

Effects of a combined treatment with tamoxifen and estrogen receptor β agonists on human breast cancer cell lines

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Abstract

Introduction Coexpression of estrogen receptors (ER) α and β is present in about half of all breast cancer cases. Whereas ER α is a well-established target for endocrine therapy with the selective estrogen receptor modulator tamoxifen, the applicability of ER β as target in breast cancer therapy is unclear. In this study, we examined the effects of two synthetic ER β agonists alone and in combination with tamoxifen on ER α / β -positive breast cancer cells.

Methods We treated MCF-7 and T-47D breast cancer cells with the ER β agonists ERB-041 and WAY-200070 and measured the effects on cell growth. In addition, transcriptome analyses were performed by means of Affymetrix GeneChip arrays.

Results When given alone, ER β agonists ERB-041 and WAY-200070 did not affect the growth of MCF-7 or T-47D cells. In contrast, addition of these drugs to tamoxifen increased its growth-inhibitory effect on both cell lines. This effect was more pronounced under serum-free conditions, but was also observed in the presence of serum in T-47D cells. Transcriptome analyses revealed a

set of genes regulated after addition of ER β agonists including S100A8 and CD177.

Conclusion The observed enhanced growth-inhibitory effects of a combination of tamoxifen and ER β agonists in vitro encourage further studies to test its possible use in the clinical setting.

Keywords Estrogen receptor beta · Breast cancer cell line · Tamoxifen · Combined treatment

Introduction

Estrogens are essential for growth and development of the mammary gland, and have been associated with promotion and growth of breast cancer. The expression of sexual steroid hormone receptors like estrogen receptors (ER) α and β plays an important role in cell cycle regulation. These receptors are ligand-regulated transcription factors, their action is determined by cooperation and competition between estrogen receptor subtypes, coregulators and other transcription factors [1]. Whereas ER α is primarily thought to mediate the proliferative effect of estrogens in breast tissue, the function of ER β in this tissue is more complex. There is evidence that ER β exerts antagonistic effects on ER α action, resulting for example in reduction of cellular proliferation. The growth-inhibitory action of ER β and the observed decline of ER β expression during carcinogenesis have raised the hypothesis that this receptor might act as a tumor suppressor in hormone-dependent tissues like the breast [2–4].

Due to the reported antitumoral actions of ER β , activation of this receptor by specific agonists might be a feasible treatment option for breast cancer. In this study, we used the highly specific synthetic ER β agonists

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ERB-041 and WAY-200070 [5, 6]. ERB-041 is known to display a more than 200-fold selectivity for ER β (IC_{50} ER β / α = 2/1,216 nM), WAY-200070 still has a 68-fold higher selectivity for ER β than for ER α (IC_{50} ER β / α = 2/155 nM) [7]. WAY-200070 previously has been used as an ER β agonist in several studies on brain neurochemistry, but not in the context of cancer. In contrast, ERB-041 has been used to identify about 1,000 ER β binding sites in the genome of MCF-7 breast cancer cells [8]. In the same study, ERB-041 has been shown to regulate about 500 genes in MCF-7 cells via ER β in an ER α -independent manner, confirming its activity and specificity in human breast cancer cells.

The selective estrogen receptor modulator (SERM) tamoxifen is widely used for endocrine therapy of both early and advanced ER α -positive breast cancer in pre- and post-menopausal women. Tamoxifen is metabolized into its active metabolites 4-OH tamoxifen and endoxifen, which compete with estrogens for binding to ERs [9]. After binding of tamoxifen metabolites, alternative conformations of ER proteins are triggered which lead to a decreased ability to interact with coactivator proteins and to activate transcription of estrogen-responsive genes [10].

In the present study, we tested to what extent combination of tamoxifen with the ER β agonists ERB-041 and WAY-200070 would increase the antitumoral action of tamoxifen in terms of growth inhibition of different human breast cancer cell lines and examined the underlying molecular mechanisms by means of DNA microarray analysis.

Materials and methods

Material

Phenol red-free DMEM culture medium was obtained from Invitrogen (Karlsruhe, Germany), FCS was purchased from PAA (Pasching, Austria). MCF-7 and T-47D breast cancer cells were obtained from American Type Culture Collection (Manassas, USA). Serum replacement 2 (SR2) was from Sigma (Deisenhofen, Germany). 4-OH tamoxifen (TAM), ERB-041 and WAY-200070 were from Tocris (Bristol, UK). RNeasy Mini Kit, RNase Free DNase Set and Quantitect SYBR Green PCR Kit were obtained from Qiagen (Hilden, Germany). PCR primers were synthesized at Metabion (Planegg-Martinsried, Germany). Platinum Pfx Polymerase was purchased at Invitrogen (Karlsruhe, Germany). GeneChip Human Gene 1.0 ST Arrays and all kits and reagents for array processing were from Affymetrix.

Cell culture and proliferation assays

MCF-7 and T-47D breast cancer cells were maintained in DMEM/F12 medium supplemented with 10 % FCS and

10 mM sodium pyruvate. Cells were cultured with 5 % CO₂ at 37 °C in a humidified incubator. For cell proliferation assays, cells cultured in DMEM/F12 supplemented with 10 % FBS or 1 \times SR2 were seeded in 96-well plates in triplicates (1,000 cells/well). One day later, cells growing in the presence of 1 nM E2 were treated with TAM (100/1,000 nM), 10 nM ERB-041, 10 nM WAY200070 or combinations of TAM with an ER β agonist. On days 0, 3, 4, 5 and 6, relative numbers of viable cells were measured using the fluorimetric, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer's instructions at 560Ex/590Em nm in a Victor3 multilabel counter (PerkinElmer, Germany). Cell growth was expressed as percentage of day 0.

Reverse transcription and qPCR

Total RNA from the tumor cell line was isolated by means of the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. From 0.3 μ g total RNA, cDNA was synthesized using 100 U M-MLV-P reverse transcriptase (Promega), 2.5 mM dNTP mixture and 50 pM random primers (Invitrogen). For real time PCR detection of gene expression in an intron-spanning manner, 2 μ l cDNA were amplified using Light Cycler[®]FastStart DNA master mix SYBR Green I and the LightCycler 2.0 PCR device (Roche Diagnostics, Mannheim, Germany). The PCR program was 95 °C for 15 min, followed by 45 PCR cycles (95 °C for 10 s, 56 °C for 30 s, 72 °C for 30 s) and a final extension for 5 min at 72 °C, followed by a standard melting curve analysis. In all RT-PCR experiments, a 190-bp β -actin fragment was amplified as reference gene using intron-spanning primers actin-2573 and actin-2876. Data were analyzed using the comparative $\Delta\Delta C_T$ method [11] calculating the difference between the threshold cycle (C_T) values of the target and reference gene of each sample and then comparing the resulting ΔC_T values between different samples. In these experiments, mRNA not subjected to reverse transcription was used as a negative control to distinguish cDNA and vector or genomic DNA amplification.

GeneChip[™] microarray assay

Processing of RNA samples (two biological replicates from MCF-7 cells treated with 1 nM E2, 1 nM E2 + 100 nM TAM, 1 nM E2 + 100 nM TAM + 10 nM ERB-041 or 1 nM E2 + 100 nM TAM + 10 nM WAY200070) was performed at the local Affymetrix Service Provider and Genomics Core Facility, "KFB—Center of Excellence for Fluorescent Bioanalytics" (Regensburg, Germany; <http://www.kfb-regensburg.de>). Sample preparation for microarray hybridization was carried out as described in the

Affymetrix GeneChip® Whole Transcript (WT) Sense Target Labeling Assay manual. 300 ng of total RNA was used to generate double-stranded cDNA, omitting the initial ribosomal RNA (rRNA) reduction procedure. Subsequently synthesized cRNA (WT cDNA Synthesis and Amplification Kit, Affymetrix) was purified and reverse transcribed into single-stranded (ss) DNA. After purification, the ssDNA was fragmented using a combination of uracil DNA glycosylase (UDG) and apurinic/aprimidinic endonuclease 1 (APE 1). Fragmented DNA was labeled with biotin (WT Terminal Labeling Kit, Affymetrix), and 2.3 µg DNA was hybridized to the GeneChip Human Gene 1.0 ST Array (Affymetrix) for 16 h at 45 °C in a rotating chamber. Hybridized arrays were washed and stained in an Affymetrix Washing Station FS450 using preformulated solutions (Hyb, Wash & Stain Kit, Affymetrix), and the fluorescent signals were measured with an Affymetrix GeneChip® Scanner 3000-7G.

Microarray data analysis

Summarized probe signals were created using the RMA algorithm in the Affymetrix GeneChip Expression Console Software and exported into Microsoft Excel. Data was then analyzed using Ingenuity IPA Software (Ingenuity Systems, Stanford, USA) and the GeneMANIA prediction server [12]. Genes with more than 2-fold changed mRNA levels after treatment with ERβ agonists were considered to be differentially expressed and were included in the analyses.

Results

Effect of a combination of ERB-041 and WAY-200070 with 4-OH tamoxifen on breast cancer cell growth

First, we verified ERβ expression in the employed cell lines MCF-7 and T-47D, which are known to express both ERα and β, and observed similar ERβ transcript levels in both cell lines (Fig. 1a). We now tested to what extent treatment with the single substances ERB-041 and WAY-200070 would affect proliferation of these breast cancer cell lines. For this purpose, we treated both cell lines cultured in E2-containing medium with 10 nM of each drug for up to 6 days. Neither MCF-7 nor T-47D cells showed a significant response to this treatment in terms of growth reduction (Fig. 1b). To rule out the possibility that growth effects of the ERβ agonists would be covered by growth factors present in the medium supplement FBS, we repeated these experiments in the absence of serum, using defined growth factor-free serum replacement SR2 (Sigma, Deisenhofen, Germany). Under these growth factor-free conditions, we

again did not observe any effects of both cell lines to treatment with ERB-041 and WAY-200070 (data not shown).

As expected, single treatment with 4-OH tamoxifen (TAM, 0.1 and 1 µM) for 6 days in E2-containing, serum-

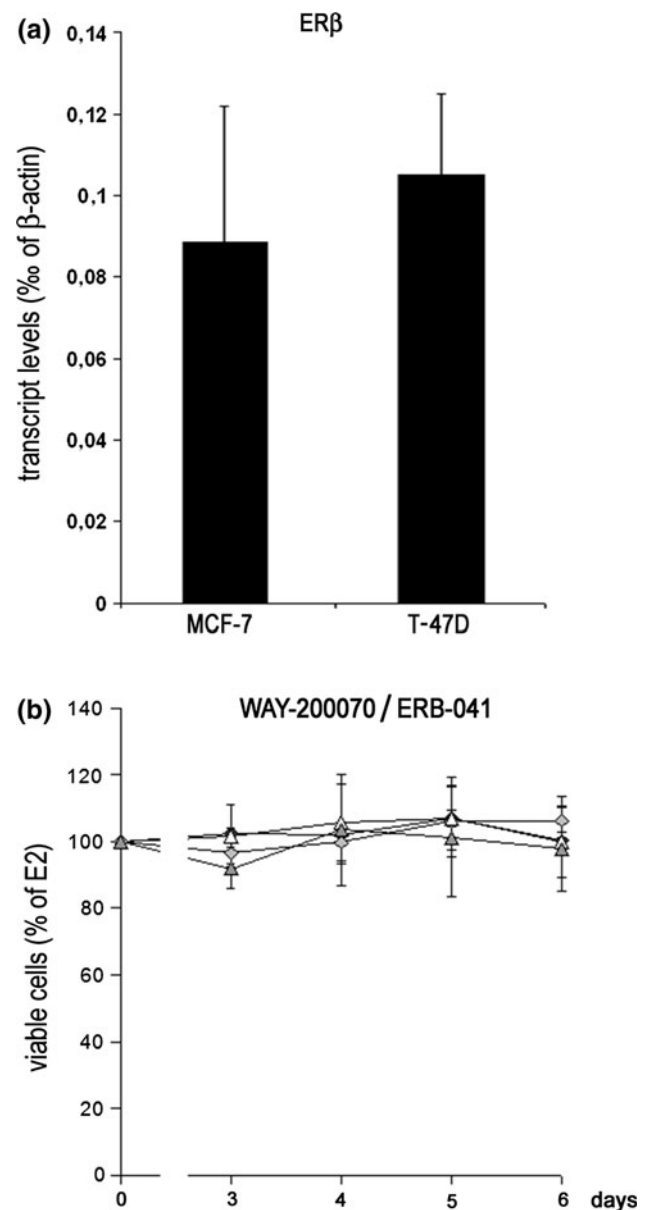


Fig. 1 Characterization of the breast cancer cell lines used in this study. **a** ERβ transcript levels in MCF-7 and T-47D cells as detected by RT-qPCR as described in the “Materials and methods” section. **b** Absence of significant effects of a single treatment with ERβ agonists on growth of MCF-7 and T-47D breast cancer cells. Cell lines cultured in SR2 medium supplemented with 1 nM E2 were treated with 10 nM of ERB-041 or WAY-200070 for up to 6 days. Relative numbers of viable cells were measured by means of the “Cell Titer Blue” assay as described in the “Materials and methods” section and are expressed in percent of E2 + vehicle EtOH. Triangles MCF-7 cells (white ERB-041, grey WAY-200070). Rhombs T-47D cells (white ERB-041, grey WAY-200070). (n = 4)

free medium significantly reduced viable numbers of MCF-7 cells down to 33.7 % and T-47D cell numbers to 60.7 % (Fig. 2). Both cell lines reacted in a similar manner to treatment with TAM when cultured with standard medium supplement 10 % FBS (data not shown).

We then performed combined treatment with 4-OH tamoxifen and ER β agonists and observed a significantly stronger growth reduction than achieved by treatment with the SERM alone. This effect was more pronounced under defined, growth factor-free conditions (Fig. 2). In MCF-7 cells, combined treatment with ERB-041 after 3 days decreased viable cell numbers from 59.4 (100 nM TAM) to 45.7 % and from 56.0 (1 μ M TAM) to 40.3 %. A significant difference to single treatment with TAM was still observed 6 days after treatment. Combined treatment with WAY-200070 exerted similar effects on this cell line. After 3 days of treatment, addition of WAY-200070 reduced the number of viable cells from 59.4 (100 nM TAM) to 36.2 %, whereas on day six, viable cell numbers were still found to be decreased from 33.6 (100 nM TAM) to 20.7 % (Fig. 2). In T-47D cells, both ER β agonists increased the growth-inhibitory effect of TAM to a similar extent. After 4–6 days of treatment, combined treatment exerted significantly larger effects than treatment with TAM alone. On day six, addition of ER β agonists decreased the number of viable cells from

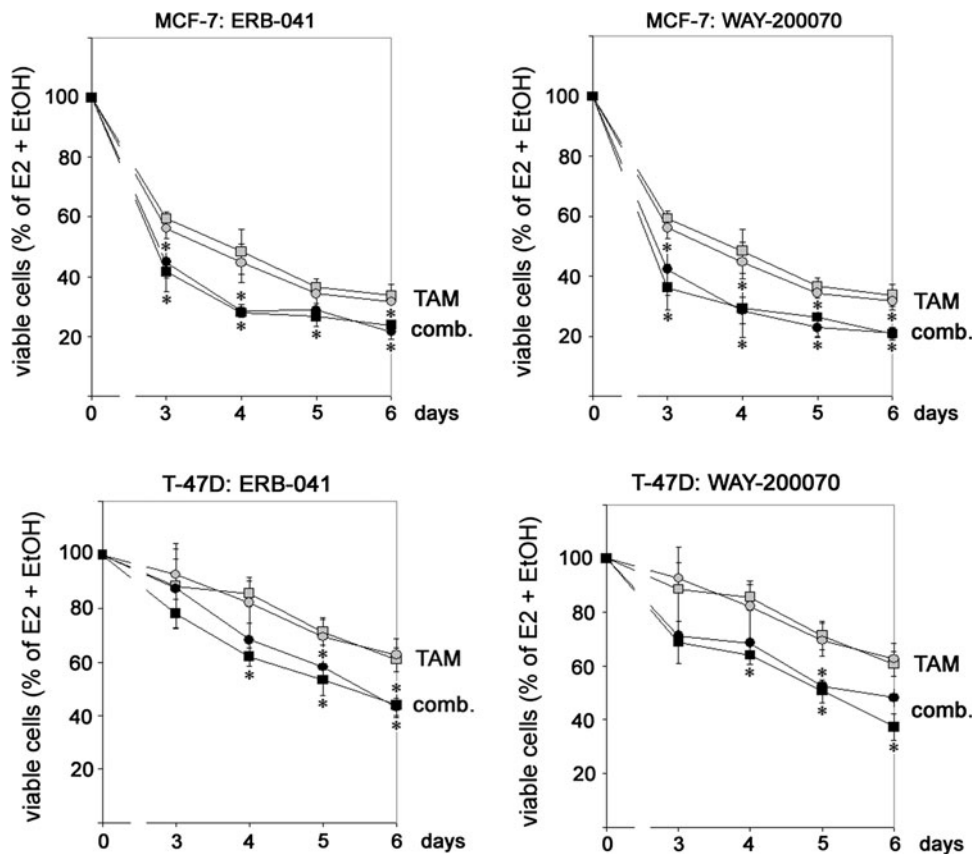
60.7 (100 nM TAM) to 43.9 % (ERB-041) or 37.5 % (WAY-200070) (Fig. 2).

To examine whether the presence of serum growth factors might affect the superior action of combined treatment on breast cancer cells, we repeated these experiments in DMEM/F12 supplemented with 10 % FBS. In T-47D cells, addition of ERB-041 increased the growth-inhibitory effect of TAM after 3 and 4 days after treatment (Fig. 3). However, this difference declined 1 day later and disappeared on day six. Addition of the second ER β agonist, WAY-200070, did not affect the antiproliferative action of TAM on T-47D cells in the presence of serum. In MCF-7 cells, we observed small differences between single treatment with TAM and combined treatment, which did not reach statistical significance (data not shown).

Transcriptome analysis

To examine the molecular mechanisms underlying the observed superior effect of a combination of TAM with ER β agonists on breast cancer cells, we analyzed changes of the MCF-7 transcriptome triggered by these treatments. Analyzing two biological replicates of RNA isolated 48 h after treatment with TAM alone or in combination with the ER β agonists by means of GeneChip Human Gene 1.0 ST DNA microarrays (Affymetrix), we found the genes

Fig. 2 Enhanced antiproliferative effects of a combination of ER β agonists with tamoxifen. The indicated cell lines cultured in SR2 medium supplemented with 1 nM E2 were treated with 0.1 or 1 μ M of 4-OH tamoxifen alone or in combination with 10 nM of ERB-041 or WAY-200070 for up to 6 days. Relative numbers of viable cells were measured by means of the “Cell Titer Blue” assay as described in the “Materials and methods” section and are expressed in percent of E2 + vehicle EtOH. Squares 100 nM 4-OH tamoxifen (TAM) (grey single drug treatment, black combination with 10 nM of the indicated ER β agonist). Circles 1 μ M 4-OH tamoxifen (TAM) (grey single drug treatment, black combination with 10 nM of the indicated ER β agonist). ($n = 4$) * $p < 0.05$ vs tamoxifen single drug treatment



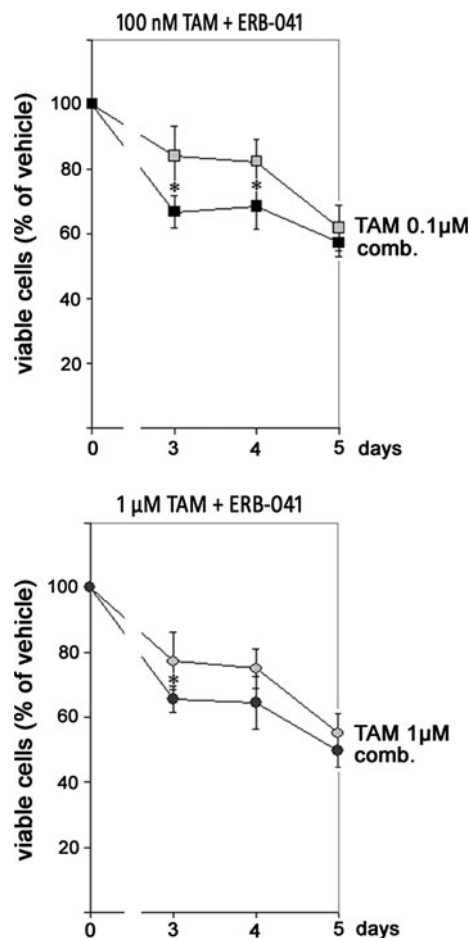


Fig. 3 Growth-inhibitory effect of a combination of 4-OH tamoxifen and ERB-041 on T-47D cells in the presence of 10 % FBS. Cells were treated with 0.1 or 1 µM of 4-OH tamoxifen alone or in combination with 10 nM of ERB-041 for up to five days. Relative numbers of viable cells were measured by means of the “Cell Titer Blue” assay as described in the “Materials and methods” section and are expressed in percent of E2 + vehicle EtOH. *Squares* 100 nM 4-OH tamoxifen (TAM) (grey single drug treatment, *black* combination with 10 nM of ERB-041). *Circles* 1 µM 4-OH tamoxifen (TAM) (grey single drug treatment, *black* combination with 10 nM of ERB-041). ($n = 4$) * $p < 0.05$ vs tamoxifen single drug treatment

ASCL1, C3, SDK2, PTGES, DDR2, LRRFIP1, NKAIN1, RHOBTB1 and FCGR2C to be more than 2-fold upregulated after treatment with TAM alone, whereas the genes SYTL4, ADAMTS9, SEMA3D, ART4, MYBL1, FHL1, RBMY2EP, UGT2B15 and CEACAM6 were at least 2-fold downregulated. Expression of these TAM-regulated genes was not significantly affected by the ER β agonists tested (Table 1). For identification of genes differentially regulated by the ER β agonists and TAM, we decided to lower the cut-off value to 1.8-fold, because the differences were smaller than expected. Genes upregulated by at least one ER β agonist when compared to TAM-triggered expression were CD177, RNU5A, ROCK1P1, GAGE12J, CYP4Z1, CYP4A11, LCN8 and FAM99A, whereas the

genes S100A8, LOC284861, SNORD13P1, DNAH14 and DPPA3 were downregulated in comparison to TAM (Table 2).

To verify the DNA microarray results, we performed qRT-PCR analyses of selected genes. In these experiments, we first confirmed regulation of MYBL1 gene triggered by TAM and ER β agonists. Whereas treatment with 1 nM E2 led to 3.5-fold increase of MYBL1 transcript levels, addition of TAM was able to block this effect (Fig. 4). Notably, addition of 10 nM ERB-041 or WAY-200070 further reduced MYBL1 expression down to 66.3 or 65.1 % of vehicle control. Transcript levels of S100A8 gene were confirmed to be downregulated in MCF-7 cells treated with a combination of 100 nM TAM and ER β agonists, but only combined treatment with WAY-200070 exerted a statistically significant effect on expression of this gene. Like in DNA microarray analysis, expression of CEACAM6 was verified to be downregulated after treatment with TAM. In contrast to our GeneChip data, qPCR analysis revealed further reduction of CEACAM6 mRNA levels after combined treatment ($p < 0.05$) (Fig. 4).

Discussion

In this study, we observed increased antitumoral effects of a combined treatment with tamoxifen and ER β agonists on human breast cancer cell lines in vitro. Whereas neither ERB-041 nor WAY-200070 alone were able to affect proliferation of the ER α/β -positive breast cancer cell lines employed in this study, their combination with the well-established SERM triggered an additional decline of cell growth.

The selective ER β agonists ERB-041 and WAY-200070 were used in a 10-nM concentration only, because their IC₅₀ values for ER β are 5 and 2 nM, respectively, and we wanted to rule out unspecific activation of ER α which is known to occur at concentrations in a 100-nM range [5, 7].

The enhanced antitumoral effect we observed after addition of these ER β agonists is in line with a previous study demonstrating ER β overexpression to increase the growth-inhibitory effect of tamoxifen on MCF-7 cells [13]. The authors concluded that the G1 cell cycle arrest triggered by tamoxifen together with a G2 arrest resulting from ER β overexpression led to a potent blockade of cell cycle. However, in our setting, the molecular mechanisms underlying the increased growth arrest are proposed to be different, because we did not observe any effect of the employed ER β agonists on cell cycle when used alone.

It is well known that ER β is able to exert antitumoral actions in hormone-dependent tissues like the breast. Knockdown of ER β has been reported to increase proliferation, but to decrease apoptosis of breast cancer or

Table 1 Genes affected by treatment of MCF-7 breast cancer cells with 4-OH tamoxifen (TAM) as assessed by DNA microarray analysis

Gene	Description	TAM vs E2	β 41 + TAM vs TAM	WAY + TAM vs TAM
ASCL1	Achaete-scute complex homolog 1	3.89	1.07	1.02
C3	Complement component 3	3.28	1.08	1.00
SDK2	Sidekick homolog 2 (chicken)	3.13	-1.10	-1.09
PTGES	Prostaglandin E synthase	2.61	1.07	1.17
DDR2	Discoidin domain receptor tyrosine kinase 2	2.35	-1.36	-1.42
LRRFIP1	Leucine-rich repeat (in FLII) interacting protein 1	2.27	-1.64	-1.08
NKAIN1	Na ⁺ /K ⁺ transporting ATPase interacting 1	2.21	-1.02	-1.11
RHOBTB1	Rho-related BTB domain containing 1	2.20	1.11	1.05
ADAMTS9	ADAM metalloproteinase with thrombospondin type 1 motif, 9	-2.06	-1.05	-1.17
SEMA3D	Sema domain, immunoglobulin domain (Ig), (semaphorin) 3D	-2.11	1.14	-1.05
ART4	ADP-ribosyltransferase 4 (Dombrock blood group)	-2.12	1.02	-1.18
MYBL1	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	-2.14	-1.39*	-1.42*
FHL1	Four and a half LIM domains 1	-2.20	-1.22	-1.23
UGT2B15	UDP glucuronosyltransferase 2 family, polypeptide B15	-2.26	1.07	-1.15
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	-2.43	1.08	-1.01

Co-treatment with ER β agonists ERB-041 (β 41) or WAY200070 (WAY) did not significantly change the TAM-specific gene regulation pattern. The shown values demonstrate the -fold change of gene transcript levels after 48 h of treatment in comparison to TAM or E2, respectively. The cut-off value was set to 2.0-fold change (bold values)

* Downregulation of MYBL1 expression was found to be small but statistically significant in subsequent RT-qPCR experiments

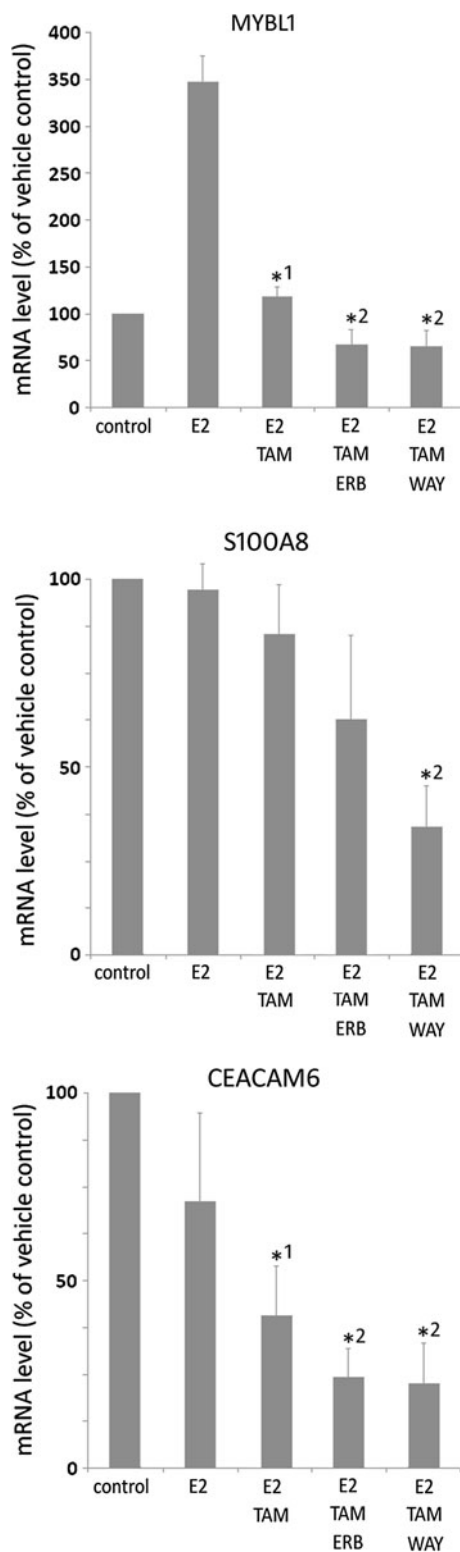
Table 2 Genes affected by addition of ER β agonists ERB-041 (β 41) or WAY200070 (WAY) to tamoxifen (TAM) in MCF-7 cells, as assessed by DNA microarray analysis

Gene	Description	β 41 +TAM vs TAM	WAY + TAM vs TAM	TAM vs E2
CD177	CD177 molecule	2.45	1.85	1.04
RNU5A	RNA, U5A small nuclear	1.75	1.93	-1.35
ROCK1P1	Rho-associated, coiled-coil containing protein kinase 1 pseudogene 1	2.61	1.05	-1.04
GAGE12 J	G antigen 12 J	2.46	1.14	-1.14
CYP4Z1	Cytochrome P450, family 4, subfamily Z, polypeptide 1	1.56	2.00	-1.71
CYP4A11	Cytochrome P450, family 4, subfamily A, polypeptide 11	1.82	1.64	-1.23
LCN8	Lipocalin 8	1.06	1.93	-1.08
FAM99A	Family with sequence similarity 99, member A	1.95	-1.02	-1.32
LOC284861	Hypothetical LOC284861	-1.86	-1.08	1.63
SNORD13P1	Small nucleolar RNA, C/D box 13 pseudogene 1	-1.84	-1.41	1.21
DNAH14	Dynein, axonemal, heavy chain 14	-1.54	-1.86	1.85
DPPA3	Developmental pluripotency associated 3	-2.19	-1.30	1.37
S100A8	S100 calcium binding protein A8	-1.92	-1.81	1.38

The shown values demonstrate the -fold change of gene transcript levels after 48 h of treatment in comparison to TAM or E2, respectively. The cut-off value was set to 1.8-fold change (bold values)

mammary epithelial cells, whereas ER β overexpression induced growth arrest and apoptosis [14, 15]. In contrast, to the best of our knowledge there is no report demonstrating antitumoral effects triggered by ER β agonists, which is in line with our observations after single drug treatment of ER α / β -positive breast cancer cells with ERB-041 or WAY-200070.

Increased growth inhibition triggered by addition of ER β agonists to tamoxifen was most pronounced in the absence of growth factors, but in the case of ERB-041 was also evident in serum-containing culture medium. Thus, it is tempting to speculate that phosphorylation of ER β triggered by serum growth factors might hamper the effects of WAY-200070, and to a lesser extent of ERB-041 [16].



◀**Fig. 4** Validation of Affymetrix GeneChip Data by means of qRT-PCR. Transcript levels of MYBL1, S100A8 and CEACAM6 were examined 48 h after treatment of MCF-7 cells with E2 (1 nM), 4-OH tamoxifen (TAM, 100 nM) or ER β agonists (10 nM) in the indicated combinations. Transcript levels have been normalized using GAPDH and are presented in percent of the respective levels in vehicle-treated MCF-7 cells. WAY WAY-200070, ERB ERB-041. *1 $p < 0.05$ vs E2; *2 $p < 0.05$ vs E2 + TAM ($n = 4$)

tamoxifen resulted in more than 2.5-fold upregulation of the genes ASCL1, C3, SDK2 or PTGES, while the four top downregulated genes were CEACAM6, UGT2B15, FHL1 and MYBL1. Our qPCR data confirming increased downregulation of MYBL1 (A-Myb) expression by a combination of TAM and ER β agonists might point at a potential molecular mechanism underlying the observed effects on cellular proliferation, since this transcription factor has been reported to promote cancer cell growth [17]. CEACAM6 gene is not only known to be downregulated after tamoxifen treatment, but also to predict breast cancer recurrence following adjuvant tamoxifen [18].

Apart from MYBL1 and CEACAM6, addition of ERB-041 or WAY-200070 did not significantly change expression of genes found to be regulated by 4-OH tamoxifen. In contrast, the combination with ER β agonists altered mRNA expression of other genes not being regulated by 4-OH tamoxifen alone. CD177 was the gene exhibiting the strongest induction factor after treatment with ERB-041 or WAY-200070 when compared to the tamoxifen-triggered transcriptome. Although the function of CD177 (NB1) gene, coding for a glycosyl-phosphatidylinositol-linked surface protein, normally secreted by neutrophils, in cancer remains to be solved, it has been reported to be part of a two-gene classifier system predicting clinical outcome of colon cancer patients [19]. A more plausible candidate to explain the observed superior effect of combined treatment on breast cancer cells might be the gene coding for the S100 calcium binding protein A8 (S100A8), which was downregulated both by co-treatment with ERB-041 and WAY200070. S100A8 expression has been reported to be overexpressed in cancer and to play an important role in progression of breast cancer, especially in proliferation and metastasis. In a recent study, S100A8 expression was shown to be elevated in higher grade, ER α -negative, basal-type breast cancer [20]. Finally, we used GeneMANIA Software to find pathways linking ER β to genes regulated by co-treatment with ER β agonists [12, 21, 22]. The first gene network linked ER β gene to S100A8 via binding of the proline-rich nuclear receptor coactivator 1 (PNRC1) to ER β , thereby forming a bridge to retinoic acid receptors γ and β , leading to regulation of S100A8 gene expression [23, 24]. The observed small downregulation of MYBL1 by ER β agonists might result from interaction of the steroid hormone receptor with CREBBP via coactivator NCOA1,

To elucidate molecular mechanisms underlying the increased growth-inhibitory effect of a combined treatment with tamoxifen and ER β agonists, we analyzed the effect of the used drugs on MCF-7 transcriptome. Treatment of MCF-7 cells growing in E2-containing medium with 4-OH

thereby regulating MYBL1 in a BCL2-dependent manner [23, 25, 26]. These interactions might provide at least one possible explanation for the observed effects of ERB-041 and WAY-200070 on MYBL1 and S100A8 expression. However, software-based pathway analysis was not able to find links between ER β and other strongly regulated genes like CD177.

In conclusion, our data demonstrating an enhanced antiproliferative effect of a combined treatment with tamoxifen and ER β agonists on breast cancer cells in vitro might encourage further studies on a potential clinical applicability of this combination. Given that this effect was more pronounced in the absence of serum, it might be important to find out to what extent inhibitors of growth factor signaling might improve the beneficial effect of ER β agonists under physiological conditions.

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Conflict of interest The authors declare that they have no conflict of interest.

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