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Retinol inhibits aromatase activity and expression *in vitro*

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Abstract

Aromatase converts androgens into estrogens and is thought to supply a local source of estrogen that facilitates the growth of hormone-responsive tumor cells. Inhibition of aromatase is therefore an important chemopreventive strategy. We investigated the effect of retinol and selected retinoids on the activity and expression of aromatase in two human carcinoma cell lines *in vitro*. Retinol (ROH) and all-*trans* retinoic acid (ATRA) significantly inhibited aromatase activity in a concentration-dependent manner in microsomes isolated from JEG-3 human placental carcinoma cells, whereas 9-*cis* and 13-*cis* retinoic acid had significant inhibitory activity only at the highest concentrations tested. Similar results were observed in an assay of cellular aromatase activity in MCF-7 human breast cancer cells. Enzyme kinetic studies by double-reciprocal plot demonstrated that ROH inhibited microsomal aromatase activity in a mixed manner. In addition, ROH suppressed both the basal and cAMP-induced expression of aromatase mRNA in MCF-7 cells and inhibited transcription controlled by a cAMP-responsive element. These results suggest that aromatase activity and expression are a molecular target of ROH and chemopreventive retinoids, an activity that may underlie, in part, their inhibitory effects on hormone-dependent cancer.

Keywords: Retinol; Aromatase; Retinoic acid; cAMP; MCF-7 cells

1. Introduction

Retinol (ROH) is obtained in the diet as retinyl esters from animal products or as β-carotene from fruits and vegetables. ROH is metabolized in cells to retinal and then to all-*trans* retinoic acid [1], which, in turn, may be isomerized to 9-*cis* retinoic acid (9-*cis* RA) or 13-*cis* retinoic acid (13-*cis* RA). Plasma ROH levels are inversely correlated with the breast cancer survival in postmenopausal women [2], and the incidence of breast cancer is inversely correlated with total ROH consumption [3–5]. Plasma ROH or carotenoid levels are also inversely associated with the risk of ovarian [6], bladder [7], lung and other cancers [8]. In clinical trials, several retinoids have significant activity in the reversal of head and neck, skin and cervical premalignancy, and in the prevention of second primary tumors associated with breast, head and neck, skin and non-small cell lung cancers [9,10]. Retinoids have preventive activity in many *in vivo* experimental systems against breast, skin, bladder, lung and oral carcinogenesis [11]. The chemopreventive and chemotherapeutic activity of retinoids is thought to be based on their ability to control cellular proliferation and differentiation. ROH has been shown to promote epithelial cell differentiation [12], inhibit cancer cell proliferation and metastasis [13,14], and retinoids have been shown to induce apoptosis [15] and inhibit cyclooxygenase expression [16], among other mechanisms [1]. Thus, ROH and its metabolites exert their chemopreventive activity through several molecular/biochemical pathways.

Aromatase is a key enzyme that catalyzes the biosynthesis of estrogens from androgens [17]. It belongs to the cytochrome P450 family and is encoded by the *CYP19* gene. The expression of aromatase is particularly high in breast tumor tissue and in the surrounding stroma in postmenopausal breast cancer patients [18,19]. Such intratumoral aromatase provides a source of local estrogen production that triggers estrogen-dependent cancer cell proliferation [20–22]. Because approximately 75% of postmenopausal breast cancer is estrogen-receptive positive [23], inhibition of aromatase activity is an important strategy to reduce the growth-stimulatory effects of estrogens in estrogen-dependent breast cancer [24,25].

Because of the importance of aromatase activity to the growth of breast and other hormone-dependent tumors, and the established protective effect of retinoids, in the present study we examined the effect of naturally occurring retinoids on aromatase activity and expression *in vitro*. We demonstrate that ROH and ATRA exhibited significant inhibitory effect on aromatase activity, whereas 9-*cis* RA and 13-*cis* RA showed a significant inhibitory effect only at higher concentrations. Similar results were observed in an assay of cellular aromatase activity in MCF-7 cells. In addition, ROH suppressed both aromatase mRNA expression and cAMP-controlled transcription. Thus, our data indicate that aromatase is a molecular target of retinoids.

Abbreviations: CRE, cAMP-responsive element; DMSO, dimethylsulfoxide; RA, retinoic acid; ROH, retinol.

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2. Material and methods

2.1. Materials

Unless otherwise noted, all reagents were from Sigma (St. Louis, MO, USA). Retinoids were dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mM, aliquoted and stored at −20°C. Experiments were performed under yellow light. All culture media components and trypsin/EDTA were from BioFluids (Rockville, MD, USA).

2.2. Cell culture

JEG-3 human choriocarcinoma cells and MCF-7 human breast carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). JEG-3 cells were maintained in Eagle's Minimum Essential Medium with nonessential amino acids, supplemented with 10% fetal bovine serum and 2 mM glutamine. MCF-7 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine. Both cell lines were passed weekly using 0.05% trypsin/0.02% EDTA.

2.3. Microsomal preparation

JEG-3 cells were grown to confluence in 175-cm^2 culture flasks. The cells were trypsinized and pelleted by centrifugation, and the pellet resuspended with phosphate-buffered saline and repelleted. The cell pellet was resuspended in 10 mM Tris–HCl, pH 7.5, containing 0.25M sucrose and protease inhibitors (100 μg/ml phenylmethysulfonyl fluoride, 300 μg/ml EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin and 0.7 μg/ml pepstatin A). The cells were sonicated for 30 s on ice with a Branson sonifer, setting 2. The sonicate was then subjected to centrifugation at 10,000×*g*, 4°C, for 10 min to remove cellular debris. The supernatant was subjected to centrifugation at 500,000×*g*, 4°C, for 15 min. The supernatant was removed and the microsomal pellet was resuspended in the above buffer. Protein was measured by the method of Bradford [26] using bovine serum albumin as a standard. Microsomes were aliquoted and stored at −70°C.

2.4. Measurement of microsomal aromatase activity

Microsomal aromatase activity was measured by the ^3H_2O release method as described by Thompson and Siiteri [27]. This assay measures the amount of ^3H_2O formed during the conversion of androstenedione to estrone by aromatase. Briefly, 10 μg of JEG-3 microsomes was incubated with DMSO (control) or retinoid for 1 h at 37°C in 275 μl of PBS, pH 7.5, with 1 mM NADPH and 25 nM [1β-^{3}H(N)]-androst-4-ene-3,17-dione (NEN, Boston, MA, USA). The reaction was terminated by the addition of 75 μl of 50% trichloroacetic acid. This was subjected to centrifugation at 15,000×*g*, 4°C, for 15 min. The supernatant (275 μl) was removed and 80 μl of 10% activated charcoal and 45 μl H_2O were added. This was vortexed gently and incubated at room temperature for 30 min. It was then subjected to centrifugation at 15,000×*g*, 4°C, for 15 min. Radioactivity released as ^3H_2O was determined by scintillation counting of 100 μl of the supernatant in Aquasol scintillation fluid (Beckman, Palo Alto, CA, USA).

2.5. Mechanism-based inhibition assay

To determine the mechanism of inhibition, microsomal aromatase activity was determined as described above in the presence of different concentrations of [1β-^{3}H(N)]-androst-4-ene-3,17-dione and with DMSO or different concentrations of ROH. The kinetics of enzyme inhibition was determined by plotting the enzyme activity at various ROH concentrations as a double-reciprocal plot [28].

2.6. Measurement of cellular aromatase activity

The amount of aromatase activity in intact MCF-7 cells was measured as described in Shimodaira *et al.* [29]. Confluent MCF-7 cells in six-well culture plates were incubated at 37°C for 24 h in 1 ml of growth medium containing 25 nM [1β-^{3}H(N)]-androst-4-ene-3,17-dione in the presence of DMSO (control) or retinoid. The medium was then removed and 250 μl of 10% activated charcoal was added. This was incubated for 30 min at room temperature and subjected to centrifugation at 15,000×*g*, 4°C, for 15 min. A total of 625 μl was used for scintillation counting as described above.

2.7. Measurement of aromatase mRNA

The amount of aromatase mRNA was determined by semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR). Confluent MCF-7 cells in six-well culture plates were incubated at 37°C for 24 h in 1 ml of growth medium with or without 1 mM cAMP in the presence of DMSO (control) or ROH. Following incubation, the cells were washed with PBS and total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) as directed. cDNA was synthesized from 10 μg of total RNA using an RT-PCR kit from Stratagene (La Jolla, CA, USA) as instructed. PCR was performed using the aromatase primer sequences and method of Zhou *et al.* [30], in the presence of 1.5 μCi of [^{32}P]dATP (NEN). PCR was also run using primers for glucose-3-phosphate dehydrogenase (G-3-PDH; Clonetech, Palo Alto, CA, USA) as directed. The optimum cycle number that fell within the exponential range of response for aromatase (25 cycles) and G-3-PDH (19 cycles) was used. Following PCR, 5 μl of high-density buffer (Novex, San Diego, CA, USA) was added to the samples, and they were subjected to electrophoresis on a 10% Tris-borate EDTA gel (Novex) in 1× Tris-borate EDTA buffer (Novex) for 1.5 h at 125 V. The gel was then dried and visualized by phosphoimaging using a Bio-Rad GS-363 phosphoimager (Hercules, CA, USA). Results were graphed as the ratio of aromatase mRNA normalized to G3PDH mRNA.

2.8. cAMP-controlled transcription

MCF-7 cells were transiently transfected with a luciferase reporter vector controlled by cAMP-responsive elements (CRE; Promega, Madison, WI, USA) and a β-galactosidase reporter vector. Transfected cells were treated with phosphate-buffered saline or cAMP (1 mM) for 6 h in the presence of DMSO (control) or ROH at the indicated concentrations. Luciferase activity was determined and normalized to β-galactosidase activity.

2.9. Statistical analysis

The data were presented as means of triplicate or quadruplicate determinations with standard errors. The results were analyzed using one-way analysis of variance, followed by Bonferroni test with SPSS statistical software (Chicago, IL, USA). A P value <.05 was considered significantly different.

3. Results

3.1. Effect of retinoids on the specific activity of microsomal aromatase

Microsomes isolated from JEG-3 human placental cancer cells were used as a source of aromatase. Incubation of 10 μg of microsomes with 25 nM of the aromatase substrate [1β-^{3}H(N)]-androst-4-ene-3,17-dione in the presence of the co-factor NADPH resulted in a specific aromatase activity of 25.79±0.54 pmol/h per milligram of protein. Addition of ROH resulted in a concentration-dependent decrease in activity, with an IC_{50} of approximately 0.2 μM (Fig. 1A). Addition of the retinoid ATRA also caused a concentration-dependent decrease in activity, but with less potency than ROH (IC_{50} of approximately 2.5 μM; Fig. 1B). 13-*cis* RA and 9-*cis* RA added to the reaction decreased enzyme activity significantly only at the highest concentrations tested (Fig. 1B).

3.2. Kinetics of aromatase enzyme inhibition by ROH

The inhibition of microsomal aromatase activity by different doses of ROH in the presence of different substrate concentrations was determined and the results were analyzed by double-reciprocal plot. As shown in Fig. 2, there was a dose-dependent increase in K_m and a slight decrease in V_{max}. This indicates that ROH inhibits aromatase enzyme activity in a mixed manner, with both competitive and noncompetitive components.

3.3. Effect of retinoids on cellular aromatase activity

The MCF-7 human breast adenocarcinoma cell line, incubated with the aromatase substrate [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 24 h, possessed 18.40±0.32 fmol/h per million cells of specific aromatase activity. Co-incubation of the cells with ROH resulted in a dose-dependent decrease in aromatase activity, with an IC_{50} of approximately 2 μM (Fig. 3A). The retinoids also inhibited cellular aromatase activity in a concentration-dependent manner, with an order of potency identical to that seen for microsomal aromatase activity (Fig. 3B).

3.4. Effect of ROH on aromatase mRNA levels in MCF-7 cells

The amount of aromatase mRNA in MCF-7 cells was measured by semi-quantitative RT-PCR and normalized to G3PDH mRNA. As seen in Fig. 4, treatment of MCF-7 cells with ROH at 10 μM for 24 h resulted in a 51% reduction in basal aromatase. Addition of cAMP to the cells

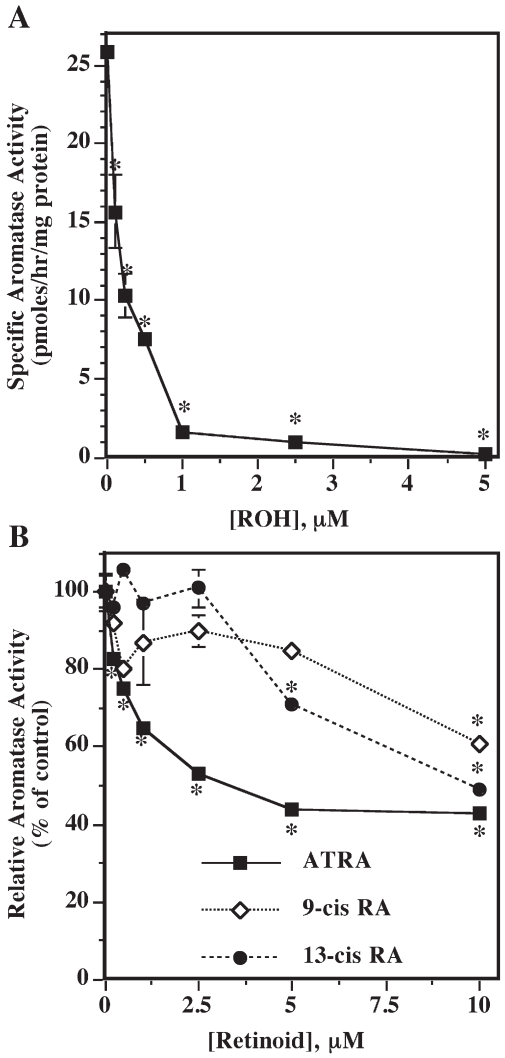

Fig. 1. Effect of retinoids on microsomal aromatase activity. Microsomes were isolated from JEG cells, and aromatase activity was assayed in the presence of DMSO (control), ROH (A) or RAs (B). $n=3\pm$S.E. * indicates a significant difference from control ($P<.05$).

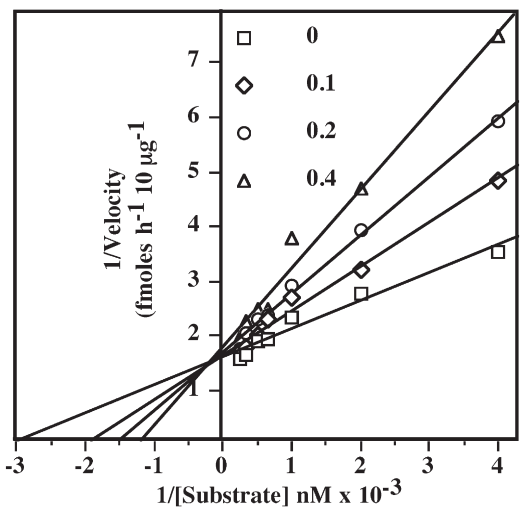

Fig. 2. Effect of ROH on microsomal aromatase activity in the presence of different substrate concentrations. Microsomal aromatase activity was assayed in the presence of DMSO (control) or the indicated concentrations of ROH (μM). Results were graphed by double-reciprocal (Lineweaver–Burk) plot. $n=4$.

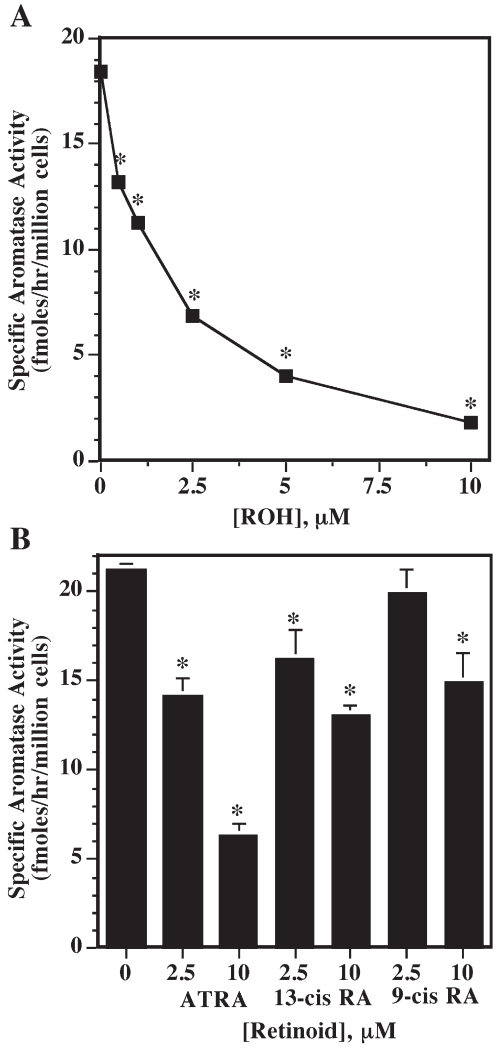

Fig. 3. Effect of retinoids on cellular aromatase activity. MCF-7 cells were incubated in DMSO (control), ROH (A) or RAs (B), and aromatase activity was measured. $n=3\pm$S.E. * indicates a significant difference from control ($P<.05$).

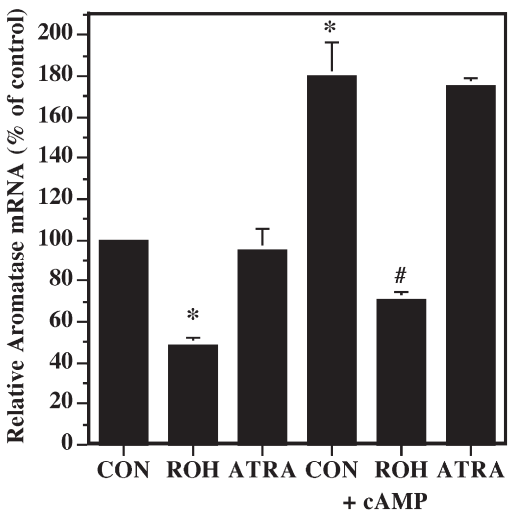

Fig. 4. Effect of ROH and ATRA on aromatase mRNA in the presence or absence of cAMP. MCF-7 cells were treated with or without cAMP to induce aromatase expression in the presence or absence of ROH or ATRA (10 μM). Aromatase mRNA levels were normalized to G3PDH mRNA levels. $n=3\pm$S.E. * indicates a significant difference from control ($P<.05$). # indicates a significant difference from Con+cAMP ($P<.05$). Con=control.

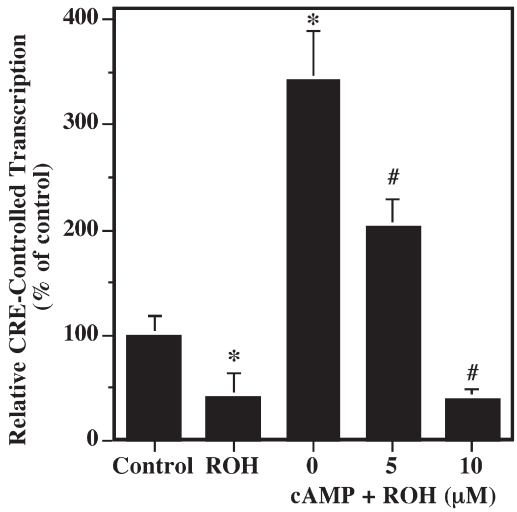

Fig. 5. Effect of ROH on CRE-controlled transcription in the presence or absence of cAMP. MCF-7 cells were transfected with a CRE-controlled luciferase reporter vector and treated with or without cAMP in the presence or absence of ROH. CRE-controlled luciferase transcription was assayed as described. $n=3\pm$S.E. * indicates a significant difference from control ($P<.05$). # indicates a significant difference from Con+cAMP ($P<.05$).

induced aromatase mRNA levels by 80%; ROH also inhibited cAMP-induced aromatase mRNA by 61%. ATRA did not significantly reduce either basal or cAMP-induced aromatase mRNA.

3.5. Effect of ROH on CRE-controlled transcription

MCF-7 cells were transfected with a reporter vector controlled by CREs similar to the one present in the aromatase gene promoter. ROH inhibited basal expression of this vector by 76% (Fig. 5). cAMP treatment of transfected cells induced transcription by 325%. In cells co-treated with ROH, there was a concentration-dependent decrease in cAMP-induced transcription.

4. Discussion

We [31] and others [32,33] previously demonstrated that the synthetic retinoid 4-hydroxyphenylretinamide (4-HPR) inhibits the activity of aromatase *in vitro*. However, despite the established chemopreventive effect of retinoids towards breast cancer, and the crucial role of aromatase in the growth of estrogen-dependent tumors, the effects of ROH and other naturally occurring retinoids on aromatase activity have not, to the best of our knowledge, been investigated. In the present study, we demonstrate that ROH was a potent inhibitor of microsomal aromatase activity, with an IC_{50} of 0.2 μM (Fig. 1A). Therefore we examined the kinetics of enzyme inhibition using a double-reciprocal plot and found that ROH, like 4-HPR [31], involved both competition for the substrate binding site and noncompetitive components (Fig. 2). ATRA also inhibited microsomal aromatase activity, albeit with less potency than ROH, while 9-*cis* and 13-*cis* RA were only weak inhibitors (Fig. 1B). β-Carotene modestly (28±1% inhibition at 10 μM) inhibited microsomal aromatase activity (data not shown). In MCF-7 cells, a similar rank order of inhibition was found, with ROH and ATRA being more effective than the other retinoids; however, it required a higher concentration of ROH to achieve an inhibition of cellular aromatase activity by 50% ($IC_{50} \approx 2$ μM). Hayden and Satre [34] showed that ROH uptake by MCF-7 cells does not occur in a linear manner, so that intracellular ROH lags behind increases in ROH concentration in the culture medium. Moreover, MCF-7 cells metabolize a small portion of ROH to ATRA [34] and other metabolites [35]. Both of these factors may result in a lower effective intracellular ROH concentration than the medium concentration would indicate and thus explains the difference in IC_{50} between the cellular and microsomal assays.

We also examined the effect of ROH on expression of aromatase in MCF-7 cells. ROH inhibited basal expression of aromatase mRNA in MCF-7 cells by 50% (Fig. 4). The regulation of aromatase expression is complex, but one of its key regulators is cAMP. cAMP activates the cAMP-responsive element binding protein (CREB), which interacts with CRE sites in the aromatase promoter I.3/II, inducing its transcription [36,37]. cAMP treatment of MCF-7 cells caused an approximately twofold increase in aromatase mRNA levels (Fig. 4). This cAMP-induced increase was also significantly suppressed by ROH. ATRA, on the other hand, did not significantly decrease basal or cAMP-induced aromatase mRNA levels. To examine this further, we transiently transfected MCF-7 cells with a luciferase reporter vector controlled by multiple CREs. In agreement with mRNA levels, ROH inhibited both basal and cAMP-induced CRE-controlled transcription (Fig. 5). Thus, ROH not only directly inhibits aromatase enzyme activity, but also decreases aromatase expression in MCF-7 cells.

The model systems used in this study, JEG-3 human placental cells used as a source of microsomal aromatase and the estrogen-receptor-positive MCF-7 human mammary cancer cell line, have been used extensively in aromatase research [30–33,37,38] and thus are appropriate *in vitro* models. Plasma concentrations of ROH reach 2–3 μM in humans [2,39]. Therefore, the concentrations used in the present study are physiological relevant.

Many of the biologic functions of ROH are thought to require its conversion to RAs, which then interact with RA receptors or retinoid X receptors to exert their effects through alterations in gene expression. However, ROH itself has many direct activities in cancer cells [13,14,40–42]. The inhibition of aromatase activity by ROH and ATRA and aromatase expression by ROH are activities that have previously not been known. Because of the critical role of aromatase activity to the continued stimulation of estrogen-responsive tumor cell proliferation, inhibition of aromatase activity and expression is an important strategy in the prevention and treatment of breast cancer. A number of very potent aromatase inhibitors have been developed [24,25]. Our results do not indicate that ROH or ATRA would be more efficacious than the currently available aromatase inhibitors, such as anastrozole, as their IC_{50} is considerably higher. Our results do suggest that the chemopreventive effects of ROH and ATRA may be more complex than previously thought, affecting not only the numerous biochemical and molecular pathways previously established, but also the production of estrogens that contribute to tumor cell growth. Thus, aromatase represents a novel molecular target of ROH and ATRA.

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