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Growth factor signaling enhances aromatase activity of breast cancer cells via post-transcriptional mechanisms

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ABSTRACT

It has been demonstrated that growth factors produced by breast cancer cells stimulate aromatase expression in both breast cancer and adjacent adipose fibroblasts and stromal cells. However, whether these growth factors affect aromatase activity by other mechanisms still remain unclear. In the current study, MCF-7aro and T47Daro aromatase transfected breast carcinoma cells were used to explore the mechanisms of post-transcriptional regulation of aromatase activity by growth factor pathways. Our study reveals that PI3K/Akt and MAPK inhibitors suppressed aromatase activity in MCF-7aro cells. However, PI3K/Akt pathway inhibitors stimulated aromatase activity in T47Daro cells. This is due to enhanced MAPK phosphorylation as compensation after the PI3K/Akt pathway has been blocked. IGF-1 treatment increased aromatase activity in both breast cancer cell lines. In addition, LTEDaro cells (long-term estrogen deprived MCF-7aro cells) which have enhanced MAPK activity, show higher aromatase activity compared to parental MCF-7aro cells, but the aromatase protein level remains the same. These results suggest that aromatase activity could be enhanced by growth factor signaling pathways via post-transcriptional mechanisms.

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1. Introduction

Estrogen plays a critical role in the growth of estrogen-dependent breast carcinoma. Approximately 60% of premenopausal and 75% of postmenopausal patients have estrogen-dependent breast cancer [1,2]. Clinical studies demonstrate that in postmenopausal breast cancer patients, concentration of estrogen in breast tumor tissues is several-fold higher than it in plasma [3]. These results support the hypothesis that estrogen is made and accumulates in the tumor. Aromatase is one of the key enzymes for the biosynthesis of estrogen. The enzyme expression is highly elevated in human breast cancer tissue than in normal breast tissue, as measured by enzyme activity assay and reverse transcription-polymerase chain reaction (RT-PCR) analysis [2,4–8]. High expression of aromatase in breast cancer cells and surrounding adipose stromal cells contributes significantly to breast tumor development and growth in the patients [2,6]. Logically, aromatase is a particularly attractive target in the treatment of estrogen receptor (ER) positive breast cancer [9].

Aromatase is encoded by the *CYP19* gene, and is expressed in a tissue-specific manner. The regulation of aromatase in various tissues is different. So far, several tissue-specific promoter regions have been identified, which include promoters PI.1, PI.3, PI.4, PI.6, PI.7, and PII [10]. PI.4 is the main promoter used in normal adipose tissue and is responsive to glucocorticoids and cytokines such as IL-1β, IL-6 and TNFα. Normal breast cell also utilizes this promoter for aromatase expression [11]. However, the increased expression of aromatase in breast cancer tissues is associated with a switch, from promoter I.4 to promoters I.3 and II, in the major promoter region utilized in *CYP19* gene expression. As a result of the switch, the regulation of estrogen biosynthesis changes from one controlled primarily by glucocorticoids and cytokines to a promoter regulated by cAMP (adenosine cyclic 3′,5′-monophosphate)-mediated pathways [7,8,11].

Prostaglandin E2 (PGE2), the product of cyclooxygenase-2 (COX-2) in the breast cancer cells, binds to EP1 (PGE2 receptor 1) and EP2 receptors to increase the cAMP level and stimulates aromatase gene expression in breast cells [12,13]. In addition, growth factors secreted by breast cancer cells could also stimulate aromatase expression in both breast cancer and adjacent adipose fibrob-

Abbreviations: PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; MAPK, mitogen activated protein kinase; COX2, cyclooxygenase 2; PGE2, prostaglandin E2; EP, PGE2 receptor; ER, estrogen receptor; EGF, epidermal growth factor; TGFα, transforming growth factor α; IGF-1, insulin-like growth factor-1; TNFα, tumor necrosis factor α; FGF, fibroblast growth factor; IL-1β, interleukin-1β; IL-6, interleukin-6; cAMP, adenosine cyclic 3′,5′-monophosphate; Her2, human epidermal growth factor receptor; mTOR, mammalian target of rapamycin; LTED, long-term estrogen deprivation; STIs, signal transduction inhibitors.

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lasts and stromal cells [11,14]. It has been demonstrated that both insulin and insulin-like growth factor-1 are able to potentiate dexamethasone stimulated aromatase activity in human skin fibroblasts. However, they do not directly stimulate aromatase activity in these cells [15]. Epidermal growth factor (EGF) has been found to increase aromatase activity and expression in the human adrenocortical carcinoma cell line NCI-H295R [16]. TGFα is able to enhance both basal and FSH-stimulated aromatase activity after long time treatment in human granulosa cells [17]. In addition, TGFα, EGF and FGF also stimulate aromatase activity in MCF-7 and T47D breast cancer cells [18]. Through the use of a transgenic mouse model, one group found that Her2 status is a determinant of mammary aromatase activity [19]. MCF-7 cell conditioned medium can stimulate aromatase activity in breast adipose fibroblasts at the transcriptional level [20]. All the studies suggest a correlation between growth factor pathways activation and increased aromatase activity. However, it is difficult to distinguish the transcriptional or post-transcriptional regulation of aromatase by growth factors in most of the studies.

Although estrogen concentration is higher in the breast cancer tissue of ER+ breast cancer patients, aromatase protein level does not always proved to be higher than it in the normal breast tissue [5]. One of the reasons is the low efficiency of the current aromatase antibody used to check the aromatase protein level in the studies. Secondly, it is also possible that aromatase activity has been significantly improved by growth factors secreted by the cancer tissue, but aromatase protein level does not increase dramatically. In another word, growth factors might enhance aromatase activity via a post-transcriptional mechanism. If this is the case, it is normal that no high aromatase protein is detected with these ER+ breast cancer samples, but the aromatase activity and estrogen concentration both have been elevated. A systematic study is needed to elucidate the possible aromatase post-transcriptional regulation by growth factors.

In the current study, we used MCF-7aro and T47Daro aromatase transfected breast carcinoma cells to explore the mechanisms of posttranscriptional regulation of aromatase activity. The basal aromatase activity in the original cells is almost undetectable, and the high aromatase activity in the stably transfected cells is artificially controlled and the expression is less likely to be regulated by the cellular signaling pathways [21]. These cells are good model to study the post-transcriptional regulation of aromatase. EGFR and IGFR family are the major growth factor receptors expressed in the breast cancer cell surface. We will mainly focus the study on how these pathways regulate aromatase activity in breast cancer cells. Specific signal transduction inhibitors and growth factors such as IGF were used in the study to investigate how aromatase has been affected. Aromatase protein level was also checked by western blotting to exclude the possibility that aromatase protein degradation could be affected by the growth factor pathways. The results suggest that aromatase activity can be enhanced by growth factor signaling pathways via post-transcriptional mechanisms.

2. Materials and methods

2.1. Reagents

Radiolabeled [1β-^{3}H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products (Boston, MA). PI3K inhibitor LY294002, Akt inhibitor Triciribine and MAPK inhibitor U0126 were purchased from Cayman Chemical (Ann Arbor, MI). mTOR inhibitor RAD001 was from Novartis (Basel, Switzerland). IGF-1, trypsin and all enzymes were obtained from Invitrogen (Carlsbad, CA). Testosterone and 17β-estradiol were from Sigma Chemical (St. Louis, MO). For *in vitro* experiments, these agents at various concentrations were dissolved in DMSO. Mouse anti-aromatase was from Serotec (Raleigh, NC). All other antibodies were from Cell Signaling (Danvers, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). Radioactive samples were counted on a LS6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Scintillation solution ScientiSafe 30% was obtained from Fisher Scientific (Pittsburgh, PA).

2.2. Cell culture

The ER-positive aromatase-overexpressing MCF-7 and T47D cell lines, MCF-7aro and T47Daro, were prepared by stable transfection with the human placental aromatase gene and neomycin selection, as described previously [21]. Her-2-over-expressing MCF-7 cells were kindly provided by Dr. Dihua Yu (The University of Texas M.D. Anderson Cancer Center, Houston, TX). All three breast cancer cell lines were cultured in MEM, supplemented with 10% fetal bovine serum (FBS), 100 mg/L sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin-streptomycin, and 200 mg/L G418 for MCF-7aro, MCF-7/Her2 and T47Daro cells (Invitrogen, Carlsbad, CA). LTEDaro cells (long-term estrogen deprivation MCF-7aro cells) developed in our laboratory were cultured in the same media, but charcoal stripped fetal bovine serum (CSFBS) was used. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

2.3. Tritiated water-release assay in cells

Measurement of aromatase enzyme activity in cells was based on the tritium water release assay [22,23]. For all the experiments, the regular cell culture media with 10%FBS was used. For the IGF-1 treatment, cells were starved of FBS for 24 h before the treatment. Cells in 12-well plates were treated with DMSO (control), various signal transduction inhibitors at different concentrations for 24 h. Then, the cells were incubated for 1 h (24 h incubation for MCF-7 and MCF-7/Her2 cells, and 6-well plates were used) with fresh media containing the inhibitors and 2 μCi [1β-^{3}H]-androst-4-ene-3,17-dione (100 nM). Subsequently, the reaction mixture was removed, and cellular proteins were precipitated using 10% trichloroacetic acid at 42 °C for 20 min. After a brief centrifugation, the media was extracted three times with an equal amount of chloroform to extract unused substrate, and the aqueous layer subsequently treated with 1% dextran-treated charcoal. After centrifugation, a 300-μL aliquot containing the product was counted in 3 mL of liquid scintillation mixture. Each sample was prepared in triplicate and results were corrected for blanks and for the cell contents of culture flasks. One milliliter of 0.5N NaOH was added to each well and the plates were shaken overnight at room temperature to solubilize cell proteins. Protein concentrations were determined by using the Bradford assay method to normalize measured radioactivity.

2.4. Expression and Purification of NmChAro

The design of recombinant human aromatase NmChAro was described by our previous study [24]. The *Escherichia coli* BL21 (DE3) strain was used for the expression of NmChAro. Bacteria was harvested, incubated on ice for 30 min with 0.5 mg/mL lysozyme in buffer A [100 mM potassium-phosphate buffer (pH 7.4), 20% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μM androstenedione], and disrupted by sonication on ice (Branson Sonifier 450, 70% full power, 3× 1 min). NmChAro was isolated from the pelleted membranes with buffer B (buffer A containing 0.1% Tween 20 and 0.5 M NaCl), and purified by metal-ion affinity chromatography (Ni Sepharose 6 Fast Flow; Amersham). After elution of NmChAro with a linear imidozole gradient from 50 mM to 300 mM in buffer B, the red colored fractions were pooled,

desalted, applied to a hydroxyapatite (Bio-Rad) column for the elimination of minor contaminants, and eluted with a linear gradient of 0–1 M NaCl in buffer C (buffer A containing 0.1% Tween 20). Purified NmChAro was loaded on a gel-filtration column (Superdex 200) to remove aggregates and Tween 20 detergent, eluted with buffer D [25 mM Na-HEPES buffer (pH 7.4), 0.15 M NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT], and concentrated using a centrifugal device (Ultra-15 30K; Millipore, Billerica, MA). Purified and concentrated NmChAro Protein concentrations were determined by the Bradford assay method and then stored in buffer D.

2.5. Aromatase enzyme activity assay

Aromatase activity was determined according to the published tritiated water-release method [24]. The standard *in vitro* assay was reconstituted with 100 nM human NADPH-P450 reductase (BD Biosciences, Franklin Lakes, NJ) in a 500-μL reaction buffer containing 67 mM potassium phosphate (pH 7.4), compounds treatment and DMSO as control, 0.1% BSA, 0.1 μg purified recombinant aromatase, 10 μM progesterone, and 500 nM [1β-^{3}H]androstenedione at 37 C in a shaking water bath for 20 min. The incubation was initiated by the addition of 300 μM of NADPH, and terminated by the addition of 500 μL 20% trichloroacetic acid. The reaction was mixed with charcoal-dextran to remove any trace amount of unreacted substrate. After centrifugation of the mixture, the radioactivity of the supernatant was counted by a LS 6500 liquid scintillation counter.

2.6. Western blot

Cells were cultured in 60-mm culture dishes and incubated with DMSO or STIs for 24 hours and then lysed with CelLytic M (Sigma–Aldrich) supplemented with protease inhibitor tablets (Roche, Indianapolis, IN). Cell lysates were sonicated briefly to reduce viscosity. Protein concentration was determined and samples were stored at −70 °C until use. Sixty micrograms of total protein lysate for each sample were boiled with 1× loading buffer (100 mmol/L DTT plus bromophenol blue) for 5 min. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad) using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 100 mA for 90 min. The membrane was blocked for 2 h with 5% nonfat milk in 1× TBS-T at room temperature and then incubated with primary antibody, in 1% bovine serum albumin at 4 °C overnight. After the membrane was incubated with the primary antibody and washed four times with 1× TBS-T for 5 minutes each wash, it was incubated with the secondary antibody in 5% nonfat milk for 60 min at room temperature. The membrane was washed four times again for 5 min each time with 1× TBS-T and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the protocol of the manufacturer. The membrane blot was exposed to Basic Autorad Film (ISC Bioexpress, Kaysville, UT) and developed using a Konica SRX-101A (Konica, Tokyo, Japan). The blot was reprobed with rabbit anti-actin (Santa Cruz Biotechnology) at 1:2000 dilution as a loading control. Mouse anti-aromatase (Serotec, Raleigh, NC) was diluted (1:300). Mouse anti-pAkt, p-MAPK were diluted (1:1000). Rabbit anti-her2, Akt, MAPK were diluted (1:1000).

2.7. Statistical analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). Western densitometry quantification was performed with Totallab Quant Software (TotalLab Ltd).

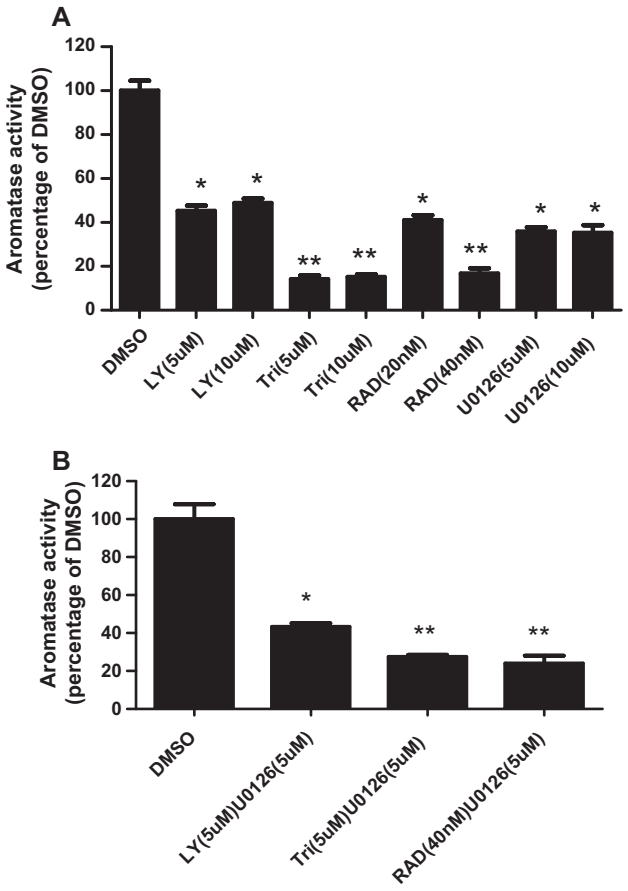

Fig. 1. Suppression of aromatase activity in MCF-7aro breast cancer cells by STIs. Cells were treated with STIs at indicated concentrations for 24 h. Aromatase activity was subsequently determined during a 1 h assay as described. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. (A) Single STIs treatment. (B) Combination of STIs treatment $^*p < 0.005$ *vs* DMSO; $^{**}p < 0.001$ *vs* DMSO by unpaired *t* test. Similar results were obtained in at least two independent experiments.

3. Results

3.1. Signaling transduction inhibitors suppressed aromatase activity in MCF-7aro cells

Several studies have demonstrated that aromatase activity can be regulated by a phosphorylation process in brain tissue [25,26]. Researchers suggest that this modification also happens in breast cancer cells [27,28]. Based on these results, we speculate that growth factor pathways controlled phosphorylation process might affect aromatase activity post-transcriptionally. The results exhibit that signal transduction inhibitors (STIs) which include PI3K inhibitor LY294002, Akt inhibitor Triciribine, mTOR inhibitor RAD001 and MAPK inhibitor U-0126 suppressed aromatase activity in MCF-7aro cells (Fig. 1A). This is consistent with other studies and our speculation [27]. However, it seems that this suppression is not dose-dependent for most of the inhibitors. Possible the lower concentration of the inhibitor saturated the corresponding kinases already, which makes the higher concentration of these inhibitors cannot further improve the effect. Since the expression of aromatase is artificially controlled in this cell line, these inhibitors can only regulate aromatase activity via post-transcriptional mechanisms. Further studies reveal that combination of inhibitors which are able to block the PI3K/Akt pathway and MAPK pathways still could not totally suppress the cellular aromatase activity (Fig. 1B). This suggests that the regulation of aromatase is very complex in

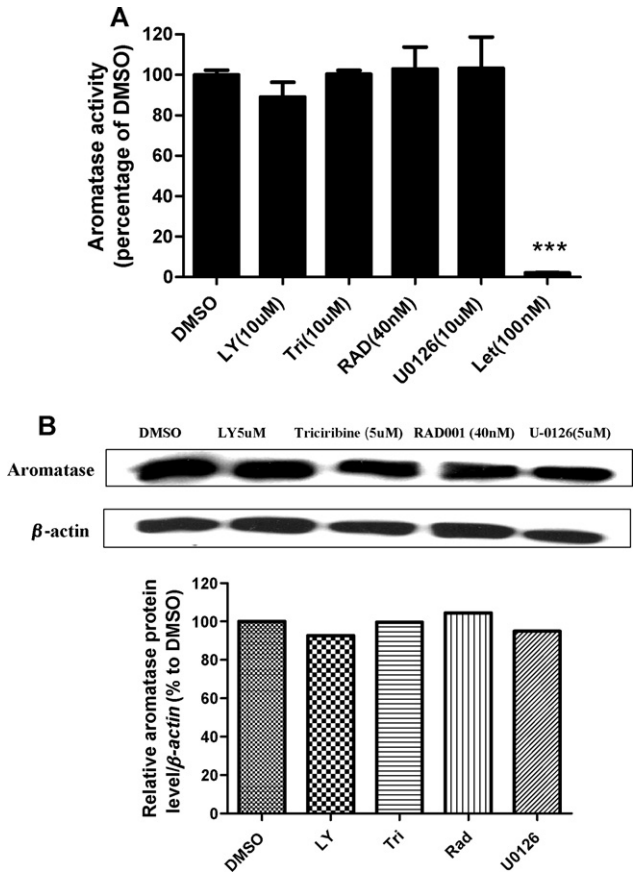

Fig. 2. STIs neither directly inhibit aromatase, nor improve the degradation of aromatase in breast cancer cells. (A) Aromatase inhibition assay. Aromatase inhibitor letrozole was used as positive control. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. (B) Aromatase enzyme level was checked by western blot in MCF-7aro cells after the cells were treated with STIs at the indicated concentrations for 24 h. ***$p < 0.0001$ *vs* DMSO by unpaired *t* test. Similar results were obtained in at least two independent experiments.

the breast cancer cells, and multiply pathways including PI3K/Akt and MAPK signaling channels might be involved. Blocking only these two pathways is not enough to erase all aromatase activity. It is also possible that growth factor pathways only enhance basal aromatase activity. STIs blocking the growth factor pathways can only erase the enhancement of aromatase, but cannot affect the basal aromatase activity.

3.2. Signal transduction inhibitors did not direct block aromatase enzyme, neither change aromatase enzyme concentration in MCF-7aro cells

It is also possible that the kinase inhibitors in our studies affect aromatase activity by direct inhibition. To rule out this possibility, recombinant human aromatase expressed and purified from *E. coli* was used to test the aromatase inhibitory activity of these inhibitors [24]. The result demonstrates that these compounds did not inhibit aromatase enzyme activity even at much higher concentrations compared with the ones used in the cell studies (Fig. 2A). As a positive control, aromatase inhibitor (AI) letrozole significantly inhibited aromatase activity. The results also exhibit that aromatase enzyme basal activity does exist which is independent of growth factor pathways. Because the recombinant human aromatase shows good enzyme activity and growth factors are not involved in the process. Furthermore, these kinase inhibitors did not change the aromatase enzyme level in MCF-7aro cells as checked by western blot (Fig. 2B), which proves that these inhibitors did not increase the aromatase enzyme degradation in breast cancer cells. The same experiments were repeated at T47Daro cells. However, the aromatase enzyme level in this cell line is too low to be detected. Artificially expressed aromatase in MCF-7aro cells is less likely to be affected by transcriptional regulation. Our results indicate that STIs did not directly inhibit aromatase, neither affect the enzyme degradation. It supports the theory that growth factors pathways post-transcriptionally modify aromatase which could be blocked by STIs.

3.3. PI3K/Akt pathway inhibitors stimulated aromatase activity in T47Daro cells

To further investigate if this phenomenon occurs in other breast cancer cells, the same experiment was repeated in T47Daro cells. Unexpectedly, the PI3K inhibitor LY294002, Akt inhibitor Triciribine and mTOR inhibitor RAD001 stimulated aromatase activity in this breast cancer cell line. LY294002 at 10 μM stimulated aromatase activity less than 5 μM, which is due to the T47Daro cellular toxic effect of LY294002 at higher concentration. MAPK inhibitor U0126 still suppressed aromatase activity similarly as in MCF-7aro cells (Fig. 3A). Further studies revealed that MAPK inhibitor U0126 could block the PI3K/Akt pathway inhibitors stimulated aromatase activity (Fig. 3B). The possible explanation for this phenomenon is that T47Daro cells might compensate for the loss of signal in the PI3K/Akt pathways by enhancing the MAPK pathway for survival. The increased MAPK signaling further enhanced the aromatase activity in T47Daro cells, which could be reversed by MAPK inhibitor U0126. However, this compensation mechanism did not happen in MCF-7aro cells. This speculation was further proved by Western blot. MAPK phosphorylation in T47Daro cells was increased after PI3K inhibitor LY294002 treatment (Fig. 3C). Akt levels remained the same, and phosphorylated Akt was too low to be detected (data not shown). These results also suggest that the aromatase post-transcriptional regulation in these two cell lines is different. In MCF-7aro cells, both PI3K/Akt and MAPK pathways are important for the post-transcriptional regulation of aromatase, and the enzyme activity decreases if either one of them is blocked. In T47Daro cells, it appears that the MAPK pathway plays a more important role in regulating aromatase activity than the PI3K/Akt pathway. In addition, T47Daro cells use the MAPK pathway as a compensation mechanism to survive when the PI3K/Akt pathway is blocked.

3.4. IGF-1 treatment increased MCF-7aro and T47Daro cell aromatase activity

To further check whether growth factor signaling can enhance breast cancer cellular aromatase activity, IGF-1 was used to treat the MCF-7aro and T47Daro cells since IGFR is expressed in both of the cell lines. Aromatase activity was significantly increased in both cell lines dose-dependently after the treatment (Fig. 4A and B). Because aromatase transcription is artificially controlled in these two cell lines, IGF-1 unlikely enhances the aromatase activity through a transcriptional mechanism.

3.5. Her2 overexpressed breast cancer cells have higher aromatase activity

Her2 transfected MCF-7 cells (MCF-7/Her2) displayed higher aromatase activity compared to MCF-7 cells (Fig. 5A). However, the level of aromatase activity in MCF-7/Her2 cells was still very low, despite Her2 signaling enhancement of aromatase activity. The aromatase activity in MCF-7 cells was too low to be detected (Fig. 5A), such that Her2 signaling could not enhance the low

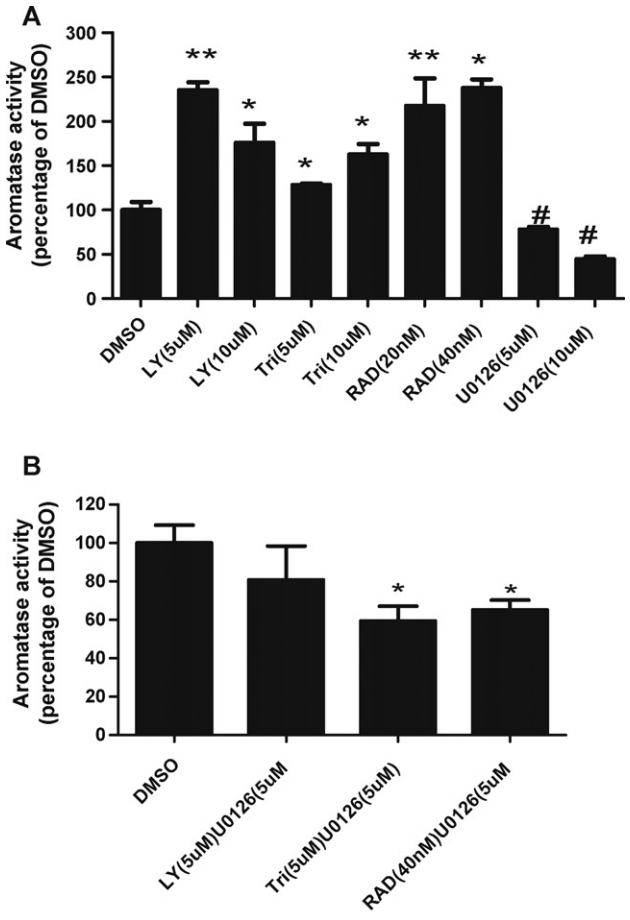

Fig. 3. Regulation of aromatase activity in T47Daro breast cancer cells by STIs. Cells were treated with STIs at indicated concentrations for 24 h. Aromatase activity was subsequently determined during a 1 h assay as described. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. (A) Single STIs treatment. (B) Combination of STIs treatment. (C) Akt and MAPK pathways were checked after PI3K inhibitor and MAPK inhibitor treatment. $^{*}p < 0.05$ *vs* DMSO; $^{**}p < 0.001$ *vs* DMSO by unpaired *t* test (stimulation); $^{\#}p < 0.05$ *vs* DMSO; $^{\#\#}p < 0.001$ *vs* DMSO by unpaired *t* test (suppression). Similar results were obtained in at least two independent experiments.

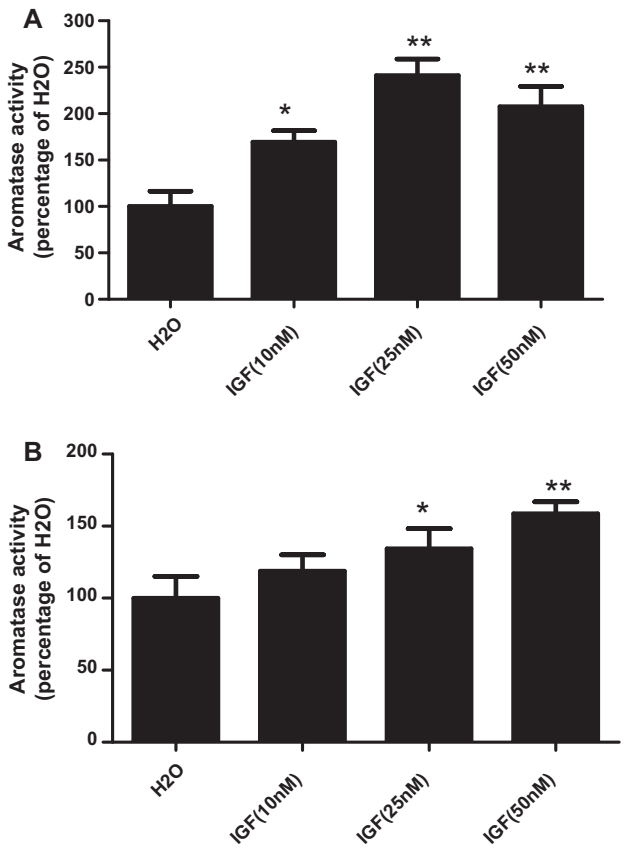

Fig. 4. Effect of IGF on MCF-7aro and T47Daro cell aromatase activity. (A) MCF-7aro cells and (B) T47Daro cells. Cells were treated with IGF at indicated concentration for 24 h. Aromatase activity was subsequently determined during a 1 h assay as described. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. $^{*}p < 0.05$ *vs* H_2O; $^{**}p < 0.001$ *vs* H_2O by unpaired *t* test. Similar results were obtained in at least two independent experiments.

aromatase activity significantly. It will be ideal if Her2 pathway amplification could enhance the aromatase activity of an aromatase stable expressed breast cancer cell line. We initially try to transfer Her2 full gene into the MCF-7aro cells which express high level of aromatase. However, this strategy does not work for this special cell line although Her2 could be easily transfected into normal MCF-7 cells. There is no good explanation for this phenomenon yet. Possible the aromatase transfection changed the MCF-7 cells somehow which make the second transfection very difficult for the cells. It has been reported that estrogen deprivation will up-regulate Her2 expression in MCF-7 cells [27,29]. We adapted this methodology to the MCF-7aro cells used in the current studies. MCF-7aro cells had very high aromatase activity because aromatase was always highly expressed in this cell line (K_m = 105.2 nM, V_m = 204.1 pmol/mg protein/h). After long-term estrogen deprivation (LTED), MCF-7aro cells became LTEDaro cells which had much higher aromatase activity (K_m = 368.2 nM, V_m = 769.2 pmol/mg protein/h) compared to the MCF-7aro cells (Fig. 5B and C). In addition, the Her2 protein level was elevated in LTEDaro cells like we predicted. The downstream kinase MAPK phosphorylation was also increased, but phosphorylated Akt was too low to be detected. Total aromatase, Akt and MAPK protein levels in LTEDaro cells were similar to that in parental MCF-7aro cells as checked by Western blot (Fig. 5D). We also used Her2 inhibitor (AG825) to check if the aromatase activity in LTEDaro cells could be suppressed. However, AG825 is toxic to LTEDaro cells. Although LTEDaro cell decreased aromatase activity (data not shown), it is difficult to check whether aromatase suppression is due to Her2 pathway blocking or lower cell viability.

4. Discussion

Estrogen binds to ER and stimulates hormone-dependent breast carcinoma growth. It has been proved that the estrogen concentration is much higher in the breast tumor tissue than it in the normal breast tissue. Several studies demonstrated that aromatase,

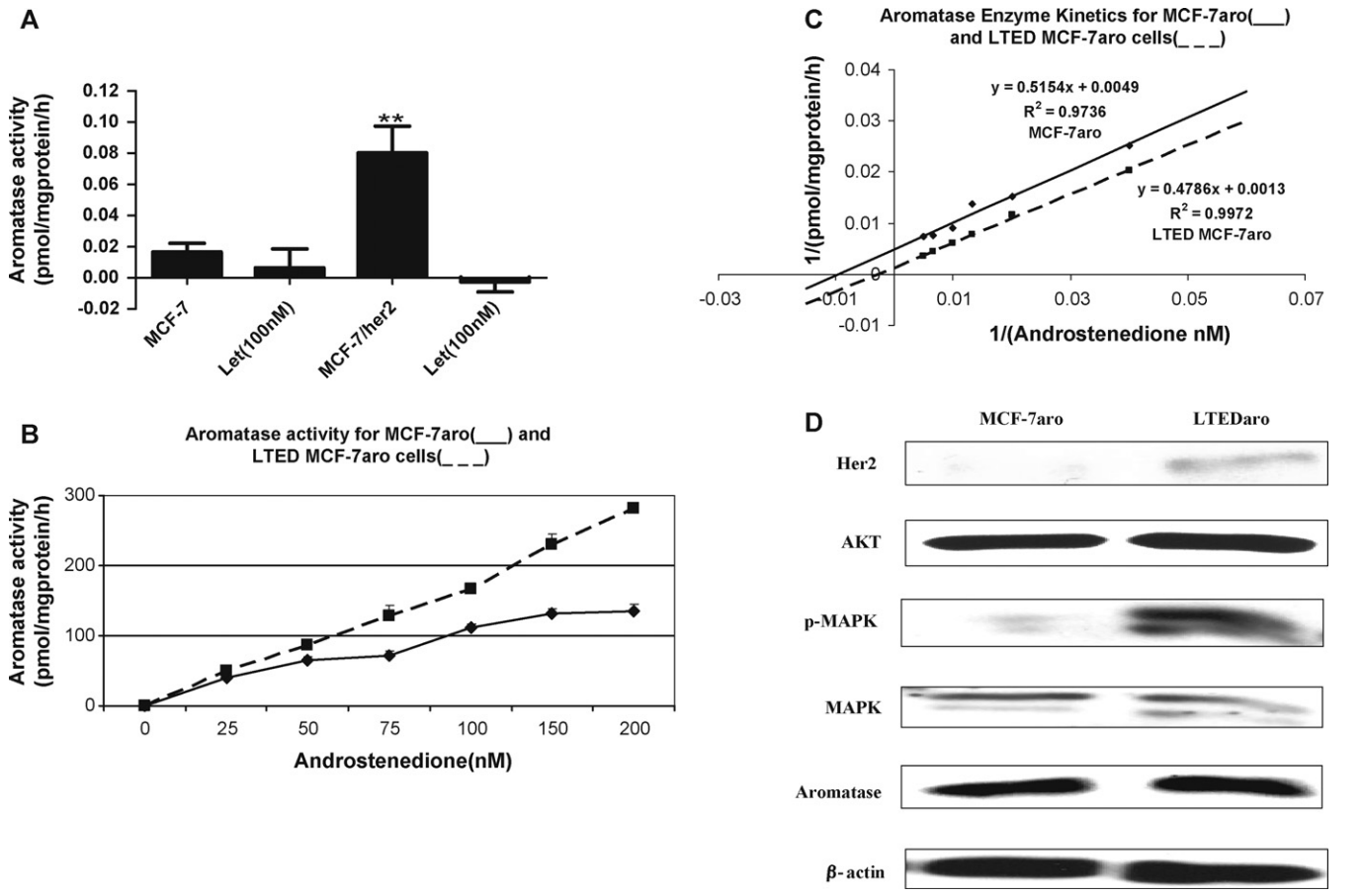

Fig. 5. Her2 pathway enhances cellular aromatase activity. (A) MCF-7 and MCF-7/Her2 cellular aromatase activity was determined during a 24 h assay. $^{**}p < 0.001$ *vs* MCF-7 by unpaired *t* test. (B) MCF-7aro and LTEDaro cells aromatase activity were carried out by incubation of cells for one hour with the androgen substrate at various concentrations (25, 50, 75, 150, and 200 nmol/L). (C) Kinetic analysis of aromatase activity in MCF-7aro and LTEDaro cells. The K_m and V_m values were determined to be 105.2 nM, 204.1 pmol/mg protein/h for MCF-7aro cells; 368.2 nM, 769.2 pmol/mg protein/h for LTEDaro cells respectively. (D) MCF-7aro and LTEDaro cell Her2 pathway and aromatase western blot.

the key enzyme for the biosynthesis of estrogen, is highly expressed in breast cancer tissue as quantified by RT-PCR [4,7,8,11]. Multiple factors attribute to the increase of aromatase expression, such as cytokines, growth factors and PGE2 produced by the COX enzyme at the cancer tissue. However, immunehistochemistry study could not provide solid evidence that aromatase protein staining is much higher in breast cancer tissue [5]. Several reasons can explain the phenomenon. Firstly, the current aromatase antibody is not sensitive enough to detect aromatase enzyme. Secondly, aromatase protein level actually is not very high in the breast tumor tissue although aromatase gene expression and activity are higher. Is it possible that aromatase could be converted into a more active pattern in breast cancer cells via a post-transcriptional modification by growth factors pathways? If this is the case, aromatase protein may not be significantly increased in breast cancer tissue, but aromatase activity could be dramatically up-regulated to produce high level of estrogen.

Our current studies focus on revealing the post-transcriptional regulation of aromatase in breast cancer cells by growth factor pathways. Both MCF-7aro and T47Daro cells have high artificially controlled aromatase expression, and the aromatase expression is less possible to be affected by growth factor pathways [21]. STIs which include PI3K inhibitor LY294002, Akt inhibitor Triciribine, mTOR inhibitor RAD001 and MAPK inhibitor U0126, decreased the aromatase activity in MCF-7aro cells. Since they did not directly inhibit or improve the degradation of aromatase as checked by enzyme assay and Western blotting, we speculate that the signaling pathways blocked by these kinase inhibitors might play a role in regulating aromatase activity via post-transcriptional mechanisms. Further, STIs which block PI3K/Akt pathways enhanced the aromatase activity in T47Daro cells. This result appears conflict with the conclusion from the MCF-7aro cell studies. However, T47Daro cells used MAPK pathways as a compensation mechanism to survive after Akt pathway was blocked, which simultaneously enhances the aromatase activity in the cells. This compensation process did not occur in MCF-7aro cells. The result also suggests that MAPK pathway may play a more important role in regulating aromatase activity than PI3K/Akt pathway in T47Daro cells. In addition, IGF-1 which could activate IGFR and downstream signaling pathways which include PI3K/Akt and MARP pathways increased aromatase activity dose-dependently in both MCF-7aro and T47Daro cells. This result further proves that activation of growth factor signaling pathways could enhance breast cancer cellular aromatase activity.

To explore whether the higher expression of growth factor receptors contribute to higher breast cancer cellular aromatase activity, we checked the effects of Her2 which is highly elevated in about 30% of breast cancer patients on aromatase activity in breast cancer cells [19]. MCF-7 cells have very low aromatase activity and Her2 transfected MCF-7 cells exhibited relatively higher aromatase activity in the study. Other studies suggest that Her2 could stimulate aromatase expression via a PGE2 mediated mechanism [19]. It appears difficult to determine whether the Her2 pathway enhances the aromatase activity by a transcriptional or post-transcriptional

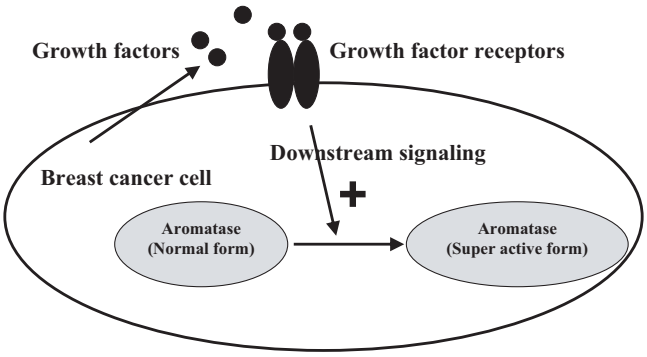

Fig. 6. A proposed model to describe the growth factor pathway that enhances breast cancer cellular aromatase activity.

manner in this model. Since we failed to transfer Her2 full gene into MCF-7aro cells, we switched to the naturally way to increase Her2 expression in MCF-7aro cells. Long term estrogen deprivation will make hormone dependent breast cancer cells switch to Her2 pathway for proliferation. Several laboratories already developed Her2 highly expressed MCF-7 cells by this strategy. We adapted it to our MCF-7aro cells. As we expected, MCF-7aro cells developed higher Her2 during the long term estrogen deprivation and became LTEDaro cells. The formed LTEDaro cells show significantly higher aromatase activity than parental MCF-7aro cells. However, the aromatase enzyme concentration remained the same between the two cell lines (Fig. 5D), which suggests that aromatase activity was enhanced through a post-transcriptional mechanism. Taking together all the results, we can conclude that Her2 pathway is able to enhance breast cancer cellular aromatase activity via post-transcriptional mechanism. However, it is still unclear which downstream kinase is regulating aromatase and whether the kinase regulates aromatase in a direct or indirect manner. We used Her2 and IGFR downstream kinase Akt plus ATP to check whether Akt could increase the activity of bio-engineered pure aromatase as suggested in other research. The negative result can only prove that Akt is not the direct regulator in our model. Her2 highly expressed MCF-7/her2 cell lysate and ATP could slightly enhanced the activity of pure aromatase in an enzyme assay (data not shown), but it is difficult to further find out which kinase is the direct player since cell lysate is such a complex mixture.

So far, we can only get to this point that growth factors contribute to the increased aromatase activity in breast cancer tissue via post-transcriptional mechanisms. This is an important supplement to the current understanding that growth factor only increased *CYP19* mRNA in breast cancer cells. Based on our results, a simplified mechanism by which growth factor pathways regulate aromatase is proposed (Fig. 6). Aromatase exists in two states: a normal form or a highly active form. The normal form could switch to a highly active form by growth factors in breast cancer cells and this switch is reversible.

It has been reported that the ER antagonist ICI182780 suppresses MCF-7aro cellular aromatase activity in a non-direct inhibitory manner [30]. There was no clear explanation in the study about the mechanism. Recently, ICI 182780 has been found to decrease Akt phosphorylation in a MCF-7aro xenograft model, which could explain why this ER antagonist agent suppresses cellular aromatase activity according to our theory [29]. These studies further support our model that by blocking growth factor signaling pathways, aromatase activity can be partially suppressed in breast cancer cells. Aromatase inhibitors are more effective for hormone dependent breast cancer than tamoxifen. However, resistance will occur after long term usage. Several laboratories' AI resistance models suggest that breast cancer cells adapt to growth factor pathways for survival [27,29]. Therefore, STIs which block these pathways can potentially overcome AI resistance. Several clinical trials are currently undergoing to evaluate whether STIs could be used to solve the AI resistance for ER positive breast cancer. Based on our results, STIs also benefit the patients due to the aromatase suppression function. More investigations are still needed to further reveal what is the exact direct regulator to post-transcriptionally affect aromatase.

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References

[1] American Cancer Society, Cancer Facts and Figures 2009, Atlanta, 2009.
[2] S.E. Bulun, T.M. Price, J. Aitken, M.S. Mahendroo, E.R. Simpson, A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription, J. Clin. Endocrinol. Metab. 77 (1993) 1622–1628.
[3] J.R. Pasqualini, G. Chetrite, C. Blacker, M.C. Feinstein, L. Delalonde, M. Talbi, C. Maloche, Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients, J. Clin. Endocrinol. Metab. 81 (1996) 1460–1464.
[4] V.H. James, J.M. McNeill, L.C. Lai, C.J. Newton, M.W. Ghilchik, M.J. Reed, Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies, Steroids 50 (1987) 269–279.
[5] H. Sasano, D.P. Edwards, T.J. Anderson, S.G. Silverberg, D.B. Evans, R.J. Santen, P. Ramage, E.R. Simpson, A.S. Bhatnagar, W.R. Miller, Validation of new aromatase monoclonal antibodies for immunohistochemistry: progress report, J. Steroid Biochem. Mol. Biol. 86 (2003) 239–244.
[6] X.Z. Sun, D. Zhou, S. Chen, Autocrine and paracrine actions of breast tumor aromatase. A three-dimensional cell culture study involving aromatase transfected MCF-7 and T-47D cells, J. Steroid Biochem. Mol. Biol. 63 (1997) 29–36.
[7] C. Zhou, D. Zhou, J. Esteban, J. Murai, P.K. Siiteri, S. Wilczynski, S. Chen, Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction, J. Steroid Biochem. Mol. Biol. 59 (1996) 163–171.
[8] D. Zhou, C. Zhou, S. Chen, Gene regulation studies of aromatase expression in breast cancer and adipose stromal cells, J. Steroid Biochem. Mol. Biol. 61 (1997) 273–280.
[9] R.W. Brueggemeier, J.C. Hackett, E.S. Az-Cruz, Aromatase inhibitors in the treatment of breast cancer, Endocr. Rev. 26 (2005) 331–345.
[10] S. Chen, Aromatase and breast cancer, Front. Biosci. 3 (1998) d922–d933.
[11] W.R. Miller, P. Mullen, P. Sourdaine, C. Watson, J.M. Dixon, J. Telford, Regulation of aromatase activity within the breast, J. Steroid Biochem. Mol. Biol. 61 (1997) 193–202.
[12] J.A. Richards, R.W. Brueggemeier, Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes, J. Clin. Endocrinol. Metab. 88 (2003) 2810–2816.
[13] Y. Zhao, V.R. Agarwal, C.R. Mendelson, E.R. Simpson, Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene, Endocrinology 137 (1996) 5739–5742.
[14] M.B Crichton, J.E. Nichols, Y. Zhao, S.E. Bulun, E.R. Simpson, Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells, Mol. Cell Endocrinol. 118 (1996) 215–220.
[15] N. Emoto, N. Ling, A. Baird, Growth factor-mediated regulation of aromatase activity in human skin fibroblasts, Proc. Soc. Exp. Biol. Med. 196 (1991) 351–358.
[16] M. Watanabe, M. Noda, S. Nakajin, Effect of epidermal growth factor and prostaglandin on the expression of aromatase (CYP19) in human adrenocortical carcinoma cell line NCI-H295R cells, J. Endocrinol. 188 (2006) 59–68.
[17] C.R. Harlow, D.J. Cahill, L.A. Maile, P.G. Wardle, M.G. Hull, Time-dependent effects of transforming growth factor alpha on aromatase activity in human granulosa cells, Hum. Reprod. 10 (1995) 2554–2559.
[18] C.M. Ryde, J.E. Nicholls, M. Dowsett, Steroid and growth factor modulation of aromatase activity in MCF7 and T47D breast carcinoma cell lines, Cancer Res. 52 (1992) 1411–1415.
[19] K. Subbaramaiah, L.R. Howe, E.R. Port, E. Brogi, J. Fishman, C.H. Liu, T. Hla, C. Hudis, A.J. Dannenberg, HER-2/neu status is a determinant of mammary aromatase activity in vivo: evidence for a cyclooxygenase-2-dependent mechanism, Cancer Res. 66 (2006) 5504–5511.
[20] S. Deb, J. Zhou, S.A. Amin, A.G. Imir, M.B. Yilmaz, Z. Lin, S.E. Bulun, A novel role of sodium butyrate in the regulation of cancer-associated aromatase promoters

I.3 and II by disrupting a transcriptional complex in breast adipose fibroblasts, J. Biol. Chem. 281 (2006) 2585–2597.
[21] D.J. Zhou, D. Pompon, S. Chen, Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening, Cancer Res. 50 (1990) 6949–6954.
[22] B. Su, E.S. Az-Cruz, S. Landini, R.W. Brueggemeier, Novel sulfonanilide analogues suppress aromatase expression and activity in breast cancer cells independent of COX-2 inhibition, J. Med. Chem. 49 (2006) 1413–1419.
[23] B. Su, S. Landini, D.D. Davis, R.W. Brueggemeier, Synthesis and biological evaluation of selective aromatase expression regulators in breast cancer cells, J. Med. Chem. 50 (2007) 1635–1644.
[24] Y. Hong, B. Yu, M. Sherman, Y.C. Yuan, D. Zhou, S. Chen, Molecular basis for the aromatization reaction and exemestane-mediated irreversible inhibition of human aromatase, Mol. Endocrinol. 21 (2007) 401–414.
[25] J. Balthazart, M. Baillien, T.D. Charlier, G.F. Ball, Calcium-dependent phosphorylation processes control brain aromatase in quail, Eur. J. Neurosci. 17 (2003) 1591–1606.
[26] J. Balthazart, M. Baillien, G.F. Ball, Interactions between kinases and phosphatases in the rapid control of brain aromatase, J. Neuroendocrinol. 17 (2005) 553–559.
[27] W. Yue, J.P. Wang, M.R. Conaway, Y. Li, R.J. Santen, Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways, J. Steroid Biochem. Mol. Biol. 86 (2003) 265–274.
[28] S. Catalano, I. Barone, C. Giordano, P. Rizza, H. Qi, G. Gu, R. Malivindi, D. Bonofiglio, S. Ando, Rapid estradiol/ERalpha signaling enhances aromatase enzymatic activity in breast cancer cells, Mol. Endocrinol. 23 (2009) 1634–1645.
[29] G. Sabnis, O. Goloubeva, D. Jelovac, A. Schayowitz, A. Brodie, Inhibition of the phosphatidylinositol 3-kinase/Akt pathway improves response of long-term estrogen-deprived breast cancer xenografts to antiestrogens, Clin Cancer Res. 13 (2007) 2751–2757.
[30] B.J. Long, S.L. Tilghman, W. Yue, A. Thiantanawat, D.N. Grigoryev, A.M. Brodie, The steroidal antiestrogen ICI 182,780 is an inhibitor of cellular aromatase activity, J. Steroid Biochem. Mol. Biol. 67 (1998) 293–304.