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Aromatase and COX in Breast Cancer: Enzyme Inhibitors and Beyond

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Introduction

Estradiol is the most potent endogenous estrogen and is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase [1]. The highest levels of enzyme are present in the ovaries of premenopausal women, in the placenta of pregnant women, and in the peripheral adipose tissues of postmenopausal women and of men. Aromatase activity has also been demonstrated in breast tissue *in vitro* [2-4]. Furthermore, expression of aromatase is highest in or near breast tumor sites [3,5], and the importance of intratumoral aromatase and local estrogen production is being unraveled [3,6,7]. Aromatase has been measured in the stromal cell component of normal breast and breast tumors, but the enzyme has also been detected in the breast epithelial cells *in vitro* [2,5,7-9]. Furthermore, expression of aromatase is highest in or near breast tumor sites [5,7]. The exact cellular location(s) of aromatase must await more rigorous analysis by several labs with a new monoclonal antibody now being developed and evaluated [10].

Regulation of aromatase in various tissues is complex, and several tissue-specific promoter regions have been identified upstream from the *CYP19* gene [11-13]. These tissue-specific promoters include promoter PI.1, PI.3, PI.4, PI.6, PI.7, and PII. Promoter PI.1 is the major promoter used in

placental tissues and is the farthest upstream. The PII promoter is utilized in the ovary and in breast cancer tissues, and it contains a cAMP response element. Promoters PI.3, PI.4, PI.6, and PI.7 are the promoters used in extraglandular sites. Promoter PI.4 is the primary promoter used in normal adipose tissue and is responsive to glucocorticoids and cytokines such as IL-1β, IL-6 and TNFα.

The increased expression of aromatase cytochrome P450 observed in breast cancer tissues was associated with a switch in the major promoter region utilized in gene expression. In the normal breast cells, aromatase expression is primarily derived by the tissue-specific promoter I.4 for transcription, whereas expression from breast cancer patients switches from promoter I.4 to promoter I.3 and promoter II [14]. As a result of the use of the alternate promoter, the regulation of estrogen biosynthesis switches from one controlled primarily by glucocorticoids and cytokines to a promoter regulated through cAMP-mediated pathways [14]. The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis [14], whereas other autocrine factors such as IL-1β do not appear to act via PGE_2 [15].

Aromatase and cyclooxygenases in breast cancer

Prostaglandin G/H endoperoxide synthase, also referred to as cyclooxygenase (COX), is a key enzyme which catalyzes the conversion of arachidonic acid to prostaglandins. Local production of PGE_2 via the cyclooxgenase isozymes (constitutive COX-1 isozyme and inducible COX-2 isozyme) can influence estrogen biosynthesis and estrogen-dependent breast cancer. This biochemical mechanism may explain epidemiological observations of the beneficial effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on breast cancer [16–19]. Previous studies in our laboratory suggest a relationship between CYP19 gene expression and the expression of COX genes [20]. Gene expressions of CYP19, COX-1, and COX-2 were performed in 20 human breast cancer specimens and in 5 normal control breast tissue samples. A positive correlation was observed between CYP19 expression and the greater extent of breast cancer cellularity, in agreement with literature reports showing that aromatase levels were higher in tumors than in normal tissue. Furthermore, a positive linear correlation was observed between COX-2 expression breast cancer cellularity in each sample. Linear regression analysis using a bivariate model shows a strong linear association between CYP19 expression and the sum of COX-1 and COX-2 expression. Similar correlations between CYP19 expression and COX-2 expression in breast cancer patient specimens have been confirmed in other laboratories [21].

This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis. In human breast stromal cells, PGE_2 acts via two G-protein coupled receptors, EP_1 and EP_2 receptors, to stimulate aromatase gene expression

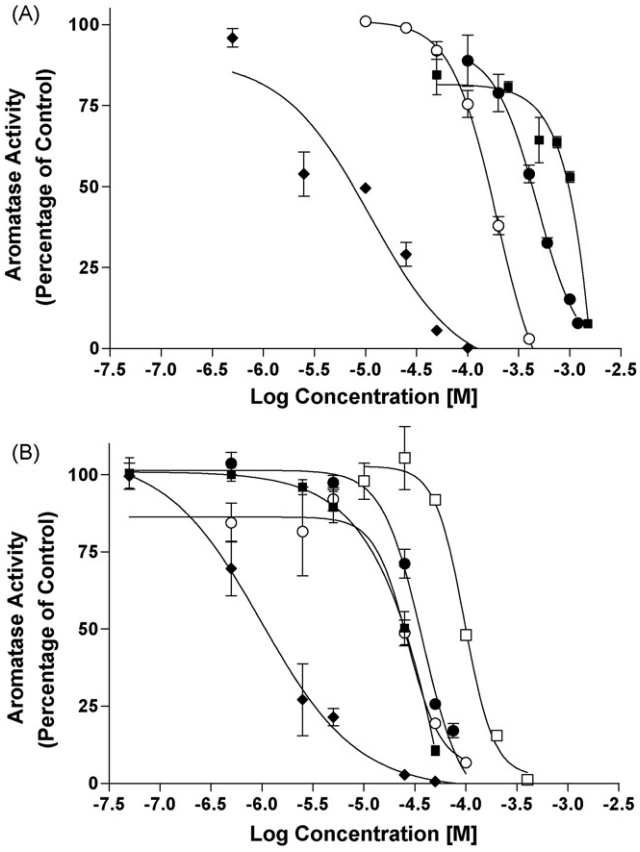


Fig. 1. Effect of NSAIDs and COX-specific inhibitors on aromatase enzyme activity. (A) SK-BR-3 cells were treated with indomethacin (◯), piroxicam (●), ibuprofen (■), or SC-560 (♦), and aromatase activity was measured using the tritiated water-release assay. (B) SK-BR-3 cells were treated with NS-398 (♦), nimesulide (◯), SC-58125 (■), celecoxib (●), or niflumic acid (□), and aromatase activity was measured using the tritiated water-release assay.

via protein kinase A and protein kinase C signaling pathways [22]. NSAIDs, COX-1 and COX-2 selective inhibitors produce dose-dependent decreases in aromatase activity in breast cancer tissues (Fig. 1) [23,24]. Real-time PCR analysis of aromatase gene expression showed a significant decrease in mRNA levels by these agents, and the effect of COX inhibitors on aromatase expression occurs through suppression at the tissue-specific promoters PI.3, PI.4, and PII. This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis (Fig. 2).

Our current research focuses on pharmacological regulation of aromatase and/or cyclooxygenases by agents that can act locally to decrease the biosynthesis of estrogen and may provide additional therapy options for patients with hormone-dependent breast cancer. Two pharmacological approaches are being developed, one involving mRNA silencing by selective short interfering RNAs (siRNA) molecules and the second utilizing small molecule drug design.

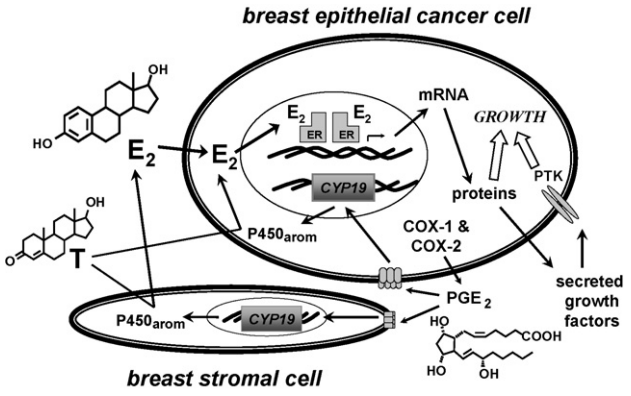


Fig. 2. Model of autocrine and paracrine pathways of aromatase and cyclooxygenases in hormone-dependent breast cancer.

Materials and methods

Reagents

The synthesis of the sulfonanilide analogs is described in our recent publication [25].

Cell culture

The MCF-7 and SK-BR-3 cell lines 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× non-essential 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. Fetal calf 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% CO_2 in a Hereaus CO_2 incubator. For all experiments, cells were plated in either T-25 flasks or 100 cm^2 plates and grown to subconfluency. Before treatment, the media was changed to a defined one containing 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

Measurement of aromatase enzyme activity was based on the tritium water-release assay [22]. Cells in T-25 flasks or 100 cm^2 plates were treated with 0.1% DMSO (control), NSAIDs (ibuprofen, piroxicam, and indomethacin), COX-1 selective inhibitor SC-560, and COX-2 selective inhibitors (SC-58125, NS-398, celecoxib, niflumic acid and nimesulide) at the indicated concentrations. After 24 h, the cells were incubated for 6 h with fresh media along with 50 nM androstenedione including 2 μCi [1β-^{3}H]-androst-4-ene-3,17-dione. 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 further dextran-treated charcoal. After centrifugation, a 250-μl aliquot containing the product was counted in 5 ml of liquid scintillation mixture. Results were corrected for blanks and for the cell contents of culture flasks, and results were expressed as picomoles of ^3H_2O formed per hour incubation time per million live cells (pmol/h/10^6 cells). To determine the amount of live cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay.

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 ng/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 1× 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 each primer (Invitrogen), 250 nM Taqman probe, 18S rRNA (Applied Biosystems, Foster City, CA), and 2.5 μl of each RT sample in a final volume of 25 μl. 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. For the specific exon I promoter regions and TATA-box-binding protein (*TBP*), the PCR reaction mixture consisted of DyNAmo Hot Start SYBR Green qPCR kit (MJ Research), 600 nM of each primer [24], and 2.5 μl of each RT sample in a final volume of 20 μl. SYBR Green uses a dye that will bind to double stranded DNA. In this methodology, the primers are carefully designed to each of the promoter regions of aromatase exon I. Cycling conditions were 95 °C for 15 min, followed

by 50 cycles at 94 °C for 10 s and 60 °C for 25 s and 72 °C for 30 s.

Statistical analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). Determination of IC_{50} values was 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

RNA interference of aromatase and cyclooxygenase-2

Newly developed RNA interference (RNAi) technology was utilized to further probe the interactions between aromatase and cyclooxygenases in breast cancer. RNAi technology permits transient suppression of the levels of endogenous proteins in mammalian cells by enhancing the degradation of target mRNA. One RNAi approach involves transfection of 21–23 nucleotide double-stranded, short interfering RNAs into mammalian cells. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC), and RISC unwinds the siRNA duplex using ATP. The unwound, single-stranded antisense strand guides RISC to mRNA that has a complementary sequence, and the complex results in the endonucleolytic cleavage of the target mRNA.

Short interfering RNAs were designed to target either human *CYP19* mRNA or human *COX-2* mRNA. The designed siRNAs were transiently transfected in SK-BR-3 cells, and several were effective in suppressing *CYP19* mRNA and in decreasing *COX-2* mRNA [23]. Two siRNA molecules, termed siAROM and siCOX2, were chosen for further investigations in SK-BR-3 cells. The transfection of siAROM resulted in suppression of *CYP19* mRNA levels by greater than 90% compared with cells transfected with a nonspecific control siRNA, termed siCTRL (Fig. 3A). The siAROM produced little suppression of *COX-2* mRNA expression (Fig. 3B). Also shown in Fig. 3B, transfection of siCOX2 suppressed *COX-2* mRNA levels by greater than 90% compared with cells transfected with siCTRL. This siCOX2 also resulted in suppression of *CYP19* mRNA levels by approximately 60% (Fig. 3A). Thus, the suppression of *COX-2* mRNA, which lowered levels of COX-2 enzyme and decreased prostaglandin production, results in suppression of *CYP19* mRNA levels.

The effects of siAROM and siCOX2 on aromatase enzyme activity in SK-BR-3 cells were also examined. The transfection of siAROM resulted in suppression of basal levels of aromatase activity by greater than 90% compared with cells transfected with a nonspecific control siRNA or untreated cells (Fig. 4A). Also shown in this figure, the siCOX2 also resulted in suppression of aromatase activity by approximately 67%. Furthermore, treatment of SK-BR-3 cells with the combination of forskolin and dexamethasone results in stimulation of aromatase activity, and the siAROM and siCOX2 both significantly suppressed the induced aromatase activity in these cells (Fig. 4B). Finally, the administration of PGE_2 to cells treated with the siRNAs results in antagonism of only the siCOX2 and restores aromatase activity to untreated levels (Fig. 5).

Novel sulfonanilide analogs for suppression of aromatase expression and activity

As shown in Fig. 1, different COX-2 inhibitors with similar IC_{50} values (concentration for 50% inhibition) for COX-2 inhibition differ significantly in their ability to suppress aromatase activity. This observation suggests differences in the mechanisms by which these COX inhibitors modulate aromatase expression in SK-BR-3 cells. It is noteworthy that the effect of aromatase suppression by the COX-2 selective inhibitor NS-398 was greater than other COX-2 inhibitors,

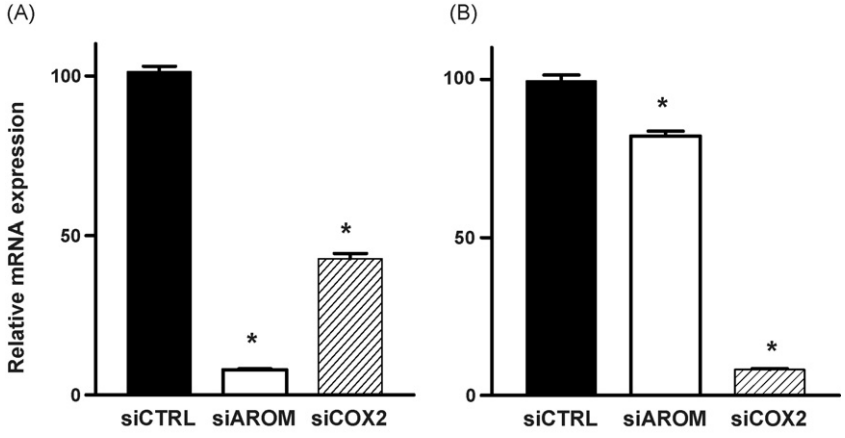


Fig. 3. Suppression of CYP19 mRNA (A) and COX-2 mRNA (B) by siRNA molecules transfected into SK-BR-3 breast cancer cells. Each data bar represents the mean results of three independent determinations. $^{*}P < 0.05$ vs. control by unpaired *t*-test.

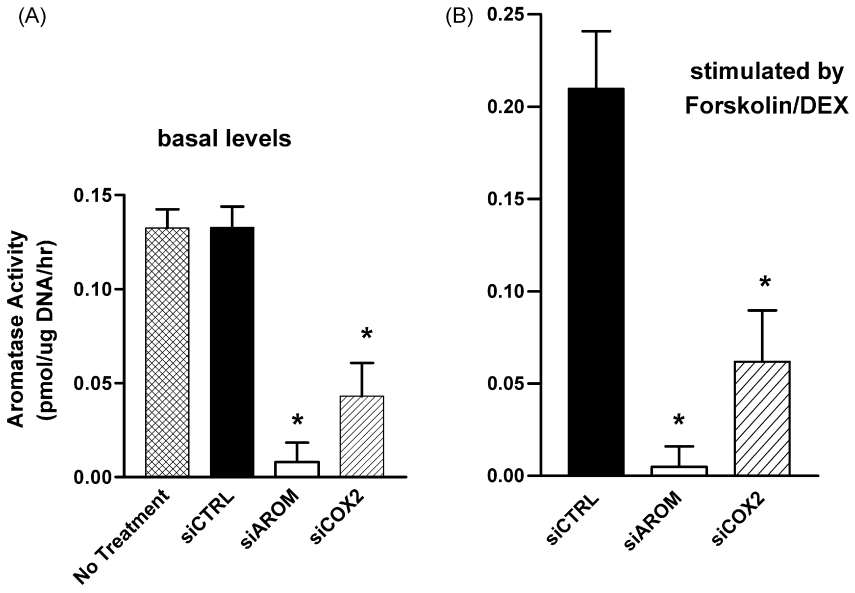


Fig. 4. Suppression of basal aromatase activity (A) and stimulated aromatase activity (B) by siRNA molecules transfected into SK-BR-3 breast cancer cells. Each data bar represents the mean results of three independent determinations. $^{*}P < 0.05$ vs. control by unpaired t-test.

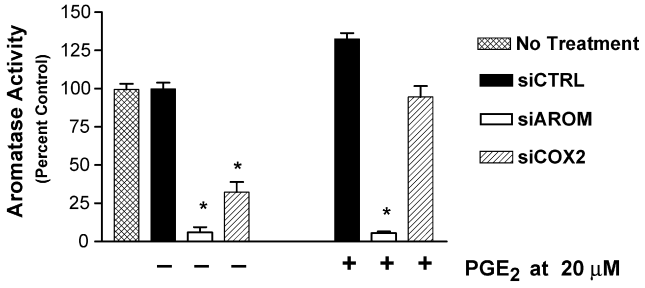
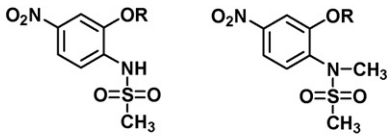


Fig. 5. Effect of PGE_2 on suppression of aromatase activity by siRNAs.

even though NS-398 has weak COX-2 inhibitory activity. To determine whether the modulation of aromatase expression by NS-398 required the inhibition of COX-2 enzyme activity, we designed and synthesized NS-398 analogs with no COX-2 inhibitory activity (Fig. 6) [25]. Introduction of a methyl group at the N atom of the sulfonamide group to the COX-2 inhibitor nimesulide resulted in no COX-2 inhibitory activity [26]. This structural modification was utilized in our drug design. The nitrate group at the 4 position of NS-398 was retained and modifications of the sulfonamide and of the 2 position alkyl group were made to generate the new compounds [25].



R group	NS-398 analogs	N-methyl analogs
cyclohexyl	NS-398	17
propyl	9	18
isopropyl	10	19
methylcyclohexyl	11	12
cyclopentyl	12	21
1-ethyl-propyl	13	22
nonyl	14	23
hexyl	15	24
methyl	16	25

Fig. 6. Synthetic novel sulfonanilides.

To investigate whether these compounds decrease aromatase activity in breast cancer cells, we performed a 1 μM bioassay in SK-BR-3 breast cancer cells, and most compounds significantly decreased aromatase activity (Fig. 7). In an effort to discriminate among compounds in this library, dose response studies of the active compounds were performed, and the resulting IC_{50} values of the compounds ranged from approximately 0.20 to 6.00 μM. Our results

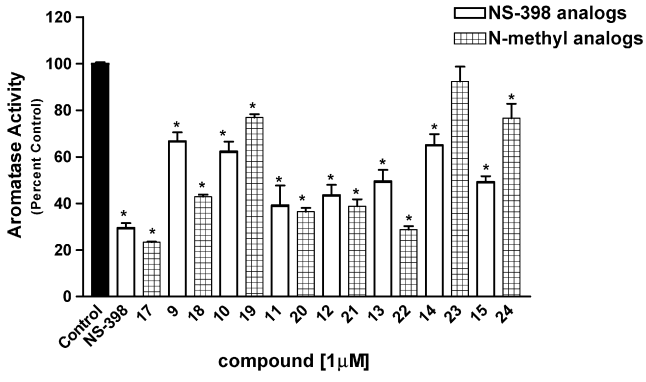


Fig. 7. Suppression of aromatase activity in SK-BR-3 breast cancer cells. SK-BR-3 cells were treated with indicated compounds (1 μM). Aromatase activity was measured as described in Section 2. The results were normalized against a control treatment with vehicle. The value of 100% is equal to 0.03 pmol/h/10^6 cells. Each data bar represents the mean results of three independent determinations. $^{*}P < 0.05$ vs. control by unpaired t-test.

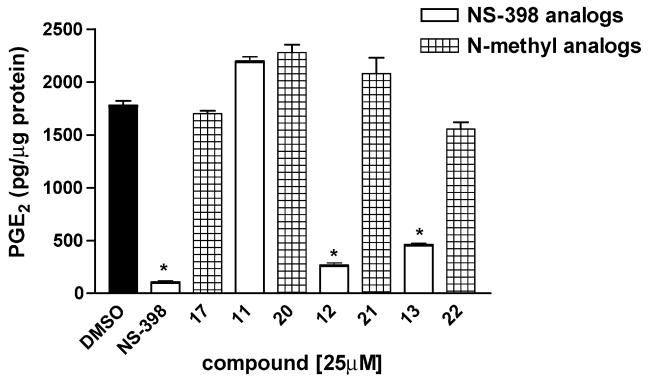


Fig. 8. Effect of NS-398 derivatives on PGE_2 production of MDA-MB-231 cells. Cells were treated for 24 h with the indicated agents at 25 μM. Results are expressed as means of the concentration of PGE_2 produced per microgram protein ± S.E.M., $^{*}P < 0.05$ vs. control by unpaired *t*-test ($n = 6$).

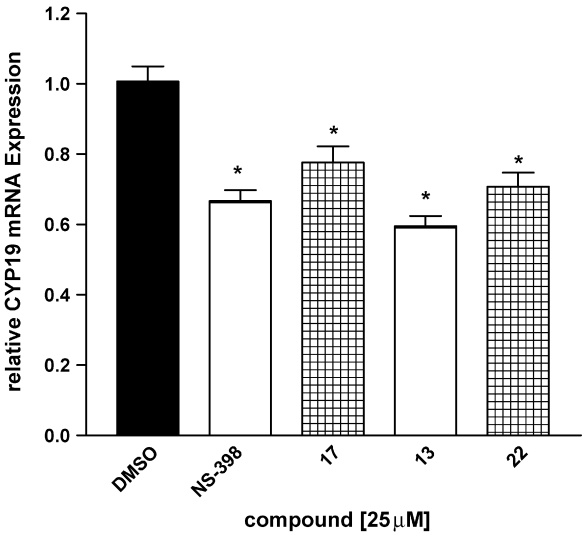


Fig. 9. Real-time RT-PCR analysis of *CYP19* mRNA expression in SK-BR-3 cells. Cells were treated for 24 h with the indicated agents at 25 μM, and total RNA was isolated. Results are expressed as means of *CYP19* (normalized to 18S rRNA) ± S.E.M., $^{*}P < 0.05$ vs. control by unpaired *t*-test ($n = 9$).

suggest that the length of the group on position 2 of the compounds is important for the suppression of aromatase activity. Compounds containing a methoxy (**16** and **25**) or an isopropyloxy (**10** and **19**), which are relatively short, have low ability to suppress aromatase activity. Extremely long chain substituents (**15**, **24**, **14** and **23**) have reduced activity as well, which may also be due to the poor solubility of the compounds. All the N-methyl compounds exhibited better activity than their corresponding unsubstituted compounds with the exception of compounds **23** and **24**. One possible explanation is the pK_a value of the reagents.

The production of PGE_2 was measured in cells treated with NS-398 and the novel sulfonanilide derivatives. NS-398, compound **12** and **13** resulted in a significantly decrease in PGE_2 production, whereas compounds **17**, **20**, **21** and **22** did not show any inhibitory activity (Fig. 8) [25]. This is consistent with our design approach that the introduction of a methyl group in to the N atom of the sulfonamide group results in analogs that cannot be deprotonated and thus loses COX-2 inhibitory activity. In addition, compound **11** did not show any COX-2 inhibitory activity, and compound **11** has one carbon longer side chain comparing with NS-398. This result suggests that the size of the side chain is very important for the COX-2 inhibitory activity and that this extension affects the binding of the compound with COX-2 and results in no COX-2 inhibitory activity.

Analysis of total *CYP19* mRNA transcripts was performed using real-time PCR in order to determine whether the decrease in aromatase activity by NS-398 in SK-BR-3 cells was due to a down-regulation of aromatase expression at transcriptional level. SK-BR-3 cells were treated with NS-398, compound **17**, **13** and **22** for 24 h at concentrations at 25 μM. Total RNA was extracted at 24 h, and *CYP19* transcript levels were compared to control (vehicle) treatment. All four compounds significantly decreased *CYP19* gene expression in SK-BR-3 cells relative to the control (Fig. 9). No effect on the expression level of the housekeeping 18S rRNA was observed with any of the compounds. Compounds **17** and **22**, which do not show COX-2 inhibitory activity, decreased aromatase expression at similar levels. This suggests that the compounds interfere with pathways affecting aromatase expression in breast cancer cells that do not involve prostaglandins and COX enzyme activities. Similar results on *CYP19* gene expression and aromatase activity have been observed in MCF-7 human breast cancer cell cultures and in primary cultures of isolated breast fibroblasts.

Conclusions

Local regulation of aromatase by both endogenous factors as well as exogenous medicinal agents will influence the levels of estrogen available for breast cancer growth. The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis, and previous studies in our laboratories have shown a strong linear association between aromatase (*CYP19*) expression and expression of the cyclooxygenases (*COX-1* and *COX-2*) in breast cancer specimens. Using selective pharmacological agents, dose-dependent decreases in aromatase activity were observed following treatment with NSAIDs, COX-1 selective inhibitor, and COX-2 selective inhibitors. Real-time PCR analysis of aromatase gene expression showed a significant decrease in *CYP19* mRNA levels in treated cells when compared to vehicle control. These results suggest that the effect of COX inhibitors on aromatase occurs at the transcriptional level.

Investigations using RNA interference technology confirmed the interactions between aromatase and cyclooxygenases in breast cancer. Short interfering RNAs were designed against either human *CYP19* mRNA or human *COX-2* mRNA. Treatment of breast cancer cells with siAROM suppressed *CYP19* mRNA and aromatase enzyme activity. Treatment with stCOX2 downregulated the expression of

COX-2 mRNA; furthermore, the siCOX-2-mediated suppression of COX-2 also resulted in suppression of aromatase mRNA. Finally, the administration of PGE_2 to cells treated with the siRNAs results in antagonism of only the siCOX2 and restores aromatase activity to untreated levels.

The small molecule drug design approach focused on the synthesis and biological evaluation of a novel series of sulfonanilide analogs derived from the COX-2 selective inhibitors. The compounds suppressed aromatase enzyme activity in SK-BR-3 breast cancer cells in a dose-dependent manner, and structure activity analysis did not find a correlation between aromatase suppression and COX inhibition. Real-time PCR analysis demonstrated that the sulfonanilide analogs decrease aromatase gene transcription in breast cells. Thus, these results suggest that the novel sulfonanilides targeting aromatase expression may be valuable tools for selective regulation of aromatase in breast cancer.

Thus, the regulation of aromatase and cyclooxygenases in breast cancer involves complex autocrine and paracrine interactions, resulting in significant consequences on the pathogenesis of hormone-dependent breast cancer via growth stimulation from local estrogen biosynthesis (Fig. 2). Higher levels of *COX-2* expression and COX-2 enzyme activity could result in higher levels of PGE_2, which in turn could increase *CYP19* expression through increases in intracellular cAMP levels and activation of promoter 1.3 and promoter II. Thus, the breast cancer tissue microenvironment can influence the extent of estrogen biosynthesis and metabolism, resulting in altered levels of hormonally active estrogens and therefore influencing breast tumor development and growth. Furthermore, siRNAs and novel sulfonanilides targeting aromatase expression may be valuable tools for selective regulation of aromatase in breast cancer.

Acknowledgements

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