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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**

Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 500 W. 12th Avenue, Columbus, OH 43210, USA

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ABSTRACT

Previous studies have demonstrated that cyclooxygenase-2 (COX-2) inhibitor NS-398 decrease aromatase activity at the transcript level in breast cancer cells. However, *N*-Methyl NS-398, which does not have COX-2 inhibitory activity but has very similar structure to NS-398, decreases aromatase activity and transcription in MCF-7 and MDA-MB-231 breast cells to the same extent as NS-398. This suggests that NS-398 decrease aromatase expression in breast cancer cells via other mechanism(s). Further investigations find that both compounds only decrease aromatase activity stimulated by forskolin/phorbol ester at the transcript level in both breast cancer cell lines and in breast stromal cells from patients. They do not affect aromatase expression and activity stimulated by dexamethasone. Both compounds also suppress MCF-7 cell proliferation stimulated by testosterone. Aromatase inhibition studies using placental microsomes demonstrate that the compounds show only weak direct aromatase inhibition. These results suggest that NS-398 and its *N*-methyl analog suppress aromatase expression and activity with multiple mechanisms.

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1. 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-

* *Corresponding author*. Tel.: +1 614 292 5231; fax: +1 614 292 3113.
E-mail address: brueggemeier.1@osu.edu (R.W. Brueggemeier).

Abbreviations: PGE_2, Prostaglandin E_2; COX-2, cyclooxygenase-2; NSAIDs, nonsteroidal anti-inflammatory drugs; ER, estrogen receptors; AIs, aromatase inhibitors; SERMs, selective estrogen modulators; TPA, tetradecanoyl phorbol acetate; DEX, dexamethasone; FSK, forskolin; PKA, protein kinase A; PKC, protein kinase C.

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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 5′ 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 E_2 (PGE_2) derived from tumorous epithelium and/or infiltrating macrophages. PGE_2 acts via EP_1 and EP_2 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.

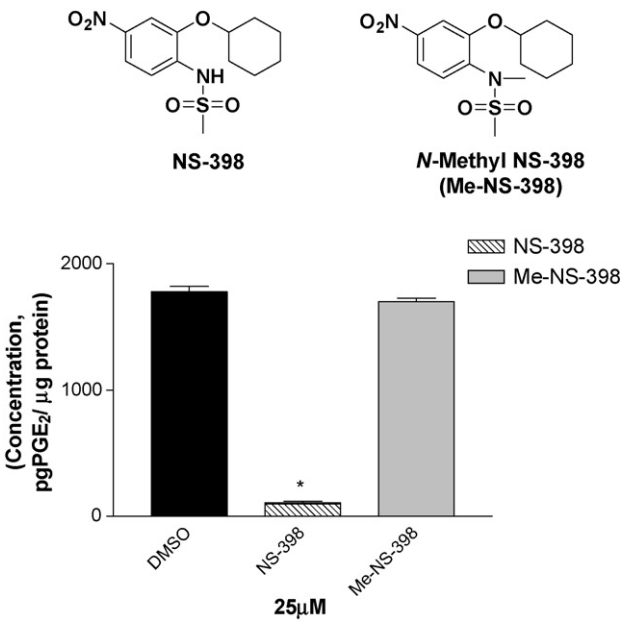

Fig. 1 – Structure of NS-398 and *N*-Methyl NS-398 and their COX-2 inhibition results in MDA-MB-231 cell line [28].

2. Materials and methods

2.1. *Reagents*

Radiolabeled [1β-^{3}H]-androst-4-ene-3,17-dione was obtained from NEN Life Science Products (Boston, MA). 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.

2.2. *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% CO_2 in a Hereaus CO_2 incubator. For all experiments, cells were plated in either T-25

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