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Suppression of Aromatase in Human Breast Cells by A Cyclooxygenase-2 Inhibitor and Its Analog Involves Multiple Mechanisms Independent of Cyclooxygenase-2 Inhibition

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Suppression of aromatase in human breast cells by a cyclooxygenase-2 inhibitor and its analog involves multiple mechanisms independent of cyclooxygenase-2 inhibition

Bin Su, Edgar S. Díaz-Cruz, Serena Landini, Robert W. Brueggemeier

Introduction

Breast cancer is the second leading cause of cancer death in women in the United States, and an estimated 212,930 women in the United States will be diagnosed with breast cancer in 2006 [1]. Approximately two-thirds of breast cancers are termed hormone-dependent breast cancers, contain estrogen receptors (ER), and require estrogen for tumor growth. Treatment of ER-positive breast cancer cases includes hormonal therapies such as aromatase inhibitors (AIs) or antiestrogens/selective estrogen modulators (SERMs) [2].

The most commonly used SERM is tamoxifen, which inhibits the growth of breast tumors by competitive antagonism of estrogen at the ER ligand binding site. However, development of drug resistance and increased uterine and endometrial cancer reduce its clinical application [3–5]. In contrast, AIs markedly suppress plasma estrogen levels by inhibiting aromatase, the cytochrome P450 enzyme responsible for the synthesis of estrogens from androgenic precursors. However, these compounds inhibit aromatase activity in a global fashion and thus could adversely impact sites where estrogen is required for normal func-
tion. The risk of important long-term reduction in bone density, including osteoporosis, may increase with the use of aromatase inhibitors [6–8]. Short-term use of letrozole has been associated with an increase in bone-resorption markers in plasma and urine, and adjuvant therapy with anastrozole appears to be associated with a higher incidence of fractures than adjuvant therapy with tamoxifen [9–11].

A new approach to reduce the risk of side effects is to develop agents that regulate aromatase expression in a tissue-specific manner. In postmenopausal women, estrogens are produced locally in the breast by aromatase. Higher levels of estrogens in breast cancer cells and breast adipose tissue stimulate tumor growth by binding to estrogen receptors in both an autocrine and a paracrine manner [12–14]. In humans, cytochrome P450 aromatase is encoded by the CYP19 gene, which contains nine coding exons (exons II–X). The expression of this gene is regulated in a tissue-specific manner by the alternative use of eight promoters, each one of them being associated with a specific S′ untranslated exon I [15]. Furthermore, due to the unique organization of tissue-specific promoters, various promoters employ different signaling pathways and different transcription factors [16–20].

One of the major stimulators of aromatase expression in cancer breast is prostaglandin E2 (PGE2) derived from tumorous epithelium and/or infiltrating macrophages. PGE2 acts via EP1 and EP2 receptor subtypes to stimulate both the protein kinase A and protein kinase C pathways to increase aromatase expression through promoter II and promoter I.3 in breast stromal cells [21–24]. Moreover, expression of the CYP19 gene is correlated with COX-1 and COX-2 expression in human breast cancer [25]. This biochemical mechanism may explain epidemiological observations of the beneficial effect of non-steroidal anti-inflammatory drugs (NSAIDs) on breast cancer. Recently, the COX-2 selective inhibitor, celecoxib, has shown strong chemopreventive activity against mammary carcinoma in rats [26].

COX-2 inhibitors can suppress aromatase activity in breast cancer cells by suppressing aromatase transcription [27]. However, the suppression varied significantly among COX-2 inhibitors. This observation suggests differences in the mechanisms by which these COX inhibitors modulate aromatase expression in breast cancer cells. Among all the tested COX-2 inhibitors, NS-398 showed the highest potency in suppressing aromatase transcription and activity compared to other COX-2 inhibitors studied. On the other hand, NS-398 is a weak inhibitor of COX-2. Thus, NS-398 may target aromatase gene regulation through other pathways independent of direct COX-2 inhibition. To investigate potential mechanisms for NS-398 suppression of aromatase, we synthesized N-Methyl NS-398 (Me-NS-398) (Fig. 1), which does not have COX-2 inhibitory activity but has very similar chemical structure to NS-398 [28]. N-Methyl NS-398 suppresses aromatase expression and activity in SK-BR-3 breast cancer cells at similar extent with NS-398. In the present study, we investigated the effects of these two agents on the regulation of aromatase expression in other human breast cancer cells and in breast stromal cells from patients.

Materials and methods

Reagents

Radiolabeled [18, 3H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products (Boston, MA). NS-398 and RU486 was purchased from Cayman Chemical (Ann Arbor, MI). Me-NS-398 was synthesized in our laboratory [28]. For in vitro experiments, these agents at various concentrations were dissolved in DMSO. Trypsin, TRIzol, and all enzymes were obtained from Invitrogen (Carlsbad, CA). Radioactive samples were counted on a LS6800 liquid scintillation counter (Beckman, Palo Alto, CA). Scintillation solution 3a70B was obtained from Research Prospect International Corp. (Mount Prospect, IL). Tetradecanoyl phorbol acetate (TPA), dexamethasone (DEX), forskolin (FSK), NADP+ and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO). Human tissues (breast cancer, placenta) were obtained through OSUCCC Tissue Procurement under IRB-approved protocols OSU #2002H0104 and OSU #2002H0105.

Cell culture

MCF-7 cells and MDA-MB-231 cells were obtained from ATCC (Rockville, MD). Cell cultures were maintained in phenol red-free custom media (MEM, Earle’s salts, 1.5× amino acids, 2× nonessential amino acids, L-glutamine, 1.5× vitamins, Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 20 mg/L gentamycin. Adipose stromal cells were obtained as described [29] and maintained in DMEM/F12 media with 10% FBS, 2 mM L-glutamine and 20 mg/L gentamycin. Fetal bovine serum was heat inactivated for 30 min in a 56 °C water bath before use. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO2 in a Hereaus CO2 incubator. For all experiments, cells were plated in either T-25
flasks or 100 nM plates and grown to subconfluence. Before treatment, the media was changed to defined media consisting of DMEM/F12 media (Sigma) with 1.0 mg/mL human albumin (OSU Hospital Pharmacy), 5.0 mg/L human transferin and 5.0 mg/L bovine insulin.

**Tritiated water-release assay in cells**

Measurement of aromatase enzyme activity in cells was based on the tritium water release assay [24]. Cells in T-25 flasks or 100 mM plates were treated with DMSO (control), NS-398 and N-methyl NS-398 for 24 h. In the combination studies, cells were pre-treated with FSK/TPA or DEX for 24 h, then in combination with the indicated compounds for 24 h. After 24 h, cells were then incubated for 6 h with fresh media containing drugs and 2 µCi [1β-3H]-androst-4-ene-3,17-dione (100 nM). Subsequently, the reaction mixture was removed, and 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, 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 250 µL aliquot containing the product was counted in 5 mL of liquid scintillation mixture. Each sample was run in triplicate and background values were determined with microsomal protein inactivated by boiling.

**Preparation of human placental microsomes**

Human placentas were processed immediately after delivery from The Ohio State University Hospitals at 4 °C. The placenta was washed with normal saline, and connective and vascular tissue was removed. Microsomes were prepared from the remaining tissue using the method described by Kellis and Vickery [31]. Microsomal suspensions were stored at −80 °C until required.

**RNA extraction**

Total RNA was isolated using the TRIzol reagent according to the manufacturer’s protocol. Total RNA pellets were dissolved in DNase, RNase-free water and quantitated using a spectrophotometer. The quality of RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide; the 18S and 28S rRNA bands were visualized under ultraviolet light.

**cDNA synthesis**

Isolated total RNA (2 µg) was treated with DNase I Amplification grade, according to the recommended protocol to eliminate any DNA before reverse transcription. Treated total RNA was denatured at 65 °C for 5 min in the presence of 2.5 mg/µL random hexamers and 0.5 mM dNTP mix. The samples were snap-cooled on ice and centrifuged briefly. Complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase according to the recommended protocol. Briefly, the reactions were conducted in the presence of 1X First-Strand Buffer and 20 mM DTT at 42 °C for 50 min and consequently inactivated at 70 °C for 15 min. The cDNA generated was used as a template in real-time PCR reactions.

**Real-time PCR**

Real-time PCR was performed using the Opticon™ 2 system from MJ Research (Waltham, MA). For the CYP19 total gene the PCR reaction mixture consisted of Taqman® Universal PCR Master Mix (Applied Biosystems), 600 nM of CYP19 primer (sense: 5′-TGT CTC TTT GTC CTT CA-TCT ATT TCT C-3′; antisense: 5′-CTA CCA ATA ACA GTC TGG ATT TCG-3′); 250 nM Taqman probe (6FAM 5′-TGC TGG CAC CAG ACT TGC CCT C-3′; TAMRA), and 2.0 µL of each cDNA sample in a final volume of 20 µL. For the 18S housekeeping total gene the PCR reaction mixture consisted of Taqman® Universal PCR Master Mix (Applied Biosystems), 500 nM of 18S primer (sense: 5′-CAC TTT GGA GGA ACA TCA GAC-3′; antisense: 5′-ATT TGG CCT GGA GAA CAG CCC TG-3′); 50 nM Taqman probe (6FAM 5′-TGC TGG CAC CAG ACT TGC CCT C-3′; TAMRA) (Invitrogen), and 2.0 µL of each cDNA sample in a final volume of 20 µL. The Taqman probes for aromatase and 18S were designed to anneal to a specific sequence of the aromatase and 18S gene correspondingly between the forward and the reverse primers. Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min.

**Cell proliferation assay**

MCF-7 cells were harvested, counted, and plated at a concentration of 1 × 10^4 cells/well in 400 µL total volume/well in 24-well plates. After 24 h, the culture medium was removed.
and changed to DMEM/F12 media supplemented with 5% charcoal stripped FBS. After 24 h, culture wells (n = 6) were treated with the compounds combined with or without Testosterone (100 nM) (400 μL volume) every 2 days for a total of 6 days. Twenty-four hours after the last treatment, 3, (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and phenazine methosulfate were prepared in PBS at a final assay concentrations of 333 μg/mL and 25 μM, respectively. These solutions were combined and 50 μL of this mixture were added to each well. After 1 h of incubation at 37 °C, absorbance at 490 nm was measured using a SPECTRAMax plate reader.

**Statistical analysis**

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). Determination of IC50 values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s t-test and P values reported at 95% confidence intervals.

**Results**

**Decrease of aromatase enzymatic activity in MCF-7 and MDA-MB-231 breast cancer cells**

PGE2 produced in breast cancer tissue regulates aromatase expression through PKA/PKC driven pathway in an autocrine and paracrine manner in breast cells [23,24]. COX inhibitors were able to suppress aromatase in breast cancer cells at the transcriptional level. However, COX-2 inhibitors which have similar IC50 values for COX-2 inhibition differ significantly in their ability to suppress aromatase activity. NS-398 showed the highest potency in suppressing aromatase activity compared to other agents tested such as celecoxib [27]. We hypothesize that NS-398 may target regulation of aromatase gene transcription more effectively through other pathways than direct COX-2 inhibition. To test this hypothesis, we synthesized NS-398 derivative Me-NS-398(N-methyl-N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide), which does not inhibit PGE2 production (Fig. 1) [28].

The effects of NS-398 and Me-NS-398 on aromatase activity are determined in MCF-7 and MDA-MB-231 breast cancer cells. At 25 μM, both compounds significantly decreased aromatase activity in the two cell lines (Fig. 2). At 5 μM, both compounds only decreased aromatase activity in MCF-7 cells. Thus, Me-NS-398 and NS-398 exhibit similar effects on the suppression of aromatase activity in both breast cancer cell lines.

**Aromatase activity assay in human placental microsomes**

The compounds were tested for potential direct aromatase inhibition in the microsomal aromatase assay to determine if the decrease of aromatase activity is due to direct enzyme inhibition. These investigations find that NS-398 and Me-NS-398 produce only weak inhibition of aromatase with IC50 values of 26.5 and 19.3 μM, respectively (Fig. 3). The results suggest that the suppression of aromatase activity by NS-398 and Me-NS-398 in MCF-7 and MDA-MB-231 cells by direct enzyme inhibition may only be observed at high concentrations.

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Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). Determination of IC50 values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student’s t-test and P values reported at 95% confidence intervals.

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![Aromatase inhibition of microsomal activities of compounds NS-398 (■) and Me-NS-398 (▲). Error bars represent standard error (n = 3), and the data were statistically analyzed by a nonlinear regression analysis method.](image-url)

**Fig. 3 – Aromatase inhibition of microsomal activities of compounds NS-398 (■) and Me-NS-398 (▲). Error bars represent standard error (n = 3), and the data were statistically analyzed by a nonlinear regression analysis method.**

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Fig. 4 – Real-time RT-PCR analysis of CYP19 mRNA expression in MCF-7 (A) and MDA-MB-231 cells (B). Cells were treated for 24 h with the test agents at different concentrations, and total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M. *P < 0.05 vs. control by unpaired t test (n = 9).

CYP19 mRNA expression by real-time PCR in MCF-7 and MDA-MB-231 cells

Analysis of total CYP19 mRNA transcripts was performed using real-time PCR in order to assess the extent of suppression in aromatase activity by NS-398 and Me-NS-398 through down-regulation of aromatase expression at transcriptional level. Cells were treated with NS-398 and Me-NS-398 for 24 h at 5 and 25 μM. Total RNA was extracted after 24 h, and CYP19 transcript levels are compared to control (vehicle) treatment. Both compounds significantly decreased CYP19 gene expression in the two cell lines relative to the control at 25 μM (Fig. 4). No effect on the expression level of the housekeeping gene 18S rRNA was observed with any of the compounds. At 5 μM, both compounds did not decrease CYP19 gene expression, but they suppressed aromatase activity in MCF-7 cells (Fig. 3A). This suppression of enzyme activity is not likely to occur through direct enzyme inhibition, because the drug concentration in this experiment is much lower than the IC50 value from the microsomal aromatase inhibition study. This suggests that a nongenomic mechanism may also be involved in suppression of cellular aromatase activity. In all the experiments, Me-NS-398 decrease aromatase activity and expression to a similar extent; thus, NS-398 and Me-NS-398 apparently target other pathway(s) to suppress aromatase.

NS-398 and Me-NS-398 suppress aromatase stimulated by FSK/TPA in breast cancer and stromal cells

FSK/TPA can stimulate aromatase activity by activating PKA and PKC pathways, which are the major pathways to stimulate aromatase expression in breast cancer. DEX stimulates aromatase expression by binding to glucocorticoid receptor, which is the main pathway for aromatase expression in normal breast, skin and bone tissue [18,22,23]. The effect of NS-398 and Me-NS-398 on the aromatase activity stimulated by FSK (25 μM)/TPA (10 nM) or DEX (200 nM) in MCF-7 and MDA-MB-231 cells was examined. NS-398 and Me-NS-398 at 2 μM did not suppress aromatase activity stimulated by DEX, whereas DEX stimulation could be suppressed by RU486 (200 nM), a glucocorticoid receptor antagonist, in MDA-MB-231 cells (Fig. 5B). On the other hand, the two agents suppress aromatase activity stimulated by FSK/TPA in MCF-7 cells at 2 μM.
Fig. 6 – Effect of NS-398 and Me-NS-398 on induced CYP19 gene expression in MCF-7 breast cancer cells. Cells were pre-treated with FSK(25 μM)/TPA (10 nM) for 24 h, then with the indicated compounds (2 μM) and FSK/TPA for 24 h. Total RNA was isolated. Results are expressed as means of CYP19 (normalized to 18S rRNA) ± S.E.M.; *p < 0.05 vs. FSK/TPA treatment for suppression of induced aromatase transcription; **p < 0.05 vs. DMSO treatment for stimulation of aromatase transcription by unpaired t-test (n = 9). Similar results were obtained in at least two independent experiments.

(Fig. 5A). Furthermore, we find that this suppression of aromatase activity occurs at the transcriptional level (Fig. 6). This suggests that these agents affect PKA/PKC-driven transcription of aromatase. FSK/TPA does not stimulate aromatase in MDA-MB-231 cells, and DEX does not stimulate aromatase in MCF-7 cells (Fig. 5B lane 5; Fig. 5A lane 5). For MCF-7 cells, the aromatase transcription occurs primarily via promoter I.3 and II. Promoter I.4 is not detected in this cell line [17], which may explain why DEX cannot stimulate promoter I.4 driven aromatase transcription.

In addition, NS-398 and Me-NS-398 at 2 μM decreased the aromatase activity stimulated by FSK (10 μM)/TPA (10 nM) in breast stromal cells, and this suppression occurred at the transcriptional level (Figs. 7 and 8). NS-398 and Me-NS-398 at 2 μM do not suppress aromatase activity stimulated by DEX (200 nM).

NS-398 and Me-NS-398 significantly suppressed testosterone induced MCF-7 cell proliferation

Treatment of MCF-7 cells with NS-398 or Me-NS-398 did not significantly affect normal MCF-7 cell proliferation at 2 μM (Fig. 9). Since the aromatase enzyme is present in MCF-7 cells, testosterone can be converted to estradiol and stimulate MCF-7 cell growth [33,34]. In this present study, testosterone (100 nM) significantly increase MCF-7 cell proliferation. Both NS-398 and Me-NS-398 suppressed testosterone-induced cell proliferation in MCF-7 cells, presumably by suppression of aromatase expression and subsequently lack of conversion of testosterone to estradiol.

Discussion

Increased aromatase expression in breast cancer tissue is associated with a switch in promoter usage from the normal adipose-specific promoter I.4 to the PGE2-responsive promoter II and promoter I.3. While promoter I.4 requires the
synergistic actions of glucocorticoids and class I cytokines [19], promoters I.3 and II are both transactivated via PKA and PKC dependent signaling pathways [19,35]. This switch in the regulatory mechanism of aromatase expression from normal breast tissue to cancerous tissue has been extensively investigated [36]. Our hypothesis is that higher levels of COX-2 expression result in higher levels of PGE2, which in turn results in increased CYP19 expression. Potentially, COX-2 inhibitors could inhibit aromatase expression selectively in cancer breast tissue; therefore, COX-2 inhibitors may be classified as the first generation of selective aromatase expression regulators for ER positive breast cancer.

In our previous study [27], the COX-2 selective inhibitor NS-398 suppressed aromatase transcription and activity in breast cancer cells significantly better than other COX-2 inhibitors, even though NS-398 has weaker COX-2 inhibitory activity. These results suggested that other mechanisms may also be involved in regulation of aromatase expression by COX inhibitors. Various NSAIDs can influence the expression of certain transcription factors and other pathway mediators that could result in the suppression of aromatase.

To elucidate the phenomenon, Me-NS-398, which is devoid of COX-2 inhibitory activity, was synthesized and studied previously in SK-BR-3 breast cancer cells [28]. The biological activities of NS-398 and Me-NS-398 on aromatase were investigated here in MCF-7 and MDA-MB-231 breast cancer cells, normal breast stromal cells and also in microsomal aromatase assays. The results showed that Me-NS-398 and NS-398 suppressed aromatase activity and expression in these breast cancer cells. Microsomal aromatase inhibition studies further demonstrated that the compounds only inhibit aromatase enzyme at high concentrations. NS-398 and Me-NS-398 suppressed CYP19 gene expression in both MCF-7 and MDA-MB-231 cell lines as measured by real time PCR. These results suggest that the compounds suppress aromatase activity in breast cancer cells via multiple mechanisms.

To further investigate the possible mechanism(s) in suppression of aromatase expression, the compounds were tested for their ability to suppress aromatase in breast cancer and stromal cells stimulated by FSK/TPA or DEX, which induce aromatase expression via different pathways and promoters. FSK/TPA stimulates aromatase via PKA and PKC pathways, and DEX induces aromatase via glucocorticoid receptor mediated transcription. The results demonstrated that NS-398 and Me-NS-398 suppressed aromatase activity by decreasing PKA/PKC driven transcription (FSK/TPA stimulated) and did not interfere with normal breast aromatase expression mediated through glucocorticoid receptor (DEX stimulated) in both breast epithelial and stromal cells. Additionally, the compounds inhibited testosterone stimulated MCF-7 cell proliferation, which also suggests that the compounds are able to decrease aromatase activity.

In summary, though N-methyl NS-398 does not show COX-2 inhibitory activity, it decreases aromatase activity and expression in breast cancer cells to a similar extent as NS-398. This suggests NS-398 decreases aromatase expression in breast cells by other mechanism(s) other than COX-2 inhibition. These two compounds selectively affected aromatase stimulated from FSK/TPA or DEX, which are the major pathways involved in aromatase expression in breast cancer. Consequently, our current research focuses on discerning the specific molecular target of the compounds and identifying a novel molecular basis for the development of selective aromatase expression regulators. In addition, investigations of structural requirements for aromatase suppression of NS-398 and analogs are on-going.

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