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Robert W. Brueggemeier
Ohio State University

Bin Su
Cleveland State University, B.SU@csuohio.edu

Michael V. Darby
Ohio State University

Yasuro Sugimoto
Ohio State University

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Selective regulation of aromatase expression for drug discovery*

Robert W. Brueggemeier¹, Bin Su², Michael V. Darby³, Yasuro Sugimoto⁴

Introduction

Estrogens are biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase [1,2]. 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 [3-5] and expression of aromatase is highest in or near breast tumor sites [4,6]. Regulation of aromatase in various tissues is complex, and several tissue-specific promoter regions have been identified upstream from the *CYP19* gene [2,7,8]. Promoter 11 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 contains a cAMP response element. Promoters 13, 14, 16, and 17 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-1 β , IL-6 and TNF α .

In breast cancer tissues, an increased expression of aromatase cytochrome P450 is observed due to utilization of multiple promoter regions for gene expression. In the normal breast cells, aromatase expression is primarily derived by the tissue-specific promoter 14 for transcription, whereas expression from breast can-

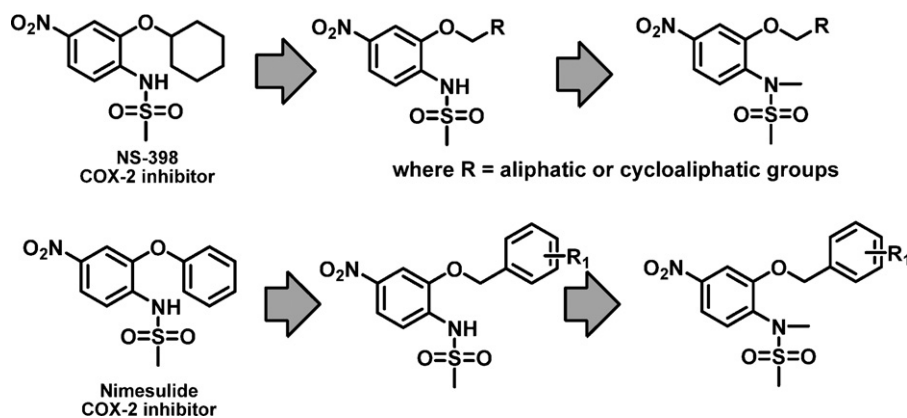


Fig. 1. Drug design approach for novel sulfonanilides as selective aromatase modulators.

cer patients is primarily derived from the utilization of promoter I.3 and promoter II [9]. As a result of the use of the alternate promoters, the regulation of estrogen biosynthesis switches from one controlled primarily by glucocorticoids and cytokines to a promoter regulated through cAMP-mediated pathways, and the prostaglandin PGE₂ increases intracellular cAMP levels and stimulates estrogen biosynthesis [9].

Research in our laboratories focuses on the development of novel therapeutic interventions targeting tissue-specific expression of aromatase. Our small molecule approach utilizes drug discovery technologies to develop novel sulfonanilides as selective agents regulating aromatase expression [10–14]. A second pharmacological approach involves the development of siRNA molecules that interfere with aromatase expression [10]. This manuscript reviews our recent publications in this area and presents preliminary results.

Novel sulfonanilide analogs for suppression of aromatase expression and activity

Previously, we demonstrated that COX-2 inhibitors nimesulide, N-(2-phenoxy-4-nitrophenyl)-methanesulfonamide, and NS-398, N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide, suppress aromatase activity in breast cancer cells by suppressing aromatase transcription [15]. Interestingly, these two agents share very similar chemical structures (Fig. 1). In addition, 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 [16]. This structural modification was utilized in our drug design of novel sulfonanilide derivatives of nimesulide and NS-398. The nitro group at the 4 position of the sulfonanilides was retained and modifications of the sulfonamide and of the 2-position group were made to generate the targeted libraries of new compounds (Fig. 1) [11].

Evaluations of the synthetic compounds were performed in both SK-BR-3 breast cancer cells, which produce aromatase expression from promoter II and I.3 mediated by cAMP [12]. SK-BR-3 cells grown in defined media containing DMEM/F12 media, 1.0 mg/mL human albumin, 5.0 mg/L human transferrin and 5.0 mg/L bovine insulin were treated with the synthetic compounds for 24 h. Cells were then incubated for 3 h with fresh media, and aromatase activity measured by the tritiated water release assay [12]. Many compounds significantly decreased aromatase activity in SK-BR-3 cells at 2.5 μ M (Fig. 2), and those compounds with enhanced activities were further evaluated for selective regulation of aromatase expression.

Dose–response studies of the effects on aromatase activity were performed in SK-BR-3 cells on six compounds. All six compounds

exhibited dose–response curves for suppression of aromatase activity (Fig. 3), and the corresponding IC₅₀ values range from 0.33 to 2.68 μ M. Furthermore, real-time PCR experiments demonstrated that these compounds significantly decreased *CYP19* gene expression at 5 μ M in SK-BR-3 cells [12]. The results of the cellular aromatase assay demonstrated that a one carbon extension at the 2-position of nimesulide results in significant increase in suppression of aromatase in breast cancer cells compared with nimesulide.

A combinatorial chemistry approach was used to generate diversely substituted nimesulide derivatives (Fig. 4) by parallel syn-

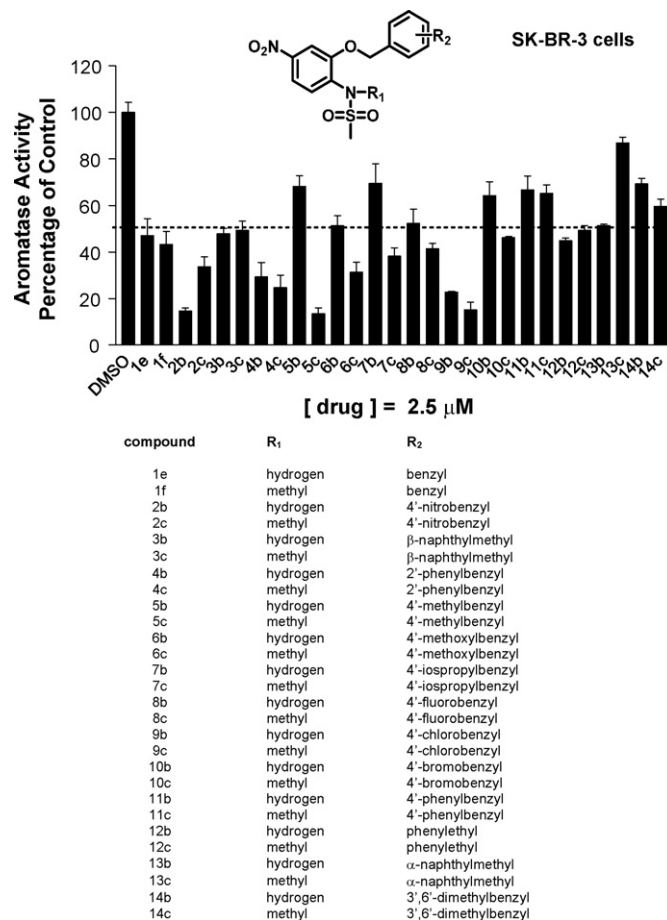


Fig. 2. Suppression of aromatase activity by novel sulfonanilides in SK-BR-3 cells (adapted from [12]). SK-BR-3 cells were treated with compounds (2.5 μ M) and aromatase activity was measured. The value of 100% is equal to 0.03 pmol/h/10⁶ cells; n = 3.

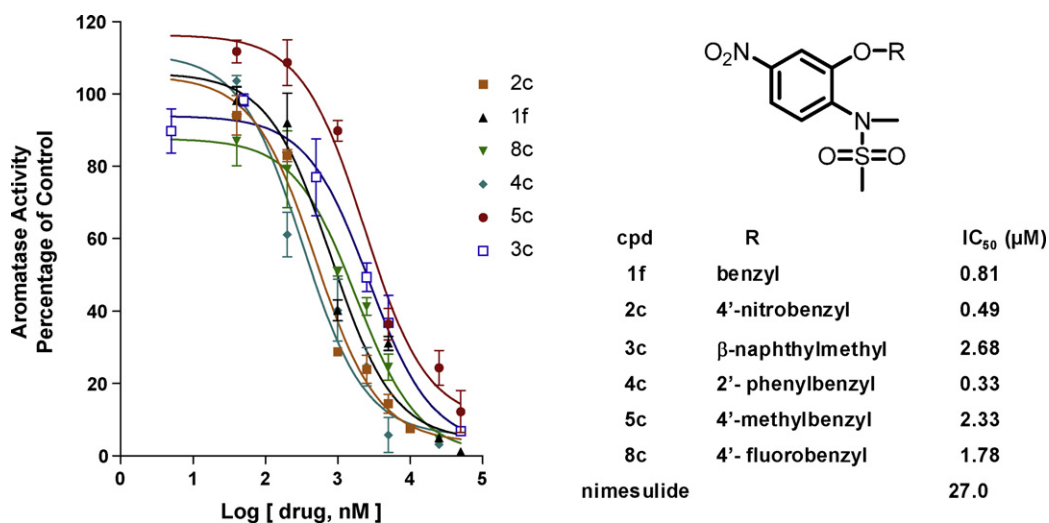


Fig. 3. Dose–response curves and IC₅₀ values for selected novel sulfonanilides (adapted from [12]). The value of 100% is equal to 0.03 pmol/h/10⁶ cells. Each data point represents the mean results of three independent determinations, and the data were statistically analyzed by a nonlinear regression analysis method.

thesis [13,14]. This innovative technology of producing libraries of structurally related compounds is particularly beneficial in the step of lead optimization, facilitating structural modifications of a lead scaffold to enhance pharmacological activity, improve pharmacokinetic properties, and/or reduce unwanted side effects. Their pharmacological evaluation as agents for suppression of aromatase activity in breast cancer cells was further explored in the SK-BR-3 breast cancer cell lines and in MCF-7 cells transfected with the aromatase gene. A ligand-based pharmacophore model identified four chemical features critical for biological activity, namely, the central aromatic ring, one hydrophobic function (Fig. 4, position A) and two hydrogen bond acceptors (Fig. 4, positions C and D).

RNA interference of aromatase expression

Newly developed RNA interference (RNAi) technology was utilized to further probe the interactions between aromatase and cyclooxygenases in breast cancer [10]. 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 (siRNA) 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 (siRNA) were designed to target either human *CYP19* mRNA or human *COX-2* mRNA. The effects of siAROM and siCOX2 on aromatase enzyme activity in SK-BR-3 cells were examined (Fig. 5). 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

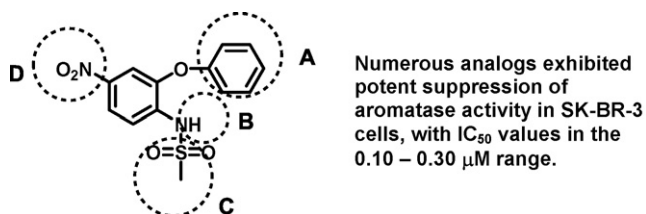


Fig. 4. Targeted libraries of novel sulfonanilides with modifications at positions A–D.

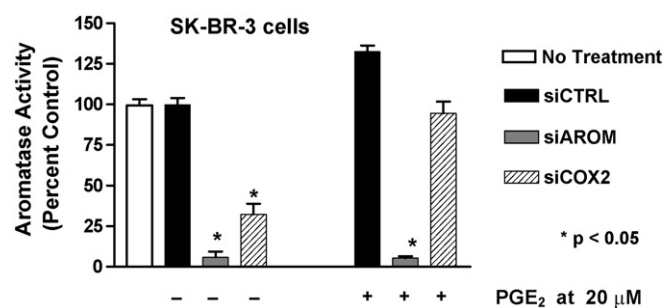


Fig. 5. Effect of PGE₂ on suppression of aromatase activity by siRNAs in SK-BR-3 cells [10]. The value of 100% is equal to 0.025 pmol/h/10⁶ cells; *n* = 4.

untreated cells. Also shown in this figure, the siCOX2 also resulted in suppression of aromatase activity by approximately 67%. Furthermore, the administration of PGE₂ to cells treated with the siRNAs results in antagonism of only the siCOX2 and restores aromatase activity to untreated levels (Fig. 5) [10].

The effect of the siAROM on aromatase enzyme activity in several cancer cells was also examined (Fig. 6). The siAROM silenced aromatase enzyme activity by greater than 90% in the SK-BR-3 human breast cancer cell line, in MCF-7 human breast cancer cells transfected with the aromatase gene, and in the JAr human choriocarcinoma cell line. The suppression of aromatase enzyme activity was greater than 80% in the JEG-3 human choriocarcinoma cell line.

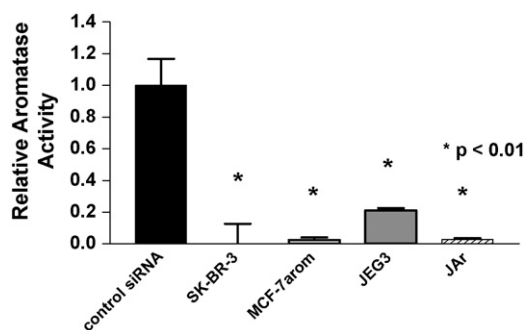


Fig. 6. Suppression of aromatase activity by siRNAs in various human cancer cell lines. The value of 1.0 is equal to 0.025 pmol/h/10⁶ cells; *n* = 3.

Discussion

Two pharmacological approaches of novel therapeutic interventions targeting tissue-specific expression of aromatase have been recently developed in our laboratories. Our small molecule approach utilizes drug discovery technologies to develop novel sulfonanilides as selective agents regulating aromatase expression. Targeted synthetic libraries of novel sulfonanilide analogs, derived from COX-2 inhibitors nimesulide and NS-398, decreased *CYP19* mRNA expression and suppressed aromatase enzyme activity in SK-BR-3 breast cancer cells in a dose dependent manner. Furthermore, N-methyl sulfonanilide analogs without COX-2 inhibitory activity showed similar effects of decreased *CYP19* mRNA expression and suppression of aromatase activity. Thus, these results suggest that the novel sulfonanilides targeting aromatase expression may be valuable tools for selective regulation of aromatase in breast cancer.

Investigations using RNA interference (RNAi) technology confirmed the interactions between aromatase and cyclooxygenases in breast cancer. Short interfering RNAs (siRNA) 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 siCOX2 down-regulated the expression of *COX-2* mRNA and also resulted in suppression of aromatase mRNA. The administration of PGE₂ to cells treated with the siRNAs results in antagonism of only the siCOX2 and restores aromatase activity to untreated levels. Finally, the siAROM was also very effective in silencing aromatase enzyme activity in human breast cancer cells in culture and in human choriocarcinoma cells in culture.

The regulation of aromatase in breast cancer involves complex autocrine and paracrine interactions, resulting in significant consequences on the pathogenesis of hormone-dependent breast cancer. 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, novel sulfonanilides and various RNAi technologies targeting aromatase expression may be valuable tools for selective regulation of aromatase in breast cancer and could be developed into the first generation of Selective Aromatase Modulators for the treatment of hormone-dependent breast cancer.

Acknowledgements

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