the expression levels of genes related to TAM sensitivity (Fig. 5a), 1.0 μM Dox significantly increased the expression levels of 4 of 15 genes related to TAM resistance, EphA-2, ER-β, Fos and VEGF (Fig. 5b).

To clarify the results obtained by Low Density Arrays, the TaqMan Real-Time RT-PCR system using triplicate samples per gene was applied to the four genes related to TAM resistance. Treatment with Dox (0.5–2.0 μM) for 16 h dose-dependently increased mRNA expression levels of all four genes as expected (Fig. 6).

To further investigate the effects of Dox on the protein expression levels of the four genes, Western blot analysis was performed. Although ER-β, Fos and VEGF were undetectable by Western blotting in either control or Dox-treated cell lysates, the protein expression of EphA-2 was significantly increased by exposure to 2.0 μM Dox for 16 h compared with the control. The increased expression of EphA-2 was also observed 24 h after treatment with either 1.0 or 2.0 μM Dox (Fig. 7). In addition, changes in VEGF secretion from KPL-1 cells into medium were investigated. VEGF secretion

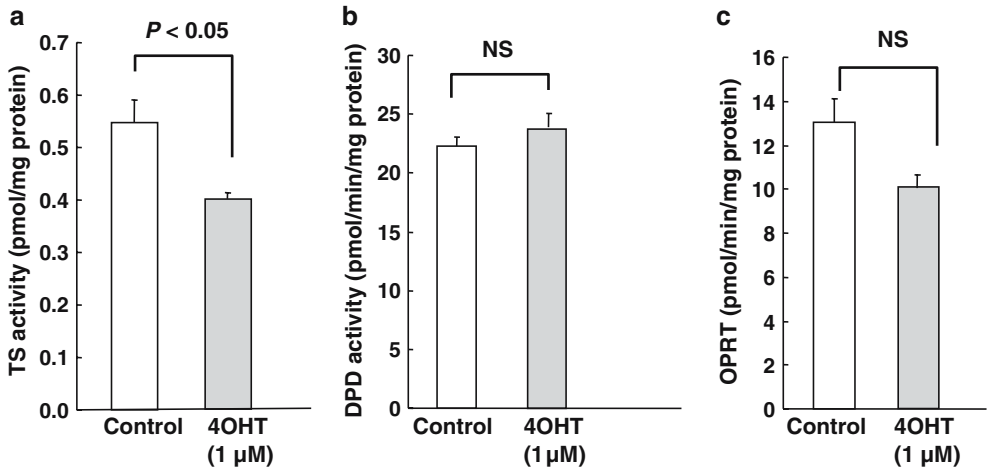

Fig. 9 Changes in enzyme activity of TS (**a**), DPD (**b**) and OPRT (**c**) after exposure to 4OHT in KPL-1 cells. The cells were treated with vehicle (*control*) or 1.0 μM 4OHT for 16 h. Enzyme activities in crude extract from collected cells were measured as described in Materials and methods. *NS* not significant

was significantly increased by exposure to either 1.0 or 2.0 μM Dox but not 1.0 μM 5-FU for 16 h compared with the control (Fig. 8).

Effects of 4OHT on the expression levels of genes related to sensitivity and resistance to 5-FU or Dox

To test the hypothesis that 4OHT influences the antitumor activity of 5-FU or Dox in ER-α-positive breast cancer cells, changes in the expression levels of genes related to 5-FU metabolism and sensitivity or resistance to Dox were tested after exposure to 1.0 μM 4OHT in KPL-1 cells. Single treatment with 4OHT did not significantly change the expression levels of three genes related to 5-FU metabolism, two genes related to Dox resistance and one gene related to sensitivity to Dox (Table 5).

To further investigate the effects of 4OHT on enzymes related to 5-FU metabolism, the changes in enzyme activity of TS, DPD and OPRT were investigated. Single treatment with 4OHT significantly decreased TS activity but not DPD and OPRT activities (Fig. 9a, b, c).

Discussion

Biological interaction between TAM and chemotherapy has long been investigated to clarify the rationale for their combined use in the treatment of breast cancer patients. There are at least two hypotheses to explain their synergistic or antagonistic interaction. The first hypothesis in favor of antagonism is that TAM delays G_1-S cell-cycle progression associated with a reduction in the number of proliferating tumor cells, and this cytostatic effect of TAM results in reduced sensitivity to chemotherapeutic agents [5]. The second hypothesis in favor of synergism is that TAM interacts with an efflux pump, *P*-glycoprotein, inhibits its function and increases the intracellular accumulation of chemotherapeutic agents, such as anthracyclines, and these effects lead to a synergistic antitumor effect of TAM and chemotherapeutic agents [7]. Although these two possible action mechanisms were successfully clarified in different laboratories, conflicting observations on the interaction between TAM and chemotherapeutic agents in terms of antitumor activity have been reported by different laboratories. For example, while synergistic interaction between 5-FU and TAM was reported by one group [6], antagonism was observed in other laboratories [8, 9]. Similarly, interaction between TAM and Dox has been reported to be additive or antagonistic in different laboratories [7–10]. These inconsistent findings provide clinicians with no rationale to use TAM and chemotherapeutic agents concurrently in the treatment of breast cancer patients.

Another weak point in the previous basic research on the interaction between TAM and chemotherapeutic agents is the lack of investigation into the effects of chemotherapeutic agents on TAM sensitivity. Interaction should be investigated both ways; that is, the effects of TAM on agent sensitivity as well as effects of the agent on TAM sensitivity. Several cycles of intravenous chemotherapy or continuous oral 5-FU derivative may influence the antitumor activity of TAM.

In this study, concurrent 5-FU (0.25–1.0 μM) and 4OHT additively inhibited the growth of ER-α-positive

breast cancer cells but not ER-α-negative cells (Fig. 2a–c; Table 2). In contrast, concurrent Dox (0.02–5.0 μM) and 4OHT did not additively inhibit the growth of either ER-α-positive or -negative breast cancer cells (Fig. 3a–c; Table 3). These findings indicate that the interaction between TAM and 5-FU is additive but the interaction between TAM and Dox is less than additive in terms of antitumor activity. In addition, no significant changes in the mRNA expression levels of tested genes related to TAM sensitivity or TAM resistance were observed by treatment with 0.5 μM 5-FU, at which an additive antitumor effect of TAM and 5-FU was observed (Fig. 4a, b). In contrast, 1.0 μM Dox significantly increased the mRNA expression levels of four tested genes related to TAM resistance, EphA-2, ER-β, Fos and VEGF but not those of genes related to TAM sensitivity (Figs. 5a, b, 6). In addition, the protein expression levels of EphA-2 and VEGF were significantly increased by 1.0 μM and/or 2.0 μM Dox, respectively (Figs. 7, 8). To the best of our knowledge, this is the first study suggesting the increased expression of genes related to TAM resistance by Dox.

Overexpression of either EphA-2 or Fos in estrogen-dependent breast cancer cells has been reported to produce an estrogen-independent phenotype [17, 23]. The expression levels of either ER-β or VEGF have been also reported to relate inversely to the response to endocrine therapy in breast cancer patients [18, 29]. In this study, since extensive time-course studies of these genes, such as investigating their expression levels a week after Dox exposure, were not investigated, it should be cautiously concluded that an increase in the expression levels of these TAM resistance genes by Dox is sustained for a long period and leads to less than additive interaction between TAM and Dox in terms of antitumor activity.

The effects of TAM on sensitivity or resistance to 5-FU and Dox were also investigated in this study. In the real-time RT-PCR system, no significant change in the expression levels of genes related to 5-FU metabolism and resistance or sensitivity to Dox was observed after treatment with 4OHT (Table 5); however, TS activity was significantly decreased by 4OHT (Fig. 9a). It has been reported that TS activity inversely relates to 5-FU sensitivity [33]. Decreased TS activity by 4OHT might be responsible for the additive interaction of TAM and 5-FU in terms of antitumor activity. On the other hand, it has been reported that multi-drug resistance molecules, MDR-1 and MRP, interact with 4OHT, which reduces their function through protein–protein interaction [7, 34]. Changes in the expression levels of multi-drug resistance molecules may not directly influence Dox sensitivity.

Recent clinical evidence has indicated that concurrent TAM and anthracycline-based chemotherapy is inferior to their sequential use [1, 2] and that concurrent TAM and an oral 5-FU derivative, UFT, is superior to each respective agent alone in the adjuvant setting for patients with ER-positive breast cancer [3]. Interestingly, the results of this study strongly support such clinical evidence and provide a preclinical rationale for concurrent use with 5-FU and TAM. However, interaction between two different agents in a patient's body is complicated, and it is very difficult to evaluate interaction among TAM and multiple chemotherapeutic agents in a combined chemotherapy. Further basic research is clearly needed to identify the most effective sequence of endocrine therapy and chemotherapy in the treatment of breast cancer patients.

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References

1. Albain KS, Green SJ, Ravdin PM, Cobau CD, Levine EG, Ingle JN, Pritchard KI, Schneider DJ, Abeloff MD, Norton L, Henderson IC, Lew D, Livingston RB, Martino S, Osborne CK (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 (Abstract 143)
2. 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
3. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ; Panel members (2005) Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. Ann Oncol 16:1569–1583
4. 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
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. 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

8. 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
9. 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
10. 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
11. 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
12. 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
13. 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
14. 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
15. van der Flier S, Brinkman A, Look MP, Kok EM, Meijer-van Gelder ME, Klijn JG, Dorssers LC, Foekens JA (2000) Bcar1/p130Cas protein and primary breast cancer: prognosis and response to tamoxifen treatment. J Natl Cancer Inst 92:120–127
16. Wilcken NR, Prall OW, Musgrove EA, Sutherland RL (1997) Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. Clin Cancer Res 3:849–854
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. Speirs V, Malone C, Walton DS, Kerin MJ, Atkin SL (1999) Increased expression of estrogen receptor β mRNA in tamoxifen-resistant breast cancer patients. Cancer Res 59:5421–5424
19. Reimer T, Koczan D, Muller H, Friese K, Thiesen HJ, Gerber B (2002) Tumour Fas ligand:Fas ratio greater than 1 is an independent marker of relative resistance to tamoxifen therapy in hormone receptor positive breast cancer. Breast Cancer Res 4:R9
20. Zhang L, Kharbanda S, Hanfelt J, Kern FG (1998) Both autocrine and paracrine effects of transfected acidic fibroblast growth factor are involved in the estrogen-independent and antiestrogen-resistant growth of MCF-7 breast cancer cells. Cancer Res 58:352–361
21. Vandermoere F, El Yazidi-Belkoura I, Adriaenssens E, Lemoine J, Hondermarck H (2005) The antiapoptotic effect of fibroblast growth factor-2 is mediated through nuclear factor-κB activation induced via interaction between Akt and IκB kinase-β in breast cancer cells. Oncogene 24:5482–5491
22. McLeskey SW, Kurebayashi J, Honig SF, Zwiebel J, Lippman ME, Dickson RB, Kern FG (1993) Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res 53:2168–2177
23. Gee JM, Willsher PC, Kenny FS, Robertson JF, Pinder SE, Ellis IO, Nicholson RI (1999) Endocrine response and resistance in breast cancer: a role for the transcription factor Fos. Int J Cancer 84:54–61
24. van Agthoven T, van Agthoven TL, Portengen H, Foekens JA, Dorssers LC (1992) Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. Cancer Res 52:5082–5088
25. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK (1993) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24:85–95
26. Kurebayashi J, Otsuki T, Moriya T, Sonoo H (2001) Hypoxia reduces hormone responsiveness of human breast cancer cells. Jpn J Cancer Res 92:1093–1101
27. Smith LM, Wise SC, Hendricks DT, Sabichi AL, Bos T, Reddy P, Brown PH, Birrer MJ (1999) cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. Oncogene 18:6063–6070
28. Berns EM, Klijn JG, van Putten WL, de Witte HH, Look MP, Meijer-van Gelder ME, Willman K, Portengen H, Benraad TJ, Foekens JA (1998) p53 protein accumulation predicts poor response to tamoxifen therapy of patients with recurrent breast cancer. J Clin Oncol 16:121–127
29. Manders P, Beex LV, Tjan-Heijnen VC, Span PN, Sweep CG (2003) Vascular endothelial growth factor is associated with the efficacy of endocrine therapy in patients with advanced breast carcinoma. Cancer 98:2125–2132
30. Spears CP, Shahinian AH, Moran RG, Heidelberger C, Corbett TH (1982) In vivo kinetics of thymidylate synthase inhibition in 5-fluorouracil-sensitive and resistant murine colon adenocarcinomas. Cancer Res 42:450–456
31. Takechi T, Okabe H, Fujioka A, Murakami Y, Fukushima M (1998) Relationship between protein levels and gene expression of dihydropyrimidine dehydrogenase in human tumor cells during growth in culture and in nude mice. Jpn J Cancer Res 89:1144–1153
32. Shirasaka T, Shimamoto Y, Fukushima M (1993) Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. Cancer Res 53:4004–4009
33. 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
34. Boumendjel A, Baubichon-Cortay H, Trompier D, Perrotton T, Di Pietro A (2005) Anticancer multidrug resistance mediated by MRP1: recent advances in the discovery of reversal agents. Med Res Rev 25:453–472