Toxicology in Vitro 28 (2014) 1215–1221

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Anti-aromatase effect of resveratrol and melatonin on hormonal positive breast cancer cells co-cultured with breast adipose fibroblasts

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ARTICLE INFO

Article history:
Received 14 January 2014
Accepted 29 May 2014
Available online 12 June 2014

Keywords:
Co-culture
Human fibroblasts
Breast cancer
Resveratrol
Melatonin

ABSTRACT

Targeting the estrogen pathway has been proven effective in the treatment for estrogen receptor positive breast cancer. There are currently two common groups of anti-estrogenic compounds used in the clinic; Selective Estrogen Receptor Modulators (SERMs, e.g. tamoxifen) and Selective Estrogen Enzyme Modulators (SEEMs e.g. letrozole). Among various naturally occurring, biologically active compounds, resveratrol and melatonin have been suggested to act as aromatase inhibitors, which make them potential candidates in hormonal treatment of breast cancer. Here we used a co-culture model in which we previously demonstrated that primary human breast adipose fibroblasts (BAFs) can convert testosterone to estradiol, which subsequently results in estrogen receptor-mediated breast cancer T47D cell proliferation. In the presence of testosterone in this model, we examined the effect of letrozole, resveratrol and melatonin on cell proliferation, estradiol (E_2) production and gene expression of *CYP19A1*, *pS2* and *Ki-67*. Both melatonin and resveratrol were found to be aromatase inhibitors in this co-culture system, albeit at different concentrations. Our co-culture model did not provide any indications that melatonin is also a selective estrogen receptor modulator. In the T47D-BAF co-culture, a melatonin concentration of 20 nM and resveratrol concentration of 20 μM have an aromatase inhibitory effect as potent as 20 nM letrozole, which is a clinically used anti-aromatase drug in breast cancer treatment. The SEEM mechanism of action of especially melatonin clearly offers potential advantages for breast cancer treatment.

1. Introduction

Adjuvant hormonal therapy is an effective treatment to reduce or prevent the recurrence of hormone receptor positive breast cancer (Bando, 2013; Bell et al., 2013; Higgins et al., 2012; Rao and Cobleigh, 2012; Yoshinami et al., 2013). Here, two common groups of pharmaceuticals are used for clinical hormonal therapy; Selective Estrogen Receptor Modulators (SERMs) e.g. tamoxifen (Balkenende et al., 2013; Yang et al., 2012) and Selective Estrogen Enzyme Modulators (SEEMs) such as the aromatase inhibitor letrozole (Doughty, 2008; Joerger and Thurlimann, 2009; Mouridsen et al., 2009; Thurlimann et al., 2005). SERMs have anti-estrogenic properties because of antagonistic actions on estrogen receptor α (ERα), which reduces the proliferation of estrogen responsive breast tumor cells. SEEMs reduce the synthesis of estrogens from androgens that results in lower circulating levels of estrogens and also reduced estrogen production in peripheral tissues. This subsequently leads to reduced proliferation of estrogen receptor (ER)-positive breast tumor cells. In this approach, the estrogen-stimulated pathways are among the most important targets for adjuvant breast cancer therapy (Lumachi et al., 2013; Yue et al., 2012).

Beside several pharmaceutical SERMs and SEEMs that have been developed over the last decades to treat or prevent breast cancer, a variety of naturally occurring, biologically active compounds have been identified that may be useful as chemopreventive agents for breast cancer. Two biological agents that are often suggested to have such cancer chemopreventive actions are resveratrol and melatonin. Resveratrol is a non-flavonoid phytoestrogen

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http://dx.doi.org/10.1016/j.tiv.2014.05.015
0887-2333/

found for example in grapes. Several epidemiological studies indicated that high resveratrol intake could reduce breast cancer risk (Levi et al., 2005). Multiple mechanisms of action have been identified that could explain the anti-carcinogenic properties of resveratrol, which include its role as a SERMs, aromatase inhibitor and/or anti-oxidant (Alkhalaf et al., 2008; Lee et al., 2012; Leon-Galicia et al., 2012). With respect to its properties as an aromatase (*CYP19A1*) inhibitor, several *in vivo* and *in vitro* studies have confirmed the modularity function of resveratrol in this key step in sex steroidogenesis (Le Corre et al., 2005; Lee and Safe, 2001; Wang et al., 2006; Zhang et al., 2004). It has been suggested that the anti-aromatase activity of resveratrol is its major property with respect to chemoprevention of breast cancer (Le Corre et al., 2005). In a 28-day repeated dose study, the no observed adverse effect level (NOEAL) was 300 mg/kg/day in rats (Crowell et al., 2004). Considering this low toxicity of resveratrol, it is a potential good candidate for adjuvant breast cancer treatment.

Melatonin (N-acetyl-5-methoxytryptamine) is an indolic and endogenous compound that is naturally produced by the pineal gland in the human body. Melatonin plays a primary role in the circadian pattern and is regulated by the hypothalamic suprachiasmatic nucleus (SCN). In addition, melatonin is believed to have oncostatic properties against many forms of cancers such as leukemia, breast, colorectal and prostate cancer (Dai et al., 2008; Mediavilla et al., 2010; Mills et al., 2005; Srinivasan et al., 2008). Multiple mechanisms have been proposed that might explain the breast cancer chemopreventive properties of melatonin (Blask et al., 2005; Grant et al., 2009; Korkmaz et al., 2009; Sanchez-Barcelo et al., 2005) from which two are especially important with respect to our present study. Firstly, melatonin can act as SERM by reducing estrogen binding to ERα receptors and inhibiting binding of the E_2-ERα complex to the DNA. The proposed mechanism of this anti-estrogenic effect of melatonin does not depend on its binding to the ER but depends on the high affinity binding to membrane melatonin receptors (MT1). The melatonin-MT1 complex interferes with the estrogen-binding activity of ERα without changing its affinity and reduces the ligand-receptor transactivation (Sanchez-Barcelo et al., 2005; Treeck et al., 2006; Yuan et al., 2002). Another important mechanism of action of melatonin with respect to prevention of breast cancer may be its role as aromatase inhibitor. Melatonin could decrease cAMP formation and down-regulate expression of promoter regions pII, pI.3 and p1.4-driven aromatase expression in MCF-7 cells (Martinez-Campa et al., 2009).

Previous studies from our laboratories have demonstrated the added value of using co-cultures from breast tumor cells and human primary breast fibroblasts (BAFs) in *in vitro* breast cancer studies (Heneweer et al., 2005a). In contrast to mono-cultures of breast tumor cell lines, these co-cultures have paracrine interactions between both cell types and therefore represent a more realistic approach to the actual *in vivo* situation of a breast tumor (Heneweer et al., 2005b). In our present study we examined the breast cancer chemopreventive properties of resveratrol and melatonin in co-cultures of ERα positive T47D cells and BAFs with an emphasis on the modulating effects on tumor cell proliferation and aromatase activity.

2. Materials and methods

2.1. Breast cancer cell culture and incubation

The human breast cancer cell line T47D was obtained from ATCC (Rockville, MD, USA). T47D cells were grown in culture medium comprising of RPMI 1640, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% glucose, 1% sodium pyruvate, and 0.08% insulin solution at a seeding concentration of 5×10^4 cells/ml. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Primary breast adipose fibroblasts (BAFs)

Primary breast tissue fibroblasts were obtained after informed consent from three breast cancer patients who attended the Bamrasnaradura Infectious Institute (Nontaburi, Thailand) for modified radical mastectomy. The research protocol was approved by the Medical Ethical Committee of the Bamrasnaradura Infectious Diseases Institute. About 5–10 g of macroscopically normal breast tissue was collected for this study. The remaining tissue was used for routine pathological examination. After the tissue was obtained, it was stored directly at 4 °C in a saline solution (0.9%NaCl) and immediately transported to the laboratory. BAFs were isolated from breast tissue following the method described earlier (Heneweer et al., 2005a) and cultured, as adherent cultures in RPMI 1640-medium w/o phenol red (Gibco/Invitrogen 11835) supplemented with Pen/Strep (invitrogen 15140), FCS (invitrogen 10270) and Insulin 10^{-3} M (144 mg/25 ml) (sigma I-5500) at a seeding concentration of 5×10^4 cells/ml. The cells were maintained in a humidified atmosphere of 37 °C with 5% CO_2. The BAFs were sub-cultured when the bottles were confluent, which was usually after one week (Heneweer et al., 2005a,b). The BAFs were kept for subculture. We isolated three primary BAF cultures from three patients (F1-3). In order to reduce the biological variation in the co-culture experiments, the BAFs from the patient with the highest aromatase expression (F3) (data from our previous experiments) were used for the co-culture in this study (Chottanapund et al., 2013).

2.3. Co-cultures of T47D breast cancer cells with BAFs

Approximately three weeks after isolation, the fibroblasts were used to establish a co-culture with T47D cells. On day 1, BAFs were plated at a density of approximately 5×10^3 cells/well in a 96-well plate (for proliferation) or at 5×10^4 cells/ml in a 25 cm^2 flask (for gene expression) in culture medium. At day 2, mono-cultures of breast cancer cells and plated primary BAFs were washed with phosphate buffered saline (PBS) and placed on assay medium in which heat inactivated FCS was replaced with heat-inactivated, charcoal/dextran-treated FCS (Hyclone, SH30068.03). On day 4, BAFs were washed with PBS and breast cancer cells were trypsinized and seeded on top of the BAFs at a density of 5×10^3 cells/well (for proliferation) or 2.5×10^5 cells/flask (for RNA expression). On day 5, fresh assay medium was added to the co-cultures after which the cells were exposed to the test compounds for 120 h (Birrell et al., 1995; Cops et al., 2008; Sonne-Hansen and Lykkesfeldt, 2005). Final solvent (ethanol) concentration was 0.1% v/v in the medium and concentrations of testing chemicals were respectively, 1 nM, 5 nM and 10 nM for testosterone (Sigma–Aldrich, Saint Louis, USA), 1 pM, 5 pM and 10 pM for 17β-estradiol (E_2) (Sigma–Aldrich, Saint Louis, USA), 10 nM, 20 nM and 30 nM for letrozole (Sigma–Aldrich, Saint Louis, USA), 5 μM, 10 μM and 20 μM for resveratrol (Sigma–Aldrich, Saint Louis, USA), and 1 nM, 5 nM, 10 nM and 20 nM for melatonin (Sigma–Aldrich, Saint Louis, USA).

2.4. Cell proliferation

Cell proliferation was determined after treatment with the test compounds by performing an MTT assay as described earlier (Heneweer et al., 2005a). The optimal cell number was established based on results from a cell viability assay of vehicle-control treated cells and visual inspection of the cells. After exposure to the

test compounds, 1 ml of MTT (5 mg MTT/ml in PBS) was added for 4 h. Next, the medium was aspirated and 100 µl of DMSO was added to dissolve the accumulated formazan crystals. The absorbance was measured at 550 nm using the 96-well microplate reader (Spectramax plus 384, Molecular Devices, California, USA).

2.5. Analysis of gene expression

After the cells were treated with the test compounds, total RNA was extracted using RNeasy® mini kit (Qiagen, Texas, USA). The purification and concentration of total RNA were measured by the ratio of absorbance at 260/280 and 260/230 NM using Nanodrop (Nanodrop Technologies, Inc., Delaware, USA). Obtained RNA was stored at −20 °C in aliquots of 10 ng/µl. Next, total RNA was reverse-transcribed into cDNA using the Qiagen high capacity cDNA reverse-transcription kit (Qiagen, Texas, USA). Gene expression of *aromatase* (*CYP19A1*), *pS2* and *Ki-67* was performed using a Roche-Light-cycler-480 one step RT PCR kit (Roche, Indiana, USA). The reaction mixture contained 0.1 µM of primers, 1X Quantitect SYBR Green RT-PCR Master mix, 0.2 µl QuantiTect RT mix and 2 µl template RNA (10 ng/µl) in a total volume of 20 µl. The mixture was reverse transcribed to cDNA at 50 °C for 20 min. After reverse transcription, the PCR was initiated by heating at 95 °C for 15 min, then followed by denaturation at 95 °C for 10 s, annealing at 57 °C for 25 s, extended at 68 °C for 30 s and acquisition at 82 °C for 5 s for 45 cycles. Relative quantification of gene expression was expressed as the difference in Ct values of the target gene to the housekeeping gene *beta-actin*. Primers for aromatase mRNA amplification were used as described previously by Sanderson et al. (2001). Primers coding for the estrogen-responsive *pS2* gene were used after Lee et al. (2003). *Ki-67* mRNA amplification was done in T47D cell lines as described by Chottanapund et al. (2013). Amplification of *β-ACTIN* (forward: 5′-TCTACAATGA GCTGCG-3′ and reverse: 5′-AGGTAGTCAGCTAGGT-3′) was used as a reference housekeeping gene. All primers were run through the National Center for Biotechnology Information (NCBI) blast (nucleotide nonredundant database) to confirm specificity. The efficiency was determined for a dilution range of cDNA and new primers were sought if the efficiency was below 95% or higher than 105%. After each RT-qPCR, a melt curve was run to ensure that primer-dimers and other non-specific products were omitted.

2.6. Measurement of estradiol (E_2)

After the cell proliferation assay with the test compounds, 50 µL assay medium of each treated co-cultured system was dispensed to measure the amount E_2 production using a commercially available ELISA Kit for human Estradiol according to the manufacturer's instruction (Invitrogen, CA, USA).

2.7. Statistical analysis

Each experiment was performed three times and in each experiment, every concentration was tested in triplicate, except for the estradiol production which was measured in duplicate as recommended by the manufacturer of the ELISA kit. Means and standard deviations were calculated of all nine values ($n = 9$). The statistical significance of differences in the means was calculated using the Student's *t*-test or one-way ANOVA. Differences were considered statistically significant if $P < 0.05$.

3. Results

Previous experiments in our laboratory showed that in the mono-cultured T47D breast tumor cells, testosterone caused a significant reduction (69%) in cell growth at 10 nM of testosterone (Chottanapund et al., 2013). In contrast, estradiol induced a significant proliferation of T47D cells (150%) at 1 pM after 120 h (Supplementary Fig. S1). Conversely, 1 nM testosterone induced significant proliferation of T47D cells (130%) when co-cultured in the presence of BAFs. This proliferative effect of testosterone declined significantly after the aromatase inhibitor letrozole (30 nM) was added to the T47D-BAF co-culture system. A maximum reduction of 25% cell growth was obtained at 10 nM testosterone, in the presence of letrozole (30 nM) (Supplementary Fig. S2). Exposure of mono-cultured T47D cells to letrozole, resveratrol and melatonin alone did not statistically significantly affect cell proliferation (Supplementary Fig. S3).

3.1. Aromatase gene expression in a co-culture of T47D with BAFs when exposed to testosterone combined with letrozole

The *CYP19A1* gene is encoding for the aromatase enzyme. The aromatase enzyme has been found to be inducible by androgen-like compounds in co-cultured BAFs with human breast cancer cells (Heneweer et al., 2005a,b). In this study, aromatase gene expression was concentration-dependently upregulated by testosterone in T47D-BAF co-cultures compared to vehicle control (Fig. 1). Testosterone at 1 nM induced aromatase expression up to 6.5-fold compared with vehicle control cells. At the highest concentration testosterone tested (10 nM), aromatase gene expression was induced 15.4-fold compared with vehicle-treated control cells. Additionally, we observed that aromatase gene expression could still be significantly induced by testosterone in the presence of the aromatase inhibitor letrozole (30 nM) (Fig. 1). Maximal induction of aromatase gene expression was 10.3-fold with 10 nM testosterone in combination with 30 nM letrozole, which was lower than aromatase expression by testosterone only.

3.2. Gene expression of pS2 and ki-67 in mono- and co-cultures of T47D cells with BAFs when exposed to testosterone with or without letrozole

The *pS2* gene is an E_2-responsive gene that can be easily upregulated in estrogen receptor positive breast tumor cells such as the T47D cells. In mono-cultured T47D cells, testosterone did not statistically significantly affect *pS2* expression (data not shown). However, in a T47D-BAF co-culture, testosterone increased *pS2* gene expression already 16.2-fold at 1 nM and even up to 31.9-fold at 10 nM compared with vehicle-treated control cells (Fig. 2a). Also gene expression of the cell proliferation marker *Ki-67* was increased by testosterone only in the co-culture with a maximum induction of 720 fold at 5 nM (Fig. 2a). Co-incubation of the T47D-BAF co-culture with testosterone and letrozole (30 nM) completely negated the induction of *pS2* and *ki-67* gene expression by testosterone alone (Fig. 2a and b).

3.3. Exposure of co-cultures of T47D cells and BAFs to testosterone combined with melatonin or resveratrol

Next, we studied the influence of melatonin and resveratrol on testosterone-induced cell proliferation of T47D-BAF co-culture. Melatonin reduced the testosterone induced cell proliferation in a concentration-dependent manner. At 20 nM melatonin, the proliferative effect of testosterone on the T47D cells in the co-culture system was fully abolished (Fig. 3). Also, cell proliferation in T47D-BAF co-culture was significantly reduced by resveratrol albeit at a much higher concentrations. In the presence of 20 µM resveratrol, the maximum reduction in T47D cell growth in the T47D-BAF co-culture was 27.6 (±14.0)% at 10 nM of testosterone (Fig. 4).

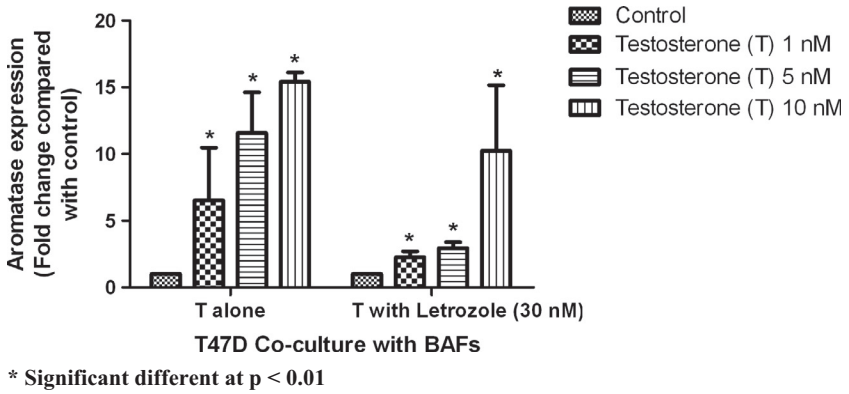


Fig. 1. Aromatase expression in T47D cells co-cultured with BAFs treated with (Left) testosterone 1, 5 and 10 nM alone or (Right) testosterone in the presence of letrozole 30 nM. Cell cultures were exposed to the test chemicals for 120 h. Data are expressed as fold change compared with vehicle-treated control cells as means ± SD ($n = 9$). *Statistically significantly different from vehicle control – treated T47D breast cancer cells co-cultured with BAFs (one-way ANOVA, $P < 0.01$).

3.4. Gene expression of aromatase, pS2 and ki-67 genes in co-cultures of T47D with BAFs exposed to testosterone combined with resveratrol or melatonin

The induced gene expression of aromatase by testosterone in the T47D-BAF co-culture was not influenced by the presence of 20 μM resveratrol or 20 nM melatonin (Fig. 5). Interestingly, both resveratrol and melatonin at these concentrations statistically significantly reduced the expression of *pS2*; (98.0 ± 1.4%, 75.8 ± 19.4%, respectively) and *ki-67* in T47D cells; (97.8 ± 0.6%, 89.2 ± 5.0%, respectively), which indicates an anti-cancer effect of both these compounds in the T47D co-culture with BAFs (Fig. 6a and b).

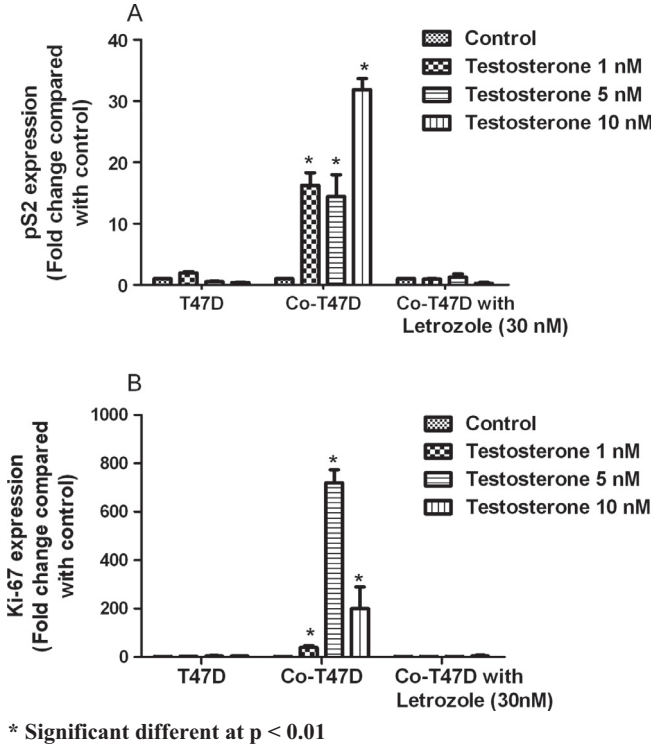


Fig. 2. Change in gene expression of (A) *pS2* and (B) *ki-67* in mono-culture T47D (left), T47D-BAF co-culture (middle) or T47D-BAF co-culture in the presence of letrozole 30 nM (right) treated with testosterone 1, 5 and 10 nM compared to vehicle-treated control. Cell cultures were exposed to the tested chemicals for 120 h. Data are expressed as means ± SD ($n = 9$). *Statistically significantly different from control – treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $P < 0.01$). The expression was diminished by adding letrozole (30 nM) into the system.

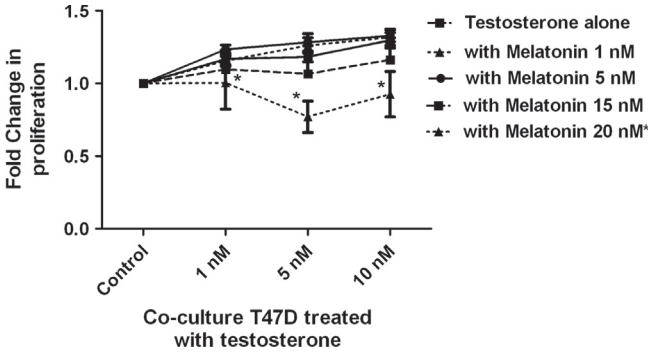


Fig. 3. Cell proliferation of T47D breast cancer cells and BAFs in co-culture with testosterone (1 nM, 5 nM or 10 nM) with or without melatonin (1, 5, 15, 20 nM) compared to the control after 120 h. There was significantly increased in cell proliferation from control to treated co-cultured T47D breast cancer cells with testosterone alone. The proliferation is significantly decreased in the presence of melatonin in the system. The maximum reduction is at 20 nM of melatonin. Data are expressed as means ± SD ($n = 9$). *Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $^*p < 0.01$).

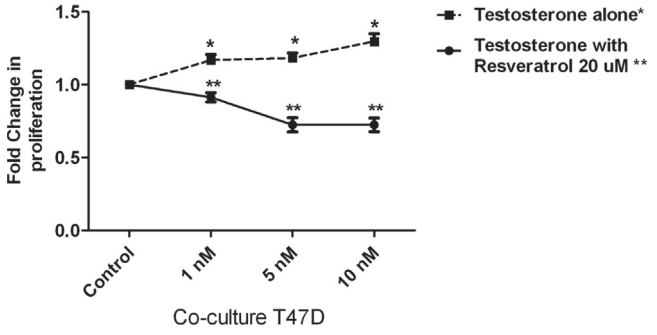
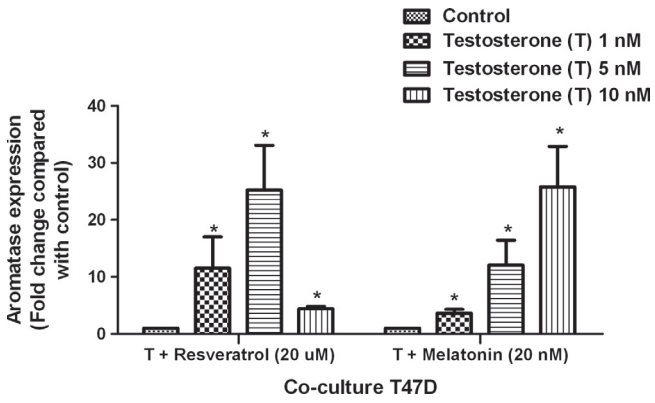
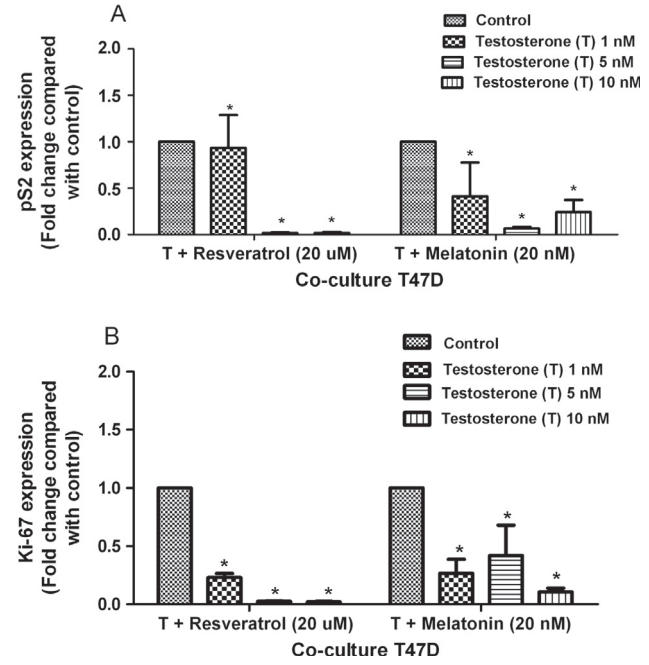


Fig. 4. Cell proliferation of T47D breast cancer cells and BAFs in co-culture with testosterone (1 nM, 5 nM or 10 nM) with or without resveratrol (20 μM) compared to the control after 120 h. There was significantly increased in cell proliferation from control to treated co-cultured T47D breast cancer cells with testosterone alone. The proliferation was significantly decreased in the presence of resveratrol (20 μM) in the system. Data are expressed as means ± SD ($n = 9$). *,**Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $p < 0.01$).



*** Significant different at p < 0.01**

Fig. 5. On the left, *Aromatase* expression in T47D cells co-culture with BAFs treated with testosterone 1, 5 and 10 nM in the presence of resveratrol (20 μM) comparing to the control. On the right, *Aromatase* expression in T47D cells co-culture with BAFs treated with testosterone 1, 5 and 10 nM in the presence of melatonin (20 nM) comparing to the control. Induction of *Aromatase* expression by testosterone in T47D cells co-cultured with BAFs in the presence of resveratrol or melatonin. Cell cultures were exposed to the tested chemicals for 120 h. Data are expressed as means ± SD ($n = 9$). *Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $P < 0.01$).

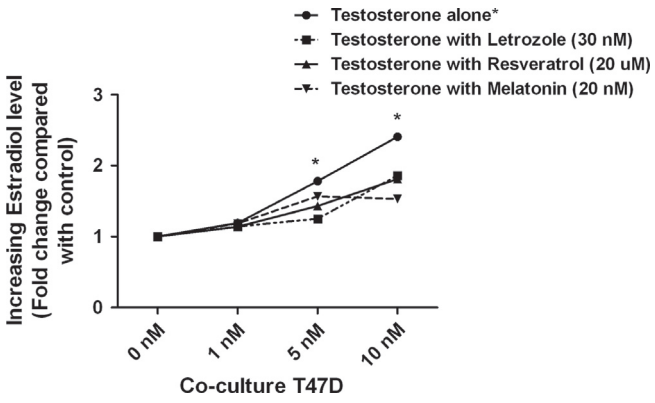


*** Significant different at p < 0.01**

Fig. 6. (A) *pS2* and (B) *ki-67* expression in co-cultured of T47D with BAFs in the presence of testosterone (1 nM, 5 nM or 10 nM) with resveratrol (20 μM) (on the left) or testosterone (1 nM, 5 nM or 10 nM) with melatonin (20 nM) (on the right) comparing to the control. Induced expression of (A) *pS2* and (B) *ki-67* by testosterone were suppressed in the presence of resveratrol and melatonin in co-culture T47D with BAFs. Cell cultures were exposed to the tested chemicals for 120 h. Data are expressed as means ± SD ($n = 9$). *Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $P < 0.01$).

3.5. Estradiol production in co-cultures of T47D with BAFs exposed testosterone alone or combined with letrozole, resveratrol or melatonin

To determine whether the increase in aromatase gene expression also results in higher aromatase activity and thus estradiol levels, the levels of E_2 in the medium of T47D-BAF co-cultures were measured using a commercially available RIA. Indeed, E_2 concentrations in the medium increased with increasing concentrations of testosterone. At 10 nM testosterone, E_2 concentration was increased 2.4-fold compared with vehicle-treated control T47D-BAF co-cultures. Letrozole (30 nM), resveratrol (20 μM) and melatonin (20 nM) all statistically significantly reduced E_2 production in the co-cultures at 5 and 10 nM of testosterone. Interestingly, melatonin was approximately equally effective as the pharmacological aromatase inhibitor letrozole (Fig. 7).



*** Significant different from Letrozole, Resveratrol and Melatonin at p < 0.01**

Fig. 7. Co-cultures T47D with BAFs were exposed to vehicle control or testosterone (1 nM, 5 nM or 10 nM) alone or in the presence of letrozole (30 nM) or resveratrol (20 μM) or melatonin (20 nM) for 120 h. The levels of E_2 in the medium of T47D-BAF co-cultures was measured using a commercially available RIA. There was an increasing of estradiol conversion according to the doses of testosterone. Data are expressed as means ± SD ($n = 6$). *Significantly different from control to treated co-cultured breast cancer cells with BAFs (one-way ANOVA, $P < 0.01$). The highest expression was in testosterone treated co-cultured breast cancer cells (●). *Significantly different from letrozole, resveratrol and melatonin treated co-cultured breast cancer cells (one-way ANOVA, $P < 0.01$).

4. Discussion

4.1. Relevance of co-culture system for in vitro breast cancer studies

There is clear evidence that stromal cells surrounding epithelial breast tumor cells play an important role in tumor cell behavior (van Duursen et al., 2011). In order to mimic the human vivo situation more closely, the tumor microenvironment should be considered in *in vitro* experiments on breast tumor cell behavior. There are two techniques for establishing co-culture systems; a direct (one-compartment) co-culture system or layering technique, and an indirect (two-compartment) co-culture system. Heneweer et al. previously described the paracrine interactions between breast carcinoma MCF7 cells (Heneweer et al., 2005a,b) and human primary BAFs in a direct cell–cell contact *in vitro* co-culture system, while the effect was not observed in two-compartment system. The modulation of aromatase in BAFs was found to be an important aspect in these paracrine interactions as stimulation of aromatase activity in BAF subsequently stimulated cell proliferation of the tumor cells (Heneweer et al., 2005a). The results of our present study clearly demonstrate the relevance of a co-culture system, as testosterone induced tumor-cell derived, estrogen-dependent *pS2* gene expression in a T47D-BAF co-culture, but not in a T47D mono-culture. This clearly demonstrates that in our co-culture system, testosterone is converted into E_2, which subsequently induced proliferation of T47D tumor cells and increase ER-dependent *pS2* expression. This is confirmed by the increased estradiol level in culture medium and the absence of E2-induced cell proliferation in combination with the aromatase inhibitor letrozole. Our results

concur with the conclusion of Miki et al., as described in their recent review (Miki et al., 2012).

In view of the estrogen receptor independent reduction in *ki-67* expression in the presence of testosterone in our co-culture observed this and our previous study indicated a possible role of testosterone, and the androgen receptor (AR), in growth inhibition of these breast tumor cells (Chottanapund et al., 2013). However, it cannot be excluded that testosterone binds to ERβ that is also expressed in T47D cells and reduces cell proliferation via this pathway (Aka and Lin, 2012). In contrast to ERα, activation of ERβ can have an anti-cell proliferative effect on tumor cells (Aka and Lin, 2012). However, it should also be noted that the expression of ERβ is much lower in T47D cells than that of the ERα and AR (Aka and Lin, 2012), which may suggest a dominant role in our experiments of the AR.

4.2. Aromatase inhibition by melatonin and resveratrol

In our present study, we used the T47D-BAF co-culture system to further study the suggested breast cancer chemopreventive effects of resveratrol and melatonin. These compounds have been suggested to act as inhibitors of aromatase and therefore can potentially reduce breast tumor cell proliferation. We have previously explored two estrogen receptor (ERα)-positive breast cancer cell lines MCF-7 and T47D to use for co-culture studies. Both cell lines express the ERα and are also positive for the androgen receptor (AR) (Aka and Lin, 2012). Theoretically, both these cell lines could be used for our co-culture studies, because conversion of testosterone by BAFs to E_2 should induce cell proliferation in the MCF-7 as well as T47D breast tumor cells. (Chottanapund et al., 2013). We decided to use the T47D cell line in this study, because there is evidence that it has a higher androgen receptor expression than MCF-7 cells (Aka and Lin, 2012). Moreover, T47D cells display lower aromatase expression and activity than MCF-7 cells. This means that the BAFs will be the major source of E2 synthesis in our co-culture system, which better reflects the *in vivo* situation (Aka and Lin, 2012).

Firstly, we studied the role of the aromatase enzyme in BAFs and subsequent ER-dependent cell proliferation of T47D cells in our co-culture system. Indeed, testosterone increased cell proliferation and *pS2* and *ki-67* gene expression, while adding the aromatase inhibitor letrozole reduced this testosterone-dependent cell proliferation and gene expression. Next, we tested resveratrol and melatonin for their breast cancer chemopreventive properties with respect to inhibition of aromatase and cell proliferation. Resveratrol displays low toxicity even at very high doses, which makes resveratrol a good candidate for breast cancer treatment or prevention (Crowell et al., 2004). We have tested only one, high concentration of resveratrol (20 μM), which was based on an earlier studies done by Wang et al. in SK-BR-3 cell line. Their study found that resveratrol suppressed promotor I.1-driven aromatase expression (Wang et al., 2008). At this concentration, proliferation of T47D cells in our co-culture system was reduced in the presence of testosterone. Together with the observations that resveratrol reduced estrogen levels in testosterone -exposed T47D-BAF co-cultures, these data suggest that resveratrol is indeed an aromatase inhibitor albeit at a high concentration (20 μM) (Le Corre et al., 2005; Lee and Safe, 2001; Wang et al., 2006, 2008).

Melatonin is an indolic compound secreted by the pineal gland, which plays a physiological role in regulation of the sleep cycle. Melatonin may have anti-tumoral actions including breast cancer, because of its function as the modulator hormone in the neuroendocrine-reproductive axis (Cos and Sanchez-Barcelo, 2000a,b). There are two proposed mechanisms of melatonin as a chemopreventive agent against breast cancer; its can act as a SERM and/or a SEEM. The SERM mechanism of action was suggested to be mediated by the MT1 melatonin receptor, but not via the ERα (Sanchez-Barcelo et al., 2012b). However, with respect to SERM action of melatonin, there are controversial results in the literature. Studies with the human MCF-7 breast carcinoma cell line indicate a decrease in cell proliferation due to the action of melatonin as a SERM at the normal physiologic dose of 1 nM (Cos and Sanchez-Barcelo, 1995; Sanchez-Barcelo et al., 2005). In contrast, other studies with both the estrogen responsive MCF-7 and T47D breast carcinoma cell lines showed no influence of melatonin on cell proliferation up to 0.1 mM concentration (Eck et al., 1998; Papazisis et al., 1998). The latter results are in agreement with our present study with mono-cultured T47D cells. However, we additionally showed in our T47D-BAF co-culture that melatonin acts as an aromatase inhibitor in a concentration-dependent manner. Melatonin has previously been shown to act like a SEEM by inhibiting the expression and activity of aromatase, estrogen sulfatase, and type 1, 17β- hydroxysteroid dehydrogenase in MCF-7 cell line (Sanchez-Barcelo et al., 2012b). In our study, the highest concentration melatonin tested (20 nM) was most effective concentration for aromatase inhibition, which is about one order of magnitude higher than physiologically relevant concentrations that lie between 45 and 200 pM (Kennaway and Voultsios, 1998). However, upon oral intake of melatonin supplements (2 mg), melatonin plasma levels in the nM range can easily be reached without any side effect (Aldhous et al., 1985). Also, in our study the effective concentration of melatonin as a SEEM was in the same concentration range as the well-known potent pharmaceutical aromatase inhibitor letrozole. Our results are in agreement with a previous study done by Grant et al. that melatonin works as SEEM (Grant et al., 2009). Together, this suggests that melatonin and its receptor may be a novel target for breast cancer therapy.

5. Conclusion

Our study again shows that the co-culture of the T47D breast tumor cells combined with breast fibroblasts is a good *in vitro* breast cancer model that can be used to study effects on aromatase, the estrogen receptor as well as the role of androgens in the paracrine interactions between both cell types. Both melatonin and resveratrol were found to be aromatase inhibitors in this co-culture system, albeit at different effect concentrations. Our co-culture model did not provide any indications that melatonin is also a selective estrogen receptor modulator. The melatonin concentration of 20 nM and resveratrol concentration of 20 μM have aromatase inhibitory effect as potent as 20 nM of letrozole, which is a clinically used anti-aromatase drug in breast cancer treatment. SEEM mechanism of action of especially melatonin may offer potential advantages for breast cancer treatment.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by the Chulabhorn Graduate Institute, Chulabhorn Research Institute, and The Institute for Risk Assessment Sciences at Utrecht University.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2014.05.015.

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