RESEARCH ARTICLE

Synergistic Effects of Tamoxifen and Tranilast on VEGF and MMP-9 Regulation in Cultured Human Breast Cancer Cells

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Abstract

**Background:** Vascular endothelial growth factor and matrix metalloproteinases are two important factors for angiogenesis associated with breast cancer growth and progression. The present study was aimed to examine the effects of tamoxifen and tranilast drugs singly or in combination on proliferation of breast cancer cells and also to evaluate VEGF and MMP-9 expression and VEGF secretion levels. **Materials and Methods:** Human breast cancer cell lines, MCF-7 and MDA-MB-231, were treated with tamoxifen and/or tranilast alone or in combination and percentage cell survival and proliferative activity were evaluated using LDH leakage and MTT assays. mRNA expression and protein levels were examined by real-time RT-PCR and ELISA assay, respectively. **Results:** LDH and MTT assays showed that the combined treatment of tamoxifen and tranilast resulted in a significant decrease in cell viability and cell proliferation compared with tamoxifen or tranilast treatment alone, with significant decrease in VEGF mRNA and protein levels. We also found that tamoxifen as a single agent rarely increased MMP-9 expression. A decrease in MMP-9 expression was seen after treatment with tranilast alone and in the combined treatment MMP-9 mRNA level was decreased. **Conclusions:** This combination treatment can able to inhibit growth, proliferation and angiogenesis of breast cancer cells.

**Keywords:** Breast cancer - tamoxifen - tranilast - angiogenesis - matrix metalloproteinase

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**Introduction**

Tumor angiogenesis, the formation of new blood capillaries from preexisting blood vessels (Bergers and Benjamin, 2003), supports growth and development of the tumor mass and is an essential process during growth and progression of breast cancer (Banerjee et al., 2007).

Vascular endothelial growth factor (VEGF) is the most well-known pro-angiogenic growth factor, and thought to be the most critical in neoplastic angiogenesis (Schneider and Sledge, 2007).

During tumor angiogenesis, high VEGF expression increase the micro-vascular density of tumor tissue (Ahluwalia et al., 2012) and has several effects including endothelial cell proliferation, migration, invasion and survival (Banerjee et al., 2007). It has been reported that VEGF stimulates both paracrine and autocrine signaling in endothelial cells, which promotes the progression and survival of tumor cells (Ferrara et al., 2003). It is also critical in tumor growth, invasiveness and metastasis of cancer (Folkman, 1990). In addition, MMPs are consistent indicators of tumor cell invasion and migration. It is known that malignant tumors increased MMP expressions compared to benign tumors, which causes degradation of the extracellular matrix, mostly implicated in promoting cancer cell invasion, metastasis and angiogenesis (Littlepage et al., 2010; Gialeli et al., 2011). Endocrine therapy is widely used in the treatment of both early stage and metastatic breast cancer. Because estrogen is essential for breast carcinogenesis (Petrangeli et al., 1994), anti-estrogens have significant therapeutic potential in endocrine therapy for breast cancer. Tamoxifen (TAM) is a non-steroidal anti-estrogen (Jordan, 2003) which used for the treatment of all stages of breast cancer (Cole et al., 1971).

Studies suggest that the combination therapy with other drugs that helps the action of tamoxifen, is supposed for getting better outcome from this anticancer treatment. On the other side, tranilast, N-(3’,4’-dimethoxy-cinnamoyl) anthranilic acid, is an antiallergic drug (Azuma et al., 1976) has been shown to exert anti-tumor and anti-metastatic effects in various cancers (Noguchi et al., 2003; Chakrabarti et al., 2009; Yamamoto et al., 2009; Izumi et al., 2010; Mitsuno et al., 2010; Subramaniam et al., 2011).

In this work, we investigated the possible synergistic effect of tranilast with anti-estrogenic compound,
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Materials and Methods

Cell culture
MCF-7 (noninvasive human breast cancer cell line, ER-positive) and MDA-MB-231 (invasive and metastatic human breast cancer cell line, ER-negative) were used in this study. The cell lines were cultured in RPMI-1640 media supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin antibiotics (100 IU ml⁻¹). Cultures were maintained at 37°C, 95% air humidity and 5% CO₂.

Drugs
Tamoxifen citrate and tranilast were procured from Enzo Life Sciences and dissolved in dimethyl sulfoxide so that the final dimethyl sulfoxide concentration did not exceed 0.5% (v/v). The concentrations of: tamoxifen were 1, 2, 5, 10 and 20 µM, tranilast concentrations was 10, 20, 50, 100 and 200 µM, and the combined treatment was examined in 1 concentration of tamoxifen (2 µM) with 5 different concentrations of tranilast: 10, 20, 50, 100, and 200 µM for 48 h.

Lactate dehydrogenase (LDH) leakage assay
Leakage of enzymes such as LDH into the culture medium is a well-known indicator of damage or injury to the cell membrane (Hsieh and Acosta, 1991). Briefly, 1x10⁴ cells/well of either MCF-7 or MDA-MB-231 cells was transferred to 96-well plates. The plates were incubated overnight at 37°C to allow the cells to attach and proliferate. On the next day, 300 µl of fresh medium containing drug concentrations were added to each well, and the plates were incubated at 37°C in 5% CO₂. All drug concentrations were tested at least in triplicate wells and the assays were repeated independently three times. After 48 h, the plates were removed from the incubator and then 100 µl of medium from each well was carefully transferred to new plates. 100 µl of LDH substrate prepared according to the manufacturer’s direction (Cytotoxicity Detection Kit, Roche Chemical Co.) was added to each well. After 20 min shaking at room temperature, the enzymatic reaction was arrested by adding 50 µl of 1M hydrochloric acid. Lactate dehydrogenase activity was determined by change in absorbance at 490 nm. For the purpose of calculating percent cytoxicity values, background LDH release from culture cells was considered as low control and triton-X 100 (0.01%) treated cells as high control.

Leakage (%)=[A₄₉₀(sample)-A₄₉₀(low control)]/A₄₉₀(high control)-A₄₉₀(low control)]x100%

Cell proliferation assay
1x10⁴ cells in 200 µl medium were plated into each well of 96-well plates and incubated overnight to allow cell adherence. After incubation with drugs for 48h at 37°C and 5% CO₂, 20 µl MTT solution (MTT Cell Growth Assay; Merck) (5 mg/ml in PBS) was added to each well. After 4 hours of incubation at 37°C, the medium with MTT were discarded and 100µl dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals at room temperature for 30min. The optical density (OD) of each well was then taken in a plate reader at 570 nm. The percentage of cell viability was calculated according to the following equation: Cell viability (%)=[A₅₇₀(sample)/A₅₇₀(control)]x100%

Measurement of VEGF secretion by ELISA assay
MCF-7 or MDA-MB-231 cell lines (5x10⁴ cell/well) were plated in 48-well plates overnight at 37°C. At near confluence, medium was then replaced with serum-free medium. Cells were treated with 2 µM tamoxifen, 200 µM tranilast or combination both and the conditioned medium was collected after 48 h.

A commercial quantitative ELISA kit (R&D Systems, Minneapolis MN, USA) was used to determine VEGF concentrations in media, according to the manufacturer’s protocol. Briefly, VEGF concentrations were determined by measuring absorbance at 420 nm, and VEGF values were normalized to the total protein concentration in each well. The VEGF protein level was expressed as ng/ml.

Real-time quantitative PCR (RQ-PCR) analysis
Total RNA was extracted from control or drug-treated cell pellets, 48 h after treatment with 2 µM tamoxifen, 200 µM tranilast or combination both, using RNeasy Mini kit (Qiagen) in accordance with the manufacturer’s protocol. The concentration and A₂₈₀/A₂₆₀ ratio were measured by UV absorbance. Quality was also determined on an ethidium bromide stained 1.5% agarose gel. First strand cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). Aliquots of the cDNA were combined with the QuantiFast SYBER Green PCR Master Mix from Qiagen and primers, and assayed in triplicate using a Rotor-Gene 6000 real-time RT-PCR (Corbett).

Analysis and fold change was calculated from the ΔΔC operators values with the 2⁻⁸ACT formula. The C operator values from samples were plotted on the standard curve and the copy number was calculated with GAPDH as the internal control.

The primers were designed using the program BioEdit and BLAST searches (http://www.ncbi.nlm.nih.gov) carried out to confirm specificity of the nucleotide sequences chosen. Primer sequences were: VEGF forward: ctacctccacatgccaga; reverse: cacacaggtgttgaaga (product size: 187 bp), MMP-9 forward: gctgtgatcctacat; reverse: aacacaggtgttcccttc (product size: 220 bp) and GAPDH forward: actctggtaagtggatattgttgc; reverse: ggaagatggtgatgggatttc (product size: 162 bp). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used as internal control for the PCR and results were expressed as mRNA percent of the respective control.

Statistical analysis
All statistical analyses were done using SPSS® software (version 19.0) and were conducted by one-way analysis of the variance (ANOVA) and Tukey test. Data
were expressed as the mean±SEM and p values<0.05 was considered significant.

Results

Cytotoxic and anti-proliferative effects of tamoxifen and/or tranilast in MCF-7 and MDA-MB-231 breast cancer cells

Cytotoxic effects of tamoxifen and tranilast alone or in combination on percent cell survival and proliferation was evaluated by monitoring LDH leakage from exposed cells and MTT test, in order to choose concentrations to be used in the single or combined treatment. The results show that the cytotoxic and anti-proliferative effects of tamoxifen and/or tranilast increased in a dose-dependent manner in both MCF-7 and MDA-MB-231 cell lines (Figure 1 and 2).

In this study, MCF-7 and MDA-MB-231 cells were treated with different doses of tamoxifen and tranilast either alone or together for 48 h. Growth and proliferation inhibition as expected, exerted by tamoxifen on breast cancer cells in presence of tranilast, demonstrating the synergistic/additive effect of both drug (p<0.05). This synergistic effect is demonstrated by the enhanced antiproliferative and cytotoxic effects of tamoxifen when tranilast was added to each cell lines (Figure 1A and 2). While tamoxifen and/or tranilast treatment does exert cytotoxic and inhibitory effects on both cell lines, MCF-7 cells (Figure 1A, 2A) showed greater sensitivity as compared with MDA-MB-231 cells (Figure 1B, 2B). Based on these observations (Figure 1, 2), the 2 µM tamoxifen and 200 µM tranilast concentrations was used to subsequent characterize the effects of tamoxifen and/or tranilast on breast cancer cells.

Effects of tamoxifen and/or tranilast on VEGF gene transcription and VEGF secretion by MCF-7 and MDA-MB-231 breast cancer cells

We determined the tamoxifen and/or tranilast effects on VEGF expression and secretion in MCF-7 and MDA-MB-231 by real-time RT-PCR and VEGF-specific ELISA, respectively. Inhibition of VEGF protein secretion by tamoxifen and/or tranilast was evaluated in both cell lines. The results shown in Figure 3 indicate that tamoxifen and tranilast moderately significantly decreased the secretion of VEGF by the breast cancer cells (p<0.05). A combination of tamoxifen and tranilast gave a considerable decrease in VEGF secreted (p<0.001 for MCF-7 and p<0.01 for MDA-MB-231 cells). A VEGF-specific ELISA revealed that inhibition effects of tamoxifen and tranilast as a single or a combination two was higher in MCF-7 cells (Figure 3A) than in MDA-MB-231 cells (Figure 3B).

We then assessed the effect of tamoxifen and/or tranilast on VEGF gene transcription in MCF-7 and MDA-MB-231 cells. As shown in Figure 4 (A, B), tamoxifen and/or tranilast were effective for downregulation of VEGF expression at 48 hours in both cell lines; however this reduction was somehow higher in combined treatment than either drugs alone (Figure 4A, B), as well inhibition effects of tamoxifen and tranilast as a single or combination was significantly higher in MCF-7 cells (Figure 4A) than in MDA-MB-231 cells (Figure 4B).

Effects of tamoxifen and/or tranilast on MMP-9 gene transcription by MCF-7 and MDA-MB-231 breast cancer cells

To determine effects of tamoxifen and/or tranilast on MMP-9 expression in breast cancer cells, we measured MMP-9 mRNA by performing real-time PCR. As seen in Figure 5, tamoxifen treatment resulted in increased MMP-9 mRNA expression in both MCF-7 and MDA-MB-231 cells compared to untreated controls although not
The cells were cultured MCF-7 and MDA-MB-231 cells were cultured without drugs (control) or in the presence of 2 µM tamoxifen, 200 µM tranilast and combination both for 48 h. A) VEGF protein levels in medium from MCF-7 cells; B) VEGF protein levels in medium from MDA-MB-231 cells. Values represent mean±SEM (*p<0.05 vs. control; **p<0.01 vs. control; ***p<0.001 vs. control)

Figure 4. The Effects of Tamoxifen and/or Tranilast on the Level of Gene Expression of VEGF in MCF-7 (A) and MDA-MB-231 Cells (B). The cells were cultured without drugs (control) or in the presence of 2 µM tamoxifen, 200 µM tranilast or a combination two and VEGF mRNA was assessed by real-time RT-PCR after 48 h treatment. Quantitative RT-PCR analysis of VEGF in MCF-7 and MDA-MB-231 cells showed that tamoxifen and tranilast as a single or in combination effectively decreased the expression of VEGF mRNA. The real-time RT-PCR results were normalized against the internal control GAPDH and are expressed as a percentage of control cells. Values are the average of three separate determinations±SEM (*p<0.05 vs. control; **p<0.01 vs. control; ***p<0.001 vs. control)

Figure 5. MMP-9 Gene Expression Changes after Treatment with Tamoxifen and/or Tranilast were Quantified using Real-time RT-PCR; (A) MCF-7 and (B) MDA-MB-231. The real-time RT-PCR results were normalized against the internal control GAPDH and are expressed as a percentage of control cells. Values are the average of three separate determinations±SEM (p=0.62 and p=0.91, respectively). The levels of MMP expression were downregulated when breast cells were treated with 200 µM tranilast (p=0.29) for MCF-7 and p=0.79 for MDA-MB-231 cells). When 2 µM tamoxifen and 200 µM tranilast were added together, MMP-9 level were decreased by about 55% (p=0.29) in MCF-7 and 20% (p=0.72) in MDA-MB-231 cells compared to control group (Figure 5).

Discussion

Breast cancer growth and metastasis are dependent on angiogenesis (Weidner et al., 1992); as well breast carcinogenesis is estrogen-dependent (Petrangeli et al., 1994).

The overexpression of VEGF in malignant breast tumors can redirect the potential of angiogenesis in the breast tumors (Perrot-Applanat et al., 2012). VEGF has been considered to be a target gene for the estrogen receptors and to contribute to breast cancer progression (Applanat et al., 2008; Khosravi Shahi et al., 2009). Estrogens are known to enhance VEGF expression (Kazi and Koos, 2007) and in breast tumors in vivo, estrogens has been shown to promote angiogenesis and increase extracellular VEGF (Dabrosin et al., 2003). Garvin et al. (2006) also demonstrated that similar events also occurred in ex vivo cultured normal human breast tissue (Garvin et al., 2006). In addition, MMP-9 are closely associated with tumor growth, invasion and metastasis (Versteeg et al., 2008) and estrogen has been shown to regulate the activity of MMP-2 and MMP-9 in breast cancer cells (Nilsson et al., 2007).

Anti-estrogens such as tamoxifen, is basis in the medical treatment of breast cancer. Tamoxifen is a competitive antagonist of estrogen, but seems to have antiangiogenic effects independent of, and in addition to, estrogen receptor content or its anti-estrogen effects (Kousidoua et al., 2008). It have been reported that tamoxifen treatment of breast cancer, induces an antiangiogenic response by an efficient decrease of VEGF both in vitro and in vivo (Garvin and Dabrosin, 2003; Garvin et al., 2005; Nilsson and Dabrosin, 2006; Kumar et al., 2013; Rajput et al., 2013).

Studies have shown that high VEGF levels in primary breast tumors correlate to shorter survival times for patients treated with tamoxifen (Linderholm et al., 2009).

Furthermore, an in vitro study by Garvin et al., was shown that tamoxifen exposure increased MMP-2 and MMP-9 levels, whereas estradiol induced a significant decrease (Nilsson et al., 2007). Thus far, the effects of estrogen and antiestrogens on MMPs have been considered with conflicting results (Van den Brule et al., 1992; Abbas et al., 1997; Philips and McFadden, 2004; Lymeratou et al., 2013). On the further hand, in addition to its anti-allergic effects (Azuma et al., 1976), tranilast has been reported to exert anti-neoplastic properties on breast cancer (Chakrabarti et al., 2009; Subramaniam et al., 2010).

In this work, we have demonstrated that the combination of tranilast and tamoxifen resulted in synergistic effects on growth, proliferation and VEGF and MMP-9 expression. Our results suggest that tranilast could play a potent role in hormone therapy in breast cancer patients, including hormone resistant or combination therapy. The present study demonstrated that the alteration of mRNA and protein level of VEGF and MMP-9 mRNA induced by tranilast and/or tamoxifen in human breast cancer MCF-7 and MDA-MB-231 cells was possibly mediated by pathways independent of estrogen receptor pathway, therefore; tamoxifen plus tranilast would be a promising
combination therapy against this malignancy.

In conclusions, we may conclude that tamoxifen plus tranilast could be a promising combination therapy for future clinical trials in breast cancer patients. Thus we believe that the present results may lead to new therapeutic options for angiogenesis inhibition of breast cancer.

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