Novel Sulfonanilide Analogues Suppress Aromatase Expression and Activity in Breast Cancer Cells Independent of COX-2 Inhibition

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Aromatase is a particularly attractive target in the treatment of estrogen receptor positive breast cancer. Aromatase levels in breast cancer cells are enhanced by prostaglandins and reduced by COX inhibitors. The synthesis and biological evaluation of a novel series of sulfonanilide analogues derived from the COX-2 selective inhibitor NS-398 are described. The compounds suppress aromatase enzyme activity in SK-BR-3 breast cancer cells in a dose- and time-dependent manner. The effect of these compounds on COX-2 inhibition is investigated in breast cancer cells as well. Structure−activity analysis does not find a correlation between aromatase suppression and COX-2 inhibition. Microsomal aromatase inhibition studies rule out the possibility of direct enzyme inhibition. Real-time PCR analysis demonstrates that the sulfonanilide analogues decrease aromatase gene transcription in SK-BR-3 cells. These studies suggest that the novel sulfonanilide compounds suppress aromatase activity and transcription in SK-BR-3 breast cancer cells independent of COX-2 inhibition.

1. Introduction

Approximately one-third of all breast cancer patients and two-thirds of postmenopausal breast cancer patients have estrogen-dependent (ER+) breast cancer, which contains estrogen receptors and requires estrogen for tumor growth.[1] Approaches for hormonal therapy of ER+ breast cancer are either blocking the mechanism of action of estrogens by selective estrogen receptor modulators (SERMs) or inhibiting estrogen biosynthesis by aromatase inhibitors (AIs).[2] For more than 30 years, the antiestrogen tamoxifen has been the mainstay of hormonal therapy in postmenopausal women with ER+ breast cancer. However, drug resistance and increased uterine and endometrial cancer limit its clinical application.[3,4] Recent reports demonstrate that the third generation of aromatase inhibitors are more effective than tamoxifen in postmenopausal breast cancer patients.[5] However, these compounds inhibit aromatase activity throughout the body and could adversely impact sites where estrogen is required for normal function, such as bone and brain.[6] As a consequence, other approaches are being investigated to develop agents that can decrease aromatase activity in a tissue selective manner.

In humans, cytochrome P450 aromatase is encoded by the *CYP19* gene, which contains nine coding exons (exons II to X). The expression of this gene is regulated in a tissue-specific manner by the alternative use of eight promoters, each one being associated with a specific 5′ untranslated exon I.[7] Furthermore, due to the unique organization of tissue-specific promoters, the various promoters employ different signaling pathways and different transcription factors.[8−12] In breast cancer tissue, aromatase expression switches from promoter I.4 to promoter I.3 and II. Thus drugs that target promoter I.3 and II-driven expression of aromatase would be useful as chemotherapeutic agents for the treatment of ER+ breast cancer. [7] Prostaglandin E_2 (PGE_2) is a major regulator driving aromatase expressing from promoter I.3 and II in breast cancer. [13] Recently, we demonstrated that NSAIDs, COX-1, and COX-2 inhibitors can suppress aromatase activity in SK-BR-3 breast cancer cells in the transcriptional level.[14] Therefore, COX selective inhibitors could serve as the first generation of selective aromatase expression regulators (SAERs).

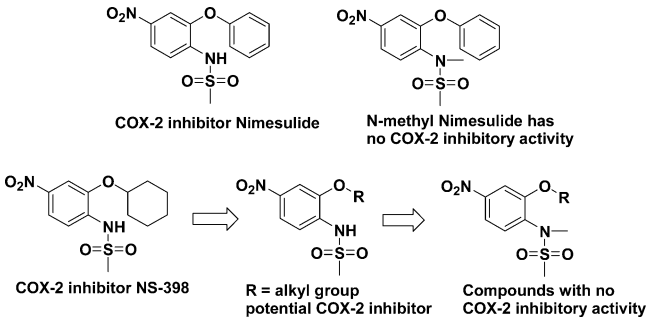

Figure 1. Compound design.

Research in our laboratory demonstrates that the COX-2 selective inhibitor NS-398 is significantly stronger in suppression of aromatase activity than other COX-2 inhibitors in breast cancer cells.[14] This manuscript describes the synthesis of a small library of novel sulfonanilide analogues and their biological activity on aromatase and COX-2 in breast cancer cells.

2. Results and Discussion

2.1. Drug Design. In our previous study, 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.[14] 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, 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 analogues with no COX-2 inhibitory activity. 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 (Figure 1).[15,16] This structural modification was utilized in our drug

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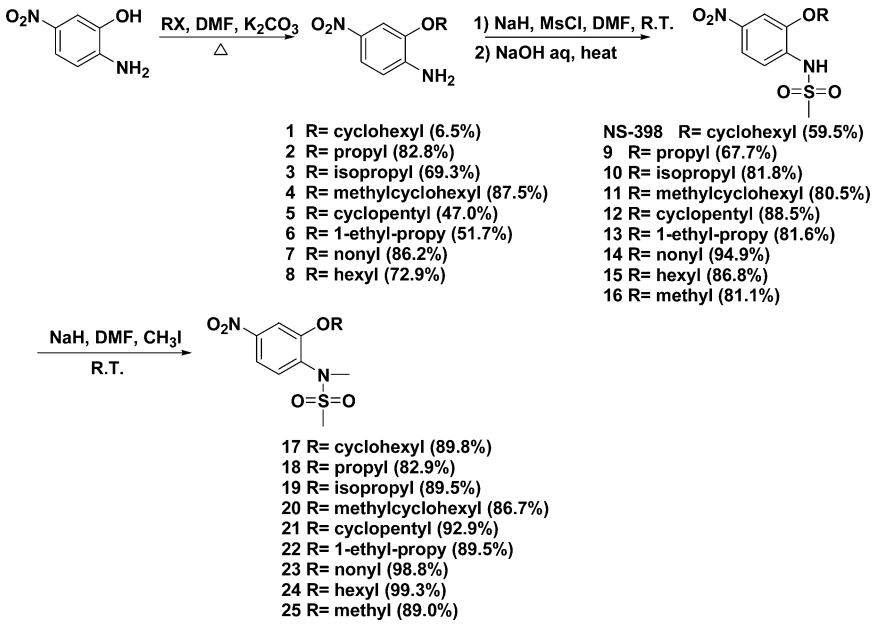
Scheme 1. Synthesis of Sulfonanilide Analogues



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.

2.2. Synthesis. All the compounds were synthesized according to a general procedure described in Scheme 1, in which R represents alkyl structures and X represents halogen atom.[15−17] The starting material 2-amino-5-nitrophenol is commercially available and was treated with K_2CO_3 and alkyl halide in DMF at room temperature or refluxed to obtain compounds **1**−**8**. Powder sodium hydride is added to compounds **1**−**8** in dry dimethylformamide (DMF) at room temperature. After the evolution of hydrogen ended, methanesulfonyl chloride was added and the reaction mixture was stirred at room-temperature overnight. After workup, the resulting mixture of *N*,*N*-bimethanesulfonamido and *N*-methanesulfonamido was hydrolyzed with 10% NaOH solution to generate NS-398 and compounds **9**−**16** as monomethanesulfonamido compounds. Methylation of NS-398 and compounds **9**−**16** yielded compounds **17**−**25**, respectively. All the synthesized compounds tested in the following biological study are confirmed by ^{1}H NMR and HRMS. Key compounds are also confirmed by elemental analysis.

2.3. Biological Evaluation. 2.3.1. Aromatase Activity Assay in SK-BR-3 Breast Cancer Cell Line. To investigate whether these compounds decrease aromatase activity in breast cancer cells, we first performed a 25 μM bioassay in SK-BR-3 breast cancer cells (data not show). All the compounds tested, with the exception of **16** and **25**, suppressed aromatase activity by almost 80−90%. At 1 μM, most compounds still significantly decrease aromatase activity (Figure 2). 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 are listed in Table 1. Our results 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.

Compounds without *N*-methyl group have low pK_a (3−4) and are deprotonated very easily in the cell culture media, with the ionization process illustrated in Figure 3. The negative charge may reduce the ability of the drug to penetrate the cell membrane. For compounds **23** and **24**, the phenomenon is reversed. They are less effective than their corresponding unsubstituted compounds **14** and **15**, respectively. The possible reason is the large side chain on the four compounds, which imparts low water solubility due to their hydrophobic effect. However, compounds **14** and **15** are easily deprotonated in cell culture media; the resulting negative charge may increase the solubility of the drugs to enhance their activity. The best three pairs of compounds, NS-398 and **17**, **12** and **21**, **13** and **22**, have a medium-sized side chain, which may bind more effectively to the target molecule. Compounds **13** and **22** with more flexible side chains are the best compounds in this library for suppression of aromatase activity.

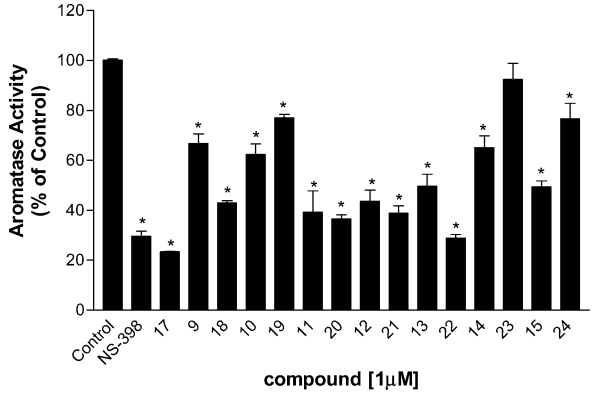
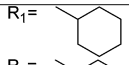
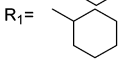
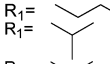
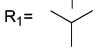
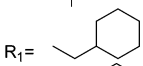
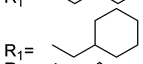
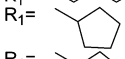
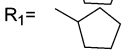
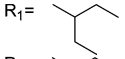
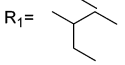


Figure 2. Suppression of aromatase activity in SK-BR-3 breast cancer cells. SK-BR-3 cells were treated with indicated compounds (1 μM). Aromatase activity was measured as described in the Experimental Section. Values are expressed as picomoles ^3H_2O formed per hour incubation time per million cells. 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.

Table 1. Suppression of Aromatase Activity in SK-BR-3 Breast Cancer Cells[a]

Compound	R_1	R_2	IC_{50} (μM)
NS-398		H	0.68
17		CH_3	0.47
9		H	2.51
18		CH_3	0.76
10		H	5.87
19		CH_3	2.81
11		H	1.21
20		CH_3	0.96
12		H	0.87
21		CH_3	0.44
13		H	0.30
22		CH_3	0.23
14		H	0.91
23		CH_3	3.96
15		H	2.67
24		CH_3	4.49
16	CH_3	H	>25
25	CH_3	CH_3	>25

[a] IC_{50} values were calculated by a nonlinear regression analysis (GraphPad Prism). Each dose−response curve contained five concentrations, each in triplicate.

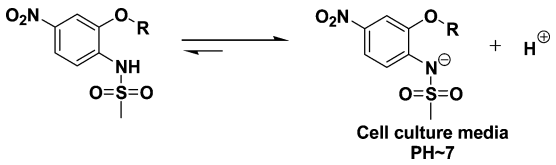


Figure 3. Ionization of the compounds in cell culture media.

2.3.2. Time-Course Studies for Suppression of Aromatase Activity in SK-BR-3 Cells. Time-course studies in SK-BR-3 cells demonstrated a prolonged reversible suppression of aromatase by NS-398 (2 μM) and compound **17** (2 μM) in a time-dependent manner. Cycloheximide (10 μM), which blocks de novo protein synthesis, showed similar results, whereas the aromatase inhibitor letrozole (10 nM) showed an acute inhibition (Figure 4A). In a separate time-course study, the suppression of aromatase activity of the four agents were shown to be reversible (Figure 4B). After removal of the drugs, aromatase activity returned in a time-dependent manner. However, in letrozole treatment, aromatase activity returned and resulted in a 150% increase after removing the drug. This is consistent with the finding that aromatase inhibitors can stabilize aromatase enzyme and thus reduce enzyme degradation.[18] In brief, time-course studies demonstrated a prolonged, reversible suppression of aromatase by NS-398 and compound **17** in SK-BR-3 cells.

2.3.3. Aromatase Activity Assay in Human Placental Microsomes. Aromatase inhibition is performed with human placental microsomes using methods previously reported by our laboratory.[19] Enzyme inhibition studies demonstrated that all the compounds cannot significantly inhibit aromatase activity at 5 μM (Figure 5), which is higher than the IC_{50} from the cell assay. The result rules out the possibility that the compounds can work as aromatase inhibitors in the cell study.

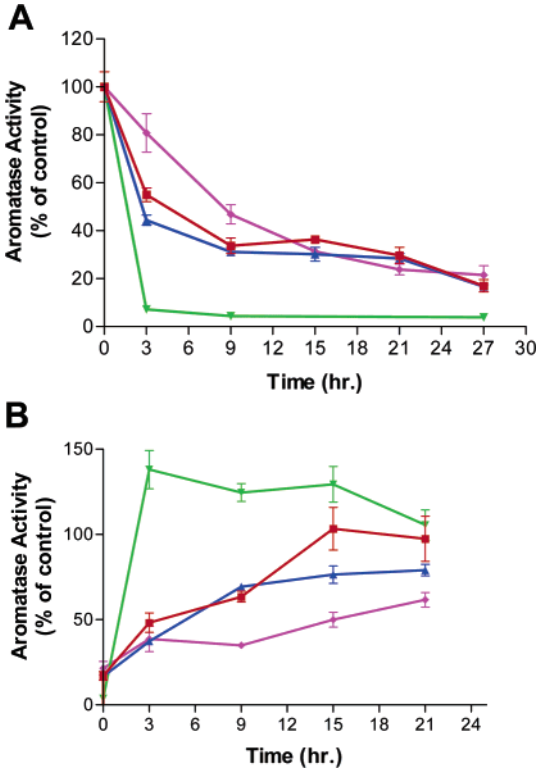


Figure 4. Time-course of aromatase suppression by NS-398 (2 μM, ■), compound 17 (2 μM, ▲), letrozole (10 nM, ▼), and cycloheximide (10 μM, ◆) in SK-BR-3 cells. (A) Cells were treated with the indicated agents for different period of times, and then aromatase activity was subsequently determined during a 3 h assay as described. The results were normalized against a corresponding control treatment with vehicle. (B) Cells were pretreated with the indicated agents for 24 h, and then the reagents were removed. The cells were also extensively washed three times with PBS, followed by incubation in fresh media without any agents for different periods of time. Aromatase activity was subsequently determined during a 3 h assay as described. The results were normalized against a control treatment with vehicle and washed as the same process. The value of 100% is equal to 0.03 pmol/h/10^6 cells.

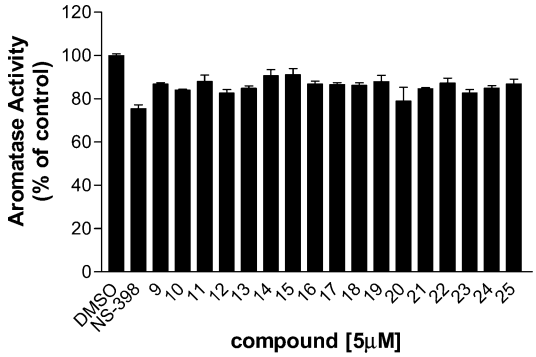


Figure 5. Microsomal aromatase inhibitory activities of NS-398 and analogues. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations.

2.3.4. Level of PGE_2 Production in MDA-MB-231 Cell Line. The production of PGE_2 was measured in cells treated with NS-398 and the novel sulfonanilide derivatives. MDA-MB-231 cell line was chosen because of its high cyclooxygenase activity. Cells were treated for 24 h with the indicated concentration (25 μM) of the agents. NS-398, compound **12**

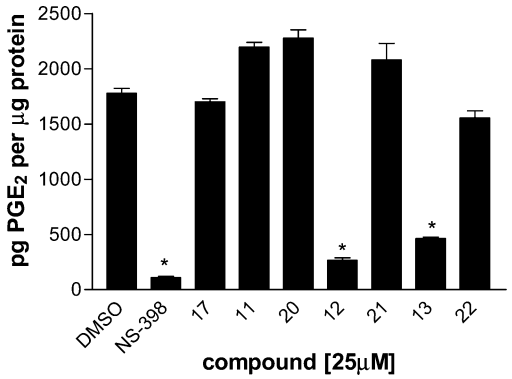


Figure 6. Effect of NS-398 derivatives on PGE_2 production of MDA-MB-231 cells. Cells were treated for 24 h with the indicated agents at 25 μM. Results are expressed as means of the concentration of PGE_2 produced per microgram protein ± SEM., *P < 0.05 vs control by unpaired t test (n = 6).

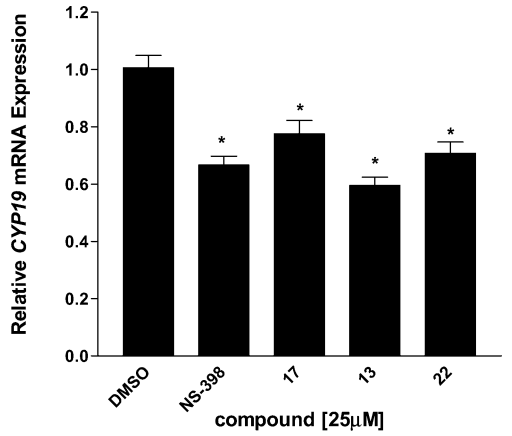


Figure 7. Real-time RT-PCR analysis of *CYP19* mRNA expression in SK-BR-3 cells. Cells were treated for 24 h with the indicated agents at 25μM, and total RNA was isolated. Results are expressed as means of *CYP19* (normalized to 18S rRNA) ± SEM., *P < 0.05 vs control by unpaired t test (n = 9).

and **13** resulted in a significantly decrease in PGE_2 production. Compounds **17**, **20**, **21**, and **22** did not show any inhibitory activity (Figure 6). 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 analogues 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.

2.3.5. *CYP19* mRNA Expression by Real-Time PCR. 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 (Figure 7). 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, decrease 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.

3. Conclusion

The selective COX-2 inhibitor, NS-398, proved to be a good lead compound for decreasing aromatase activity in breast cancer cells by suppression of *CYP19* mRNA at the transcriptional level. The potent regulatory activity of this compound suggests that COX-2 independent mechanisms may be involved in its mechanism of action. The present study reports a convenient synthetic approach for preparation of novel sulfonanilide compounds. NS-398 and the sulfonanilide analogues suppress aromatase activity in a dose- and time-dependent manner in SK-BR-3 breast cancer cells. Human placenta microsomal assay demonstrates that the compounds do not directly inhibit the aromatase enzyme reaction at concentration above the IC_{50} from cell study. In the COX-2 inhibition study, NS-398 and compound **12** and **13** showed COX-2 inhibitory activity, but their corresponding *N*-methyl compounds (**17**, **21**, and **22**, respectively) and compounds **11** and **20** did not have any COX-2 inhibitory activity. This suggests that COX-2 inhibitory activity is not necessary for the suppression of aromatase activity. Furthermore, real time PCR demonstrated that NS-398 and derivatives decreased *CYP19* gene expression. These results suggest that the novel sulfonanilide compounds suppressed aromatase activity and transcription in SK-BR-3 cells independent of COX-2 inhibition. The expression of aromatase is very complex, and researchers also found that some orphan/nuclear receptors such as ERRα-1, EAR-2, COUP-TFI, and RARγ are involved in the regulation of aromatase expression.[20,21] In addition, MAPK pathway is involved to regulate aromatase expression as well.[22] It is still too early to speculate which pathway(s) the compounds are targeting for the regulation of aromatase activity and transcription. The specific molecular target of these compounds in breast cancer cells is still under investigation. Future chemistry studies will focus on developing expanded libraries for detailed SAR studies and future drug development.

4. Experimental Section

4.1. Chemistry. Chemicals were commercially available and used as received without further purification unless otherwise noted. Moisture sensitive reactions were carried out under a dry argon atmosphere in flame-dried glassware. Solvents were distilled before use under argon. Thin-layer chromatography was performed on precoated silica gel F254 plates (Whatman). Silica gel column chromatography was performed using silica gel 60A (Merck, 230−400 Mesh). High-resolution electrospray ionization mass spectra were obtained on the Micromass QTOF Electrospray mass spectrometer at The Ohio State Chemical Instrumentation Center. All the NMR spectra were recorded on a Bruker DPX 250 and 400 MHz in either DMSO-d_6 or $CDCl_3$. Chemical shifts (δ) for ^{1}H NMR spectra are reported in parts per million to residual solvent protons.

A. General Procedure for the Preparation of 1−8. The halohydrocarbon (6 mmol, 1.2 equiv) and K_2CO_3 (0.69 g, 5 mmol) were successively added to a solution of 2-amino-5-nitrophenol (0.77 g, 5 mmol) in DMF (10 mL), and the mixture was refluxed from 2 h to 7 days. After being cooled, 20 mL of H_2O and 5 of mL saturated aqueous Na_2CO_3 was added to the mixture, and the aqueous phase was extracted with CH_2Cl_2. The organic solution was washed with saturated aqueous Na_2CO_3 solution and H_2O, dried over anhydrous $MgSO_4$, and concentrated. The residue was chromatographed on silica gel [AcOEt−hexane (1:5)] to afford desired compounds.

2-Cyclohexyloxy-4-nitroaniline (1). Cyclohexyl iodide was used and it was refluxed for 7 days. Yellow oil, 6.5%: ^{1}H NMR (400

MHz, $CDCl_3$) δ 7.76 (1H, dd, J = 8.7, 2.3 Hz), 7.66 (1H, d, J = 2.1 Hz), 6.64 (1H, d, J = 8.8 Hz), 4.66 (2H,br), 4.34 (1H, m), 2.00 (2H, m), 1.78 (2H, m), 1.56 (3H, m), 1.38 (3H, m).

2-Propyloxy-4-nitroaniline (2). 1-Iodopropane was used and it was refluxed for 2 h. Yellow solid, 82.8%: mp 59−61 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (1H, dd, J = 8.7, 2.3 Hz), 7.67 (1H, d, J = 2.1 Hz), 6.65 (1H, d, J = 8.7 Hz), 4.58 (2H,br), 4.05 (2H, dd, J = 6.5, 6.5 Hz), 1.87 (2H, m), 1.08 (3H, dd, J = 7.5, 7.5 Hz).

2-Isopropyloxy-4-nitroaniline (3). 2-Iodopropane was used and it was refluxed for 24 h. Yellow oil, 69.3%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (1H, dd, J = 8.7, 2.4 Hz), 7.62 (1H, d, J = 2.3 Hz), 6.63 (1H, d, J = 8.8 Hz), 4.77 (2H, br), 4.59 (1H, m), 1.34 (6H, d, J = 6.0 Hz).

2-Methylcyclohexyloxy-4-nitroaniline (4). Bromoethyl cyclohexane was used and it was refluxed for 6 h. Yellow solid, 87.5%: mp 54−56 °C; ^{1}H NMR (400 MHz, CDCl3) δ 7.79 (1H, dd, J = 8.7, 1.9 Hz), 7.64 (1H, d, J = 1.9 Hz), 6.64 (1H, d, J = 8.7 Hz), 4.62 (2H,br), 3.86 (2H, d, J = 6.1 Hz), 1.78 (6H, m), 1.08 (5H, m).

2-Cyclopentyloxy-4-nitroaniline (5). Cyclopentyl iodide was used and it was refluxed for 3 days. Red oil, 47%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (1H, dd, J = 8.7, 2.3 Hz), 7.66 (1H, d, J = 2.3 Hz), 6.63 (1H, d, J = 8.6 Hz), 4.87 (1H, m), 4.56 (2H,br), 1.68−2.02 (8H, m).

2-(1-Ethyl-propyloxy)-4-nitroaniline (6). 3-Bromopentane was used and it was refluxed for 5 days. Yellow solid, 51.7%: mp 62−63 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (1H, dd, J = 8.7, 2.3 Hz), 7.67 (1H, d, J = 2.2 Hz), 6.65 (1H, d, J = 8.7 Hz), 4.58 (2H,br), 4.29 (1H, m), 1.72 (4H, m), 0.98(6H, dd, J = 7.4, 7.4 Hz).

2-Nonyloxy-4-nitroaniline (7). 1-Iodononane was used and it was refluxed for 24 h. Yellow solid, 86.2%: mp 74−75 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (1H, dd, J = 8.7, 2.4 Hz), 7.67 (1H, d, J = 2.3 Hz), 6.64 (1H, d, J = 8.7 Hz), 4.57 (2H,br), 4.07 (2H, dd, J = 6.6, 6.6 Hz), 1.84 (2H, m), 1.31(14H, m), 0.89 (3H, dd, J = 6.7, 6.7 Hz).

2-Hexyloxy-4-nitroaniline (8). 1-Iodohexane was used and it was refluxed for 6 h. Yellow solid, 72.9%: mp 101−104 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (1H, dd, J = 8.7, 2.4 Hz), 7.67 (1H, d, J = 2.3 Hz), 6.64 (1H, d, J = 8.7 Hz), 4.58 (2H,br), 4.08 (2H, dd, J = 6.5, 6.5 Hz), 1.85 (2H, m), 1.48 (2H, m), 1.35 (4H, m), 0.92 (3H, dd, J = 6.9, 6.9 Hz).

B. General Procedure for the Preparation of NS-398 and Compounds 9−16. NaH (95% powder, 0.265 g, 10.5 mmol, 3.5 equiv) was added to a solution of alkyl instituted 2-amino-5-nitrophenol (3.0 mmol) in anhydrous DMF (8 mL) at room temperature. After being stirred at the same temperature for 30 min, MsCl (1.031 g, 9.0 mmol, 3 equiv) was added to the mixture, and the stirring was continued overnight at room temperature. H_2O was added to the mixture, and then it was neutralized with 5 N HCl until pH = 1−2. The intermediate precipitated as a yellow solid. It was collected by filtration and washed with H_2O, which was used to the next reaction without further purification.

The intermediate was added to a 3 N NaOH aq solution and was stirred at 80−90 °C overnight. After being cooled, it was neutralized with 5 N HCl until pH = 1−2. The precipitated solid was collected and washed with H_2O and cold ether to provide the desired product, and then it was recrystalized from ethyl acetate/hexane.

***N*-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398).** Pale yellow powder, 59.5%: mp 124−126 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.89 (dd, J = 2.3, 8.9 Hz, 1H), 7.79 (d, J = 2.4 Hz, 1H), 7.66(d, J = 8.9 Hz, 1H), 7.24(br, 1H), 4.44(m, 1H), 3.12 (s, 3H), 2.06 (m, 2H), 1.84 (m, 2H), 1.41 (m, 6H). Anal. Calcd for $C_{13}H_{18}N_2O_5S$: C, 49.67; H, 5.77; N, 8.91. Found: C, 49.75; H, 5.77; N, 8.80.

***N*-(2-Propyloxy-4-nitrophenyl)methanesulfonamide (9).** Yellow powder, 67.7%: mp 117−119 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.92 (dd, J = 2.4, 8.9 Hz, 1H), 7.79 (d, J = 2.4 Hz, 1H), 7.66 (d, J = 8.9 Hz, 1H), 7.25 (br, 1H), 4.12 (t, J = 6.6, 6.6 Hz 2H), 3.13 (s, 3H), 1.89 (m, 2H), 1.08 (t, J = 7.4, 7.4 Hz, 3H); HRMS calculated for $C_{10}H_{14}N_2NaO_5S$ $(M + Na)^+$ 297.0521, found 297.0533.

***N*-(2-Isopropyloxy-4-nitrophenyl)methanesulfonamide (10).** Yellow solid, 81.8%: mp 128−131 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (dd, J = 2.3, 9.0 Hz, 1H), 7.79 (d, J = 2.3 Hz, 1H), 7.66 (d, J = 9.0 Hz, 1H), 7.26 (br, 1H), 4.77 (m, 1H), 3.12 (s, 3H), 1.45 (d, J = 6.1 Hz, 6H); HRMS calculated for $C_{10}H_{14}N_2NaO_5S$ $(M + Na)^+$ 297.0521, found 297.0501.

***N*-(2-Methylcyclohexyloxy-4-nitrophenyl)methanesulfonamide (11).** Yellow powder, 80.5%: mp 138−142 °C; ^{1}H NMR (250 MHz, DMSO-d_6) δ 7.64 (dd, J = 2.6, 9.1 Hz, 1H), 7.41 (d, J = 2.7 Hz, 1H), 7.13 (d, J = 9.2 Hz, 1H), 3.72 (d, J = 6.4 Hz 2H), 2.71 (s, 3H), 1.69 (m, 6H), 0.99 (m, 5H); HRMS calculated for $C_{14}H_{20}N_2NaO_5S$ $(M + Na)^+$ 351.0991, found 351.1017. Anal. Calcd for $C_{14}H_{20}N_2O_5S$: C, 51.21; H, 6.14; N, 8.53. Found: C, 51.08; H, 6.10; N, 8.34.

***N*-(2-Cyclopentyloxy-4-nitrophenyl)methanesulfonamide (12).** Yellow solid 88.5%: mp 139−140 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (dd, J = 2.3, 8.9 Hz, 1H), 7.79 (d, J = 2.3 Hz, 1H), 7.65(d, J = 8.9 Hz, 1H), 7.19(br, 1H), 4.95(m, 1H), 3.12 (s, 3H), 2.06 (m, 2H), 1.59 (m, 6H); HRMS calculated for $C_{12}H_{16}N_2NaO_5S$ $(M + Na)^+$ 323.0678, found 323.0673. Anal. Calcd for $C_{12}H_{16}N_2O_5S$: C, 47.99; H, 5.37; N, 9.33. Found: C, 47.76; H, 5.45; N, 9.14.

***N*-(2-(1-Ethyl-propyloxy-4-nitrophenyl))methanesulfonamide (13).** Yellow solid, 81.6%: mp 100−102 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (dd, J = 2.4, 9.0 Hz, 1H), 7.78 (d, J = 2.3 Hz, 1H), 7.67 (d, J = 8.9 Hz, 1H), 4.38 (m, 1H), 3.12 (s, 3H), 1.75 (m, 4H), 0.98 (t, J = 7.4, 7.4 Hz, 6H); HRMS calculated for $C_{12}H_{18}N_2NaO_5S$ $(M + Na)^+$ 325.0834, found 325.0823. Anal. Calcd for $C_{12}H_{18}N_2O_5S$: C, 47.67; H, 6.00; N, 9.27. Found: C, 47.78; H, 6.11; N, 9.22.

***N*-(2-Nonyloxy-4-nitrophenyl)methanesulfonamide (14).** Yellow solid, 94.9%: mp 70−71 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (dd, J = 2.3, 8.9 Hz, 1H), 7.79 (d, J = 2.4 Hz, 1H), 7.66 (d, J = 8.9 Hz, 1H), 7.24 (br, 1H), 4.14 (t, J = 6.7, 6.7 Hz, 2H), 3.12 (s, 3H), 1.86 (m, 2H), 1.31 (m, 12H), 0.89 (t, J = 6.4, 6.4 Hz, 3H); HRMS calculated for $C_{16}H_{26}N_2NaO_5S$ $(M + Na)^+$ 381.1460, found 381.1482.

***N*-(2-Hexyloxy-4-nitrophenyl)methanesulfonamide (15).** Pale yellow solid, 86.8%: mp 74−76 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 (dd, J = 2.5, 8.6 Hz, 1H), 7.78 (d, J = 2.5 Hz, 1H), 7.65 (d, J = 8.7 Hz, 1H), 7.23 (br, 1H), 4.14 (t, J = 6.5, 6.5 Hz, 2H), 3.12 (s, 3H), 1.85 (m, 2H), 1.38 (m, 6H), 0.93 (t, J = 6.8, 6.8 Hz, 3H); HRMS calculated for $C_{13}H_{20}N_2NaO_5S$ $(M + Na)^+$ 339.0991, found 339.0986.

***N*-(2-Methoxy-4-nitrophenyl)methanesulfonamide (16).** Yellow solid, 81.1%: mp 128−130 °C; ^{1}H NMR (250 MHz, DMSO-d_6) δ 7.83 (dd, J = 2.5, 8.9 Hz, 1H), 7.72 (d, J = 2.5 Hz, 1H), 7.46 (d, J = 8.9 Hz, 1H), 3.90 (s 3H), 3.06 (s, 3H); HRMS calculated for $C_8H_{10}N_2NaO_5S$ $(M + Na)^+$ 269.0208, found 269.0223.

C. General Procedure for the Preparation of 17−25. The methanesulfonamide compound (0.5 mmol) was dissolved in 3 mL of dry DMF, and NaH powder (15.2 mg 95%, 0.6 mmol, 1.2 equiv) was added. The mixture was stirred at room temperature for 10 min, and iodomethane (0.6 mmol, 1.2 eq) was added; the stirring was kept for 2 h at room temperature. Then the mixture was taken up with 7 mL of water and 2 mL of Na_2CO_3 aq solution. The precipitated solid was collected by filtration and washed with water and cold ether to afford the desired product, and then it was recrystalized from ethyl acetate/hexane. If oil precipitated, it was extracted by using CH_2Cl_2. The organic phase was washed with water and Na_2CO_3 aq solution, dried over anhydrous $MgSO_4$, and concentrated. The residue was chromatographed on silica gel [AcOEt−hexane (1:5)] to afford the product.

***N*-Methyl-*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (17).** White solid, 89.8%: mp 129−132 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (dd, J = 2.4, 8.6 Hz, 1H), 7.80 (d, J = 2.2 Hz, 1H), 7.55 (d, J = 8.6 Hz, 1H), 4.49 (m, 1H), 3.30 (s, 3H), 2.99 (s, 3H), 2.11 (m, 2H), 1.85 (m, 2H), 1.50 (m, 6H); HRMS calculated for $C_{14}H_{20}N_2NaO_5S$ $(M + Na)^+$ 351.0991, found

351.0970. Anal. Calcd for $C_{14}H_{20}N_2O_5S$: C, 51.21; H, 6.14; N, 8.53. Found: C, 51.23; H, 6.16; N, 8.41.

***N*-Methyl-*N*-(2-propyloxy-4-nitrophenyl)methanesulfonamide (18).** Pale yellow solid, 82.9%: mp 66−69 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, J = 2.5, 8.6 Hz, 1H), 7.83 (d, J = 2.5 Hz, 1H), 7.55 (d, J = 8.5 Hz, 1H), 4.12 (t, J = 6.6, 6.6 Hz, 2H), 3.32 (s, 3H), 2.99 (s,3H), 1.92 (m, 2H), 1.12 (t, J = 7.5, 7.5 Hz, 3H); HRMS calculated for $C_{11}H_{16}N_2NaO_5S$ $(M + Na)^+$ 311.0678, found 311.0658.

***N*-Methyl-*N*-(2-isopropyloxy-4-nitrophenyl)methanesulfonamide (19).** Yellow solid, 89.5%: mp 99−100 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (dd, J = 2.5, 8.5 Hz, 1H), 7.81 (d, J = 2.5 Hz, 1H), 7.55 (d, J = 8.6 Hz, 1H), 4.80 (m, 1H), 3.30 (s, 3H), 2.99 (s,3H), 1.47 (d, J = 6.1 Hz, 6H); HRMS calculated for $C_{11}H_{16}N_2NaO_5S$ $(M + Na)^+$ 311.0678, found 311.0661.

***N*-Methyl-*N*-(2-methylcyclohexyloxy-4-nitrophenyl)methanesulfonamide (20).** Yellow powder, 86.7%: mp 105−107 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (dd, J = 2.5, 8.6 Hz, 1H), 7.83 (d, J = 2.4 Hz, 1H), 7.56 (d, J = 8.6 Hz, 1H), 3.96 (d, J = 6.0 Hz 2H), 3.33 (s, 3H), 2.98 (s, 3H), 1.76 (m, 6H), 1.13(m, 5H); HRMS calculated for $C_{15}H_{22}N_2NaO_5S$ $(M + Na)^+$ 365.1147, found 365.1169. Anal. Calcd for $C_{15}H_{22}N_2O_5S$: C, 52.62; H, 6.48; N, 8.18. Found: C, 52.62; H, 6.55; N, 8.11.

***N*-Methyl-*N*-(2-cyclopentyloxy-4-nitrophenyl)methanesulfonamide (21).** Yellow solid 92.9%: mp 102−104 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (dd, J = 2.3, 8.5 Hz, 1H), 7.82 (d, J = 2.5 Hz, 1H), 7.54 (d, J = 8.5 Hz, 1H), 4.98 (m, 1H), 3.29 (s, 3H), 2.97 (s, 3H), 2.08 (m, 2H), 1.78 (m, 6H); HRMS calculated for $C_{13}H_{18}N_2NaO_5S$ $(M + Na)^+$ 337.0834, found 337.0824. Anal. Calcd for $C_{13}H_{18}N_2O_5S$: C, 49.67; H, 5.77; N, 8.91. Found: C, 49.87; H, 5.93; N, 8.78.

***N*-Methyl-*N*-(2-(1-ethyl-propyloxy-4-nitrophenyl))methanesulfonamide (22).** Yellow solid, 89.5%: mp 89−90 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (dd, J = 2.5, 8.6 Hz, 1H), 7.80 (d, J = 2.4 Hz, 1H), 7.55 (d, J = 8.6 Hz, 1H), 4.42 (m, 1H), 3.31 (s, 3H), 2.98 (s, 3H), 1.77 (m, 4H), 1.02 (t, J = 7.4, 7.4 Hz, 6H); HRMS calculated for $C_{13}H_{20}N_2NaO_5S$ $(M + Na)^+$ 339.0991, found 339.0967. Anal. Calcd for $C_{13}H_{20}N_2O_5S$: C, 49.35; H, 6.37; N, 8.85. Found: C, 49.55; H, 6.43; N, 8.74.

***N*-Methyl-*N*-(2-nonyloxy-4-nitrophenyl)methanesulfonamide (23).** Yellow solid, 98.8%: mp 83−84 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (dd, J = 2.5, 8.6 Hz, 1H), 7.82 (d, J = 2.5 Hz, 1H), 7.55 (d, J = 8.6 Hz, 1H), 4.14 (t, J = 6.6, 6.6 Hz, 2H), 3.31 (s, 3H), 2.99 (s, 3H), 1.88 (m, 2H), 1.50 (m, 2H), 1.31 (m, 10H), 0.89 (t, J = 6.6, 6.6 Hz, 3H); HRMS calculated for $C_{17}H_{28}N_2NaO_5S$ $(M + Na)^+$ 395.1617, found 395.1612.

***N*-Methyl-*N*-(2-hexyloxy-4-nitrophenyl)methanesulfonamide (24).** Yellow oil, 99.3%: mp 44−46 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (dd, J = 2.5, 8.6 Hz, 1H), 7.82 (d, J = 2.5 Hz, 1H), 7.54 (d, J = 8.7 Hz, 1H), 4.15 (t, J = 6.6, 6.6 Hz, 2H), 3.31 (s, 3H), 2.98 (s, 3H), 1.88 (m, 2H), 1.52 (m, 2H), 1.37 (m, 4H), 0.92 (t, J = 7.0, 7.0 Hz, 3H); HRMS calculated for $C_{14}H_{22}N_2NaO_5S$ $(M + Na)^+$ 353.1147, found 353.1178.

***N*-Methyl-*N*-(2-methoxy-4-nitrophenyl)methanesulfonamide (25).** Pale yellow solid, 89%: mp 125−126 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (d, J = 2.1 Hz, 1H),7.84 (dd, J = 2.1, 8.5 Hz, 1H), 7.56 (d, J = 8.5 Hz, 1H), 3.98 (s, 3H), 3.18 (s, 3H), 3.09 (s, 3H); HRMS calculated for $C_9H_{12}N_2NaO_5S$ $(M + Na)^+$ 283.0365, found 283.0373.

4.2. Biological Study. 4.2.1. Preparation of Human Placental Microsomes. Human term 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 previously described.[23] Microsomal suspensions were stored at −80 °C until required.

4.2.2. Inhibition Study. Inhibition of human placental aromatase was determined by monitoring the amount of ^3H_2O released as the enzyme converts [1β-^{3}H]androst-4-ene-3,17-dione to estrone. All the compounds were tested at 5 μM for their potential aromatase inhibitory activity. Aromatase activity assays were carried in 0.1 M potassium phosphate buffer (pH 7.0) with 5% propylene glycol. All samples contained a NADPH regenerating system consisting of 2.85 mM glucose-6-phosphate, 1.8 mM $NADP^+$, and 1.5 units of glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MO). Samples contained 100 nM androst-4-ene-3,17-dione (400 000−450 000 dpm). Reactions were initiated with the addition of 50 μg microsomal protein. The total incubation volume was 2.0 mL. Incubations were allowed to proceed for 15 min in a shaking water bath at 37 °C. Reactions were quenched by the addition of 2.0 mL of chloroform. Samples were then vortexed and centrifuged for 5 min, and the aqueous layer was removed. The aqueous layer was subsequently extracted twice in the same manner with 2.0 mL of chloroform. A 0.5 mL aliquot of the final aqueous layer was combined with 5 mL of 3a70B scintillation cocktail (Research Products International Corp., Mt. Prospect, IL) and the amount of radioactivity determined. Each sample was run in triplicate, and background values were determined with microsomal protein inactivated by boiling. Samples containing 50 μM (±)-aminoglutethimide (Sigma, St. Louis, MO) were used a positive control. The data were analyzed with the Graphpad Prism (Version 3.0) program.

4.2.3. Aromatase Tritiated Water-Release Assay in SK-BR-3 Cell Lines. SK-BR-3 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. Measurement of aromatase enzyme activity was based on the tritium water release assay.[24] Cells in 100 mm Petri dish were treated with 0.1% DMSO (control), and inhibitors at the indicated concentrations. After 24 h, the media was changed and the cells were incubated with 100 nM [1β-^{3}H]-androst-4-ene-3,17-dione (2 Ci) for 3 h. 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 cells in each flask, the cells were trypsinized and analyzed using the diphenylamine DNA assay adapted to a 96-well plate.[25] IC_{50} sigmoidal dose−response data were analyzed with Microsoft Excel and the Graphpad Prism (Version 3.0) program.

4.2.4. Enzyme Immunoassay of PGE_2 in MDA-MB-231 Cells. 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. To study PGE_2 synthesis in cell culture media, experiments were performed in 6015 mm Petri dishes.[14] An aliquot of MDA-MB-231 cells (15×10^4 cells) were added to each dish and then incubated overnight to allow the cells to adhere to the dish. After that, cells were serum starved in defined media for 24 h. This step was followed by replacement of media with fresh media containing either vehicle (DMSO) or the indicated concentration of agents. After 24 h incubation at 37 °C the media were collected and the amount of PGE_2 was determined by ELISA (Cayman Chemical) according to the protocol provided by the manufacturer. PGE_2 concentration was normalized to total protein. Total proteins were extracted from adhered cells by 30 min treatment with 0.5 M NaOH at room temperature and shaking. Protein concentrations in these extracts were determined using a protein assay method (Bio-Rad Laboratories, Inc., Hercules, CA).

4.2.5. RNA Extraction. Total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol. Total RNA pellets were dissolved in DNase- and 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.

4.2.6. 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 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.

4.2.7. 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 GTT CTT CAT GCT ATT TCT C-3′; antisense: 5′-TCA CCA ATA ACA GTC TGG ATT TCC-3′); 250 nM Taqman probe (6FAM 5′-TGC AAA GCA CCC TAA TGT TGA AGA GGC AAT-3′TAMRA)(Invitrogen), 18S rRNA (Applied Biosystems, Foster City, CA), and 2.0 μL of each RT sample in a final volume of 20 μL. The Taqman probe was designed to anneal to a specific sequence of the aromatase gene 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.

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