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COX-2 inhibitor nimesulide analogs are aromatase suppressors in breast cancer cells

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ABSTRACT

Cyclooxygenase-2 (COX-2) inhibitor nimesulide derivatives compounds A and B decreased aromatase activity in breast cancer cells via a novel mechanism different to aromatase inhibitors (AIs), and were defined as "aromatase suppressors". Breast carcinoma cells (MCF-7aro and T47Daro) transfected with aromatase full gene were used to explore the mechanisms of the two compounds. They dose and time-dependently suppressed aromatase activity in MCF-7aro and T47Daro cells in the nanomole range. However, they neither directly inhibited aromatase, nor improved aromatase degradation even at much higher concentrations. They could also suppress androgen stimulated cell growth, but did not affect estrogen enhanced cell proliferation. These results suggest that compounds A and B selectively interfere with aromatase in breast cancer cells, but not estrogen receptor (ER) downstream to disrupt androgen mediated cell growth. Interestingly, compound B effectively inhibited LTED (long-term estrogen deprived MCF-7aro cell) cell growth, which is a model for AIs resistance, with an IC_{50} of 4.68 ± 0.54 μM. The results indicate that compound B could potentially overcome AI resistance in breast cancer cell and could be used as a lead to design more potent derivatives.

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1. Introduction

A growing body of experimental and epidemiological evidence suggests that the use of NSAIDs (non-steroidal anti-inflammatory drugs) may decrease the incidence of mammary cancer, tumor burden, and tumor volume [1–4]. Celecoxib, a COX-2 (cyclooxygenase 2) selective inhibitor, shows strong chemopreventive activity against mammary carcinoma in rats in some studies [5]. In addition to COX inhibition, these small molecules could target other molecular pathways. For example, celecoxib blocks phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase (PDK)/Akt pathway to induce prostate cancer cell apoptosis [6,7]. The COX-2 inhibitor nimesulide is able to suppress the development of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced mammary gland carcinogenesis in rats [8]. Researchers proved that nimesulide also suppresses aromatase activity and expression in several breast cancer cell lines [9]. In brief, COX-2 inhibitors benefit breast cancer patients in several ways. Firstly, they decrease PGE_2 production which subsequently promotes tumor invasiveness, angiogenesis, and progression. Secondly, they inhibit some kinases which are related with tumor growth. Thirdly, they decrease aromatase activity which is the key enzyme for the biosynthesis of estrogen.

COX-2 inhibitor nimesulide gained our attention because its structure could be easily modified to generate analogs (Fig. 1), and it significantly decreases aromatase activity in breast cancer cells. Derivatives of nimesulide have been synthesized, and their pharmacological effects on aromatase have been primarily studied in SK-BR-3 breast cancer cells [10–13]. The results indicate that nimesulide analogs are a group of new pharmaceutical agents targeting aromatase, and they are different from aromatase inhibitors (AIs). However, there are some drawbacks of these studies. Firstly, SK-BR-3 breast cancer cells are not hormone-dependent breast cancer cells although aromatase is highly expressed in this cell line, which makes them not an appropriate model to study estrogen receptor (ER) positive breast cancer. Secondly, it is difficult to distinguish the aromatase transcriptional and post-transcriptional regulation mechanisms in this cell line. Lastly, it is impossible to check how the compound affects ER mediated cell growth in this model. So far, the mechanisms by which these compounds suppress aromatase still remain unclear even after the primary investigation with SK-BR-3 breast cancer cells. These agents are just generally named "aromatase suppressor" and might potentially be a new

Abbreviations: AIs, aromatase inhibitors; PDK, phosphoinositide-dependent kinase; COX2, cyclooxygenase 2; NSAIDs, non-steroidal anti-inflammatory drugs; ER, estrogen receptor; Her2, human epidermal growth factor receptor; LTED, long-term estrogen deprivation.

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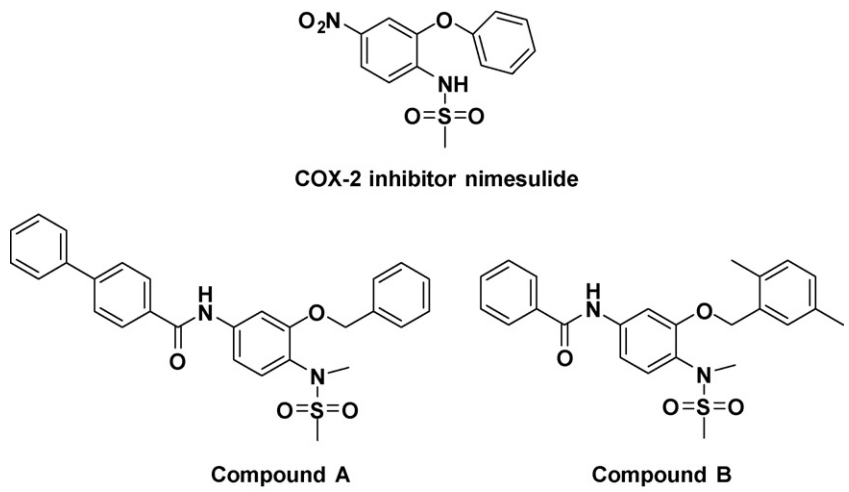

Fig. 1. Structures of nimesulide, compounds A and B.

class of endocrine disrupting agents. In this study, we systematically investigated the mechanisms of the new pharmacological active compounds in two hormone-dependent breast cancer cell lines.

Compounds A and B (Fig. 1), the most active compounds in the nimesulide derivative pool, were synthesized according to previous studies [13,14]. MCF-7 and T-47D aromatase transfected breast carcinoma cells (MCF-7aro and T47Daro) were used to explore the mechanisms of aromatase regulation by the two compounds [15]. The original aromatase activity in the cells is almost undetectable, and the high aromatase activity in the stably transfected cells is artificially controlled and the expression could not be affected by the compounds. Under this condition, we can thoroughly elucidate the mechanisms of post-transcriptional regulation of aromatase by the compounds. In addition, the compounds were further investigated with the AI resistant LTED cells (long-term estrogen deprived MCF-7aro cell).

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). Trypsin and all enzymes were obtained from Invitrogen (Carlsbad, CA). Testosterone and 17β-estradiol were from Sigma Chemical (St. Louis, MO). Mouse anti-aromatase monoclonal antibody was from Serotec (Raleigh, NC). All other antibodies were from Cell Signaling (Danvers, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). Radioactive samples were counted on a LS6500 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Scintillation solution ScientiSafe 30% was purchased from Fisher Scientific (Pittsburgh PA).

2.2. Synthesis of compounds A and B

Compounds A and B were synthesized according to the published procedure [13,14], and their structure and purity were confirmed by NMR and HPLC (Beckman HPLC, C18 column from Phenomenex, 10 mM ammonia acetate in 90% methanol as mobile phase, flow rate as 0.2 mL/min, UV detector setting up at 290 and 296 nM). Compound A (purity 98.6%), Biphenyl-4-carboxylic acid [3-benzyloxy-4-(methanesulfonyl-methyl-amino)-phenyl]-amide: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (2H, s), 8.00 (2H, d, J = 8.0 Hz), 7.76 (2H, d, J = 8.0 Hz), 7.67 (2H, d, J = 7.5 Hz), 7.53 (8H, m), 6.86 (1H, m), 5.18 (2H, s), 3.27 (3H, s), 2.80 (3H, s); Compound B (purity 97.5%), N-[3-(2,5-Dimethyl-benzyloxy)-4-(methanesulfonyl-methyl-amino)-phenyl]-benzamide: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (1H, s), 7.91 (2H, m), 7.55 (2H, m), 7.13 (5H, m), 6.87 (1H, d, J = 9.0 Hz), 5.13 (2H, s), 3.23 (3H, s), 2.72 (3H, s), 2.38 (3H, s), 2.35 (3H, s).

2.3. Cell culture

The ER-positive aromatase over-expressing MCF-7 and T-47D cell lines, MCF-7aro and T-47Daro, were prepared by stable transfection with the human placental aromatase gene and neomycin selection, as described previously [15]. All the cell lines were cultured in MEM, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin-streptomycin and 200 mg/L G418 for MCF-7aro and T47Daro cells (Invitrogen, Carlsbad, CA). LTED cells developed in our laboratory were cultured in same media but charcoal striped fetal bovine serum (CSFBS) was used [16]. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

2.4. Tritiated water-release assay in cells

Measurement of aromatase enzyme activity in cells was based on the tritium water-release assay [12]. Cells in 12 well plates were treated with DMSO (control), compound A or B at different concentrations and time period. Then the cells were incubated for 1 h with fresh media containing drugs and 2 μCi [1β-^{3}H]-androst-4-ene-3,17-dione (100 nM). 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 were extracted three times with an equal amount of chloroform to extract unused substrate, and the aqueous layer subsequently treated with 1% dextran-treated charcoal. After centrifugation, a 300-μL aliquot containing the product was counted in 3 mL of liquid scintillation mixture. Each sample was performed in triplicate and results were corrected for blanks and for the cell contents of culture flasks. 1 mL of 0.5 N NaOH was added to each well and the plates were shaken overnight at room temperature to solubilize cell proteins. Protein concentrations were determined using Bradford assay method to normalize measured radioactivity.

2.5. Expression and purification of NmChAro

The design of recombinant human aromatase NmChAro was described in our previous study [17]. The *E. coli* BL21 (DE3) strain was used for the expression of NmChAro. Bacteria was harvested, incubated on ice for 30 min with 0.5 mg/mL lysozyme in buffer A [100 mM potassium-phosphate buffer (pH 7.4), 20% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μM androstenedione], and disrupted by sonication on ice (Branson Sonifier 450, ~70% full power, 3× 1 min). NmChAro was isolated from the pelleted membranes with buffer B (buffer A containing 0.1% Tween 20 and 0.5 M NaCl), and purified by metal-ion affinity chromatography (Ni Sepharose 6 Fast Flow; Amersham). After elution of NmChAro with a linear imidazole gradient from 50 to 300 mM in buffer B, the red fractions were pooled, desalted, applied to a hydroxyapatite (Bio-Rad) column for the elimination of minor contaminants, and eluted with a linear gradient of 0–1 M NaCl in buffer C (buffer A containing 0.1% Tween 20). Purified NmChAro was loaded on a gel-filtration column (Superdex 200) to remove aggregates and Tween 20 detergent, eluted with buffer D [25 mM Na-HEPES buffer (pH 7.4), 0.15 M NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT], and concentrated using a centrifugal device (Ultra-15 30K; Millipore, Billerica, MA). Purified and concentrated NmChAro Protein concentrations were determined by the Bradford assay method and then stored in buffer D.

2.6. Aromatase enzyme activity assay

Aromatase activity was determined according to the published tritiated water-release method [17]. The standard *in vitro* assay was reconstituted with 100 nM human NADPH-P450 reductase (BD Biosciences, Franklin Lakes, NJ) in a 500-μL reaction buffer containing 67 mM potassium phosphate (pH 7.4), compounds treatment and DMSO as control, 0.1% BSA, 0.1 μg aromatase, 10 μM progesterone, and 500 nM [1β-^{3}H]androstenedione at 37 C in a shaking water bath for 20 min. The incubation was initiated by the addition of 300 μM of NADPH, and terminated by the addition of 500 μL 20% trichloroacetic acid. The reaction was mixed with charcoal-dextran to remove any trace amount of unreacted substrate. After centrifugation of the mixture, the radioactivity of the supernatant was counted by a LS 6500 liquid scintillation counter.

2.7. Western blotting

Cells were cultured in 60-mm culture dishes and incubated with DMSO or drugs for 24 h and then lysed with CelLytic M (Sigma–Aldrich) supplemented with protease inhibitor tablets (Roche, Indianapolis, IN). Cell lysates were sonicated briefly to reduce viscosity. Protein concentration was determined and samples were stored at −70 °C until use. 60 μg of proteins for each sample was boiled with 1× loading buffer (100 mmol/L DTT plus bromophenol blue) for 5 min. Samples were then electrophoresed in 10% SDS-polyacrylamide gel and transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad) using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 100 mA for 90 min. The membrane was blocked for 2 h with 5% nonfat milk in 1× TBS-T at room temperature to reduce background and then incubated with primary antibody, in 1% bovine serum albumin at 4 °C overnight. After the membrane was incubated with the primary antibody and washed four times with 1× TBS-T for 5 min each wash, it was incubated with the secondary antibody in 5% nonfat milk for 60 min at room temperature. The membrane was washed four times again for 5 min each time with 1× TBS-T and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the protocol of the manufacturer. The membrane blot was exposed to Basic Autorad Film (ISC Bioexpress, Kaysville, UT) and developed using a Konica SRX-101A (Konica, Tokyo, Japan). The blot was reprobed with anti-actin (Santa Cruz Biotechnology) at 1:2000 dilution as a loading control. Mouse anti-aromatase (Serotec, Raleigh, NC) was diluted (1:300) with 5% nonfat milk.

2.8. Cell proliferation

The effect of compounds A and B on breast cancer cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT) in six replicates. Cells were grown in culture medium in 96-well, flat-bottomed plates for 24 h, and were exposed to various concentrations of compounds A and B dissolved in DMSO (final concentration ≤0.1%) in medium for different time intervals. For androgen and estrogen stimulated cell growth, cells were hormone starved for three days before the assay. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide in fresh medium, and cells were incubated in the CO_2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide dye was solubilized in 200 μL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

2.9. 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.

3. Results

3.1. Compounds A and B suppressed aromatase activity in MCF-7aro and T47Daro cells in a dose and time-dependent manner

COX-2 inhibitor nimesulide suppresses aromatase activity and expression in SK-BR-3 breast cancer cells. However, it happens at 25 μM which is much higher than the concentration for COX-2 inhibition [9]. Nimesulide analogs which do not have COX-2 inhibitory activity still suppress aromatase activity with similar potency and even more potent than nimesulide [11]. This indicates that COX-2 independent mechanisms are involved for the aromatase suppression. Compounds A and B are the two leads from the nimesulide derivative pool [10,11,13,14]. We systematically investigate their aromatase suppression activity and try to reveal the molecular mechanism of these compounds. Previous studies showed that this series of compounds could also decrease aromatase mRNA level, but at much higher concentrations than the one for enzyme suppression in SK-BR-3 breast cancer cells. These results suggest that the suppression of aromatase activity by the compounds at low concentrations could not be explained by down regulation of aromatase expression. Post-transcriptional modulation of aromatase by the compounds may better answer the question. MCF-7aro and T47Daro cells both have stably transfected aromatase gene and express high level of aromatase, which make them a good model to study the post-transcriptional regulation of aromatase in breast

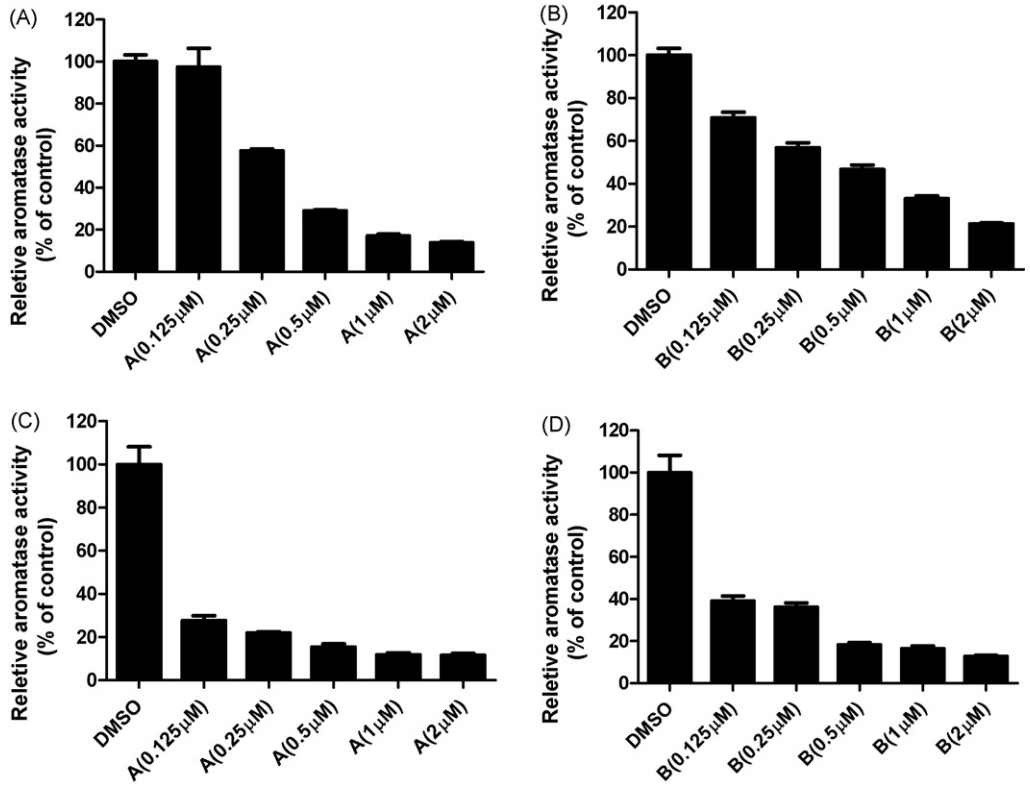

Fig. 2. Dose-dependent suppression of aromatase activity in MCF-7aro and T47Daro breast cancer cells by compounds A and B. Cells were treated with compounds A and B at indicated concentrations for 6 h. Aromatase activity was subsequently determined after a 1 h assay as described. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. Similar results were obtained in at least two independent experiments. (A) Compound A dose-dependently suppressed aromatase in MCF-7aro cells. (B) Compound B dose-dependently suppressed aromatase in MCF-7aro cells. (C) Compound A dose-dependently suppressed aromatase in T47Daro cells. (D) Compound B dose-dependently suppressed aromatase in T47Daro cells.

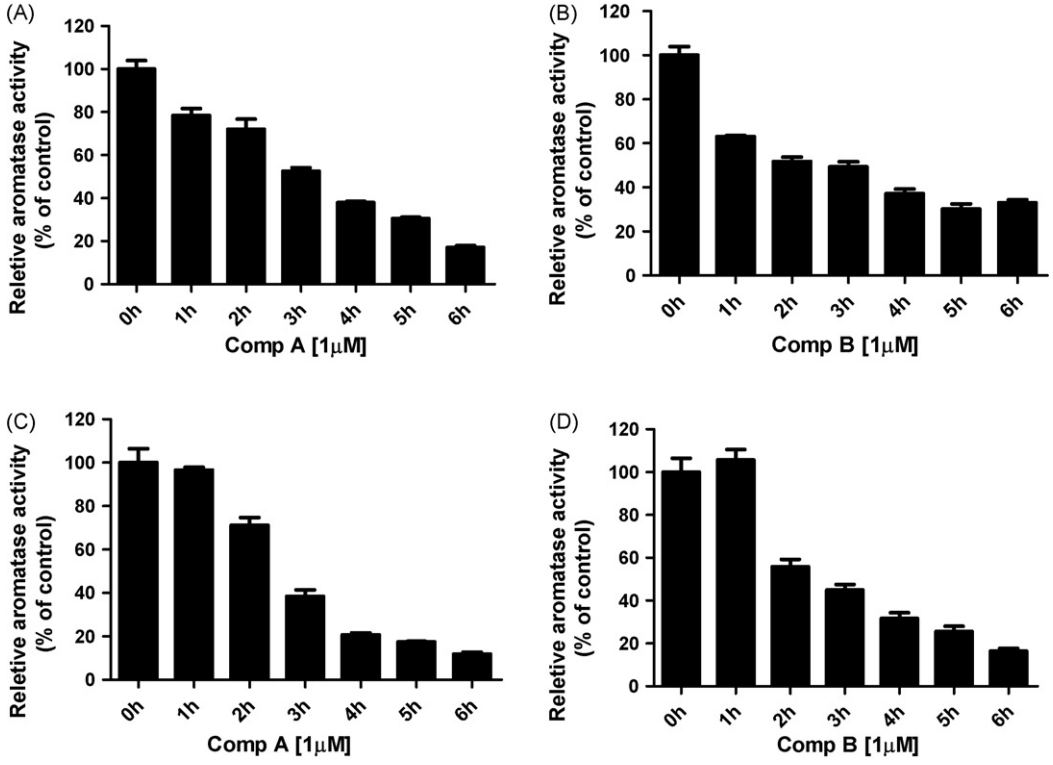

Fig. 3. Time-dependent suppression of aromatase activity in MCF-7aro and T47Daro breast cancer cells by compounds A and B. Cells were treated with compounds A and B at 1 μM for different time points. Aromatase activity was subsequently determined after a 1 h assay as described. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. Similar results were obtained in at least two independent experiments. (A) Compound A time-dependently suppressed aromatase in MCF-7aro cells. (B) Compound B time-dependently suppressed aromatase in MCF-7aro cells. (C) Compound A time-dependently suppressed aromatase in T47Daro cells. (D) Compound B time-dependently suppressed aromatase in T47Daro cells.

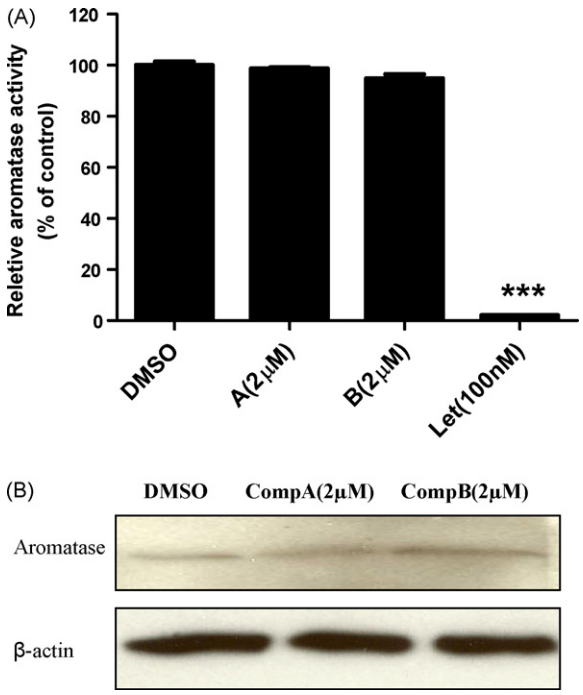

Fig. 4. Both compounds neither directly inhibited recombinant pure aromatase, nor improved aromatase degradation in MCF-7aro cells. (A) Recombinant human aromatase NmChAro was generated using the *E. coli* BL21 (DE3) strain. Aromatase activity was measured as described. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of three independent determinations ± SE. ***$P < 0.001$ vs. control by unpaired t test. Similar results were obtained in at least two independent experiments. (B) Western blotting of aromatase enzyme in MCF-7aro cells after 6 h treatment of the compounds.

cancer cells [15]. Both compounds A and B dose-dependently suppressed aromatase activity in the two cell lines (Fig. 2), with IC_{50} for compound A of 0.34 ± 0.06 and 0.10 ± 0.08 μM in MCF-7aro and T47Daro cells, respectively; for compound B of 0.49 ± 0.24 and 0.11 ± 0.02 μM in MCF-7aro and T47Daro cells, respectively. The results indicate that the compounds are more active in T47Daro cells than in MCF-7aro cells. Time course study exhibited that both compounds suppressed aromatase activity in the two cell lines with a slow manner (Fig. 3). On the other way, AIs decrease aromatase activity with an acute pattern in breast cancer cells in other studies [11].

3.2. Both compounds neither directly inhibited aromatase activity, nor improved aromatase degradation in MCF-7aro cells

To further explore the mechanisms by which the compounds decreased breast cancer cellular aromatase activity, they were tested for their biological activity on aromatase enzyme inhibition using purified aromatase protein. The results revealed that both compounds did not inhibit aromatase enzyme activity even at 2 μM (Fig. 4A), which is consistent with above study that the compounds are not AIs. As a positive control, letrozole significantly inhibited aromatase activity in the assay. Since the compounds could not affect aromatase transcription in the cells, increasing enzyme degradation level could also explain their suppression of aromatase activity. Western blotting results showed that both compounds did not increase aromatase degradation in MCF-7aro cells (Fig. 4B). All the results suggest that the compounds modulate aromatase activity in breast cancer cells via post-transcriptional mechanisms, but not direct inhibition.

3.3. Compounds A and B dose-dependently suppressed cell proliferation of MCF-7aro and T47Daro induced by testosterone, but they did not affect estradiol enhanced cell growth of MCF-7aro and T47Daro

In MCF-7aro and T47Daro cells, aromatase converts androgen to estrogen which binds to ER and stimulated cell proliferation. Treatment of MCF-7aro and T47Daro cells with testosterone significantly stimulated the cell growth, and this stimulation could be inhibited by both compounds A and B in a dose-dependent manner (Fig. 5). The results further underline the suppression of aromatase activity by the compounds. However, it is still possible that the compounds inhibited the cell proliferation by interfering with ER function or ER downstream pathways. Then the compounds were tested to check if they affect estradiol stimulated cell growth. Both compounds did not affect estrogen stimulated cell proliferation in both cell lines up to 2 μM (Fig. 6). This demonstrates that both compounds seem specifically target aromatase, not ER downstream pathways since they did not inhibit cell growth enhanced by estradiol.

3.4. Compound B significantly inhibited LTED cell proliferation

Compound that decreases aromatase activity in breast cancer cells via a different mechanism from AIs was named "aromatase suppressor" in the current study. We hypothesize that these new agents might be able to overcome AI resistance since they function differently to AIs. LTED cells were developed from MCF-7aro cells by hormone deprivation over a year, and they are not sensitive to AIs treatment anymore which make them a good model as AI resistance [16]. It would be interesting to see whether compounds A and B are able to inhibit the proliferation of these cells. The results exhibited that compound B significantly inhibited LTED cell growth with an IC_{50} of 4.68 ± 0.54 μM. Compound A only slightly suppressed the proliferation of LTED cells with an IC_{50} of 21.35 ± 4.77 μM (Fig. 7) although both compounds suppressed aromatase in breast cancer cells with similar potency. This discrepancy suggests that compound B inhibited LTED cell growth via other mechanisms besides aromatase suppression.

4. Discussion

COX-2 inhibitors have been widely studied for their anticancer activities. These biological activities are partially coming from the COX-2 inhibition of these agents. In fact, it has been reported that COX-2 inhibitors inhibit cancer cell proliferation via other mechanisms as well [1–3,5]. COX-2 inhibitors can serve as very promising lead compounds for anti-cancer drug development [7,11]. COX-2 inhibitor nimesulide is able to suppress aromatase activity in breast cancer cells. Derivatives of nimesulide show even much better activity compared with nimesulide [10–13]. However, it is still unclear about how the compounds decrease aromatase activity in breast cancer cells. Therefore, we investigated novel aromatase suppressor compounds A and B, which are COX-2 inhibitor nimesulide analogs, in aromatase transfected breast cancer cell lines MCF-7aro and T47Daro. Nimesulide analogs are able to decrease aromatase activity in SK-BR-3 breast cancer cells at sub-micromole concentration. We initially speculated that this phenomenon can be explained with transcriptional suppression of aromatase expression by these compounds. However, they only decrease mRNA of aromatase at much higher concentration (25 μM) [9,11]. This indicates that at the dosages to suppress aromatase activity, transcriptional regulation of aromatase is not the major mechanism. The compounds suppress aromatase activity, possible via post-transcriptional mechanisms. MCF-7aro and T47aro cell lines are perfect models to study the

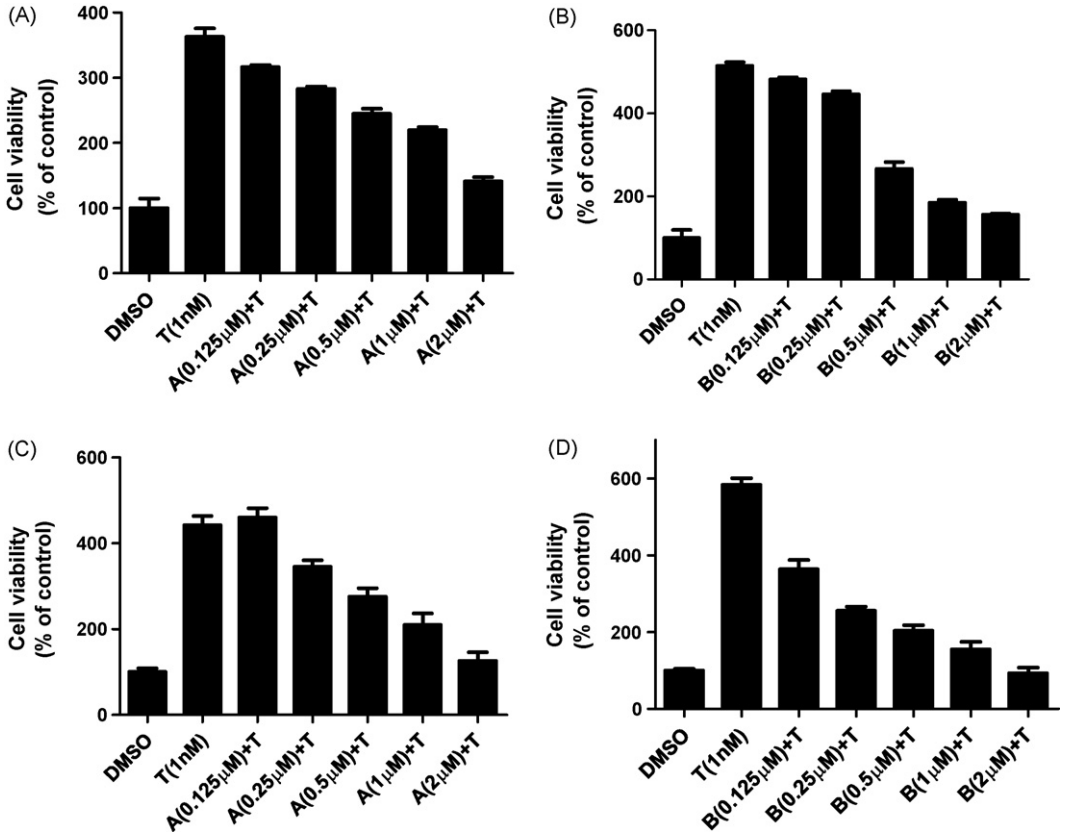

Fig. 5. Both compounds dose-dependently inhibited testosterone (T) stimulated MCF-7aro and T47Daro cells proliferation. Cells were hormone starved for three days before the assay, then exposed to T and various concentrations of the compounds for seven days, cell viability was measured by MTT assay as described in Section 2. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of six independent determinations ± SE. Similar results were obtained in at least two independent experiments. (A) Compound A dose-dependently suppressed testosterone stimulated MCF-7aro cells proliferation. (B) Compound A dose-dependently suppressed testosterone stimulated T47Daro cells proliferation. (C) Compound B dose-dependently suppressed testosterone stimulated MCF-7aro cells proliferation. (D) Compound B dose-dependently suppressed testosterone stimulated T47Daro cells proliferation.

post-transcriptional mechanisms of aromatase regulation of these compounds.

In both MCF-7aro and T47Daro cell lines, the compounds decreased aromatase activity differently to AIs. They dose and time-dependently suppressed the cellular aromatase activity with a novel post-transcriptional mechanism, and they did not inhibit aromatase activity at a non-cell assay or increase aromatase degradation as checked by enzyme assay and Western blotting. All these results indicate that the compounds are not AIs. However, the post-transcriptional regulation of aromatase in breast cancer has not been well studied yet. It has been proved that phosphorylation of aromatase plays an important role in regulating aromatase activity in brain cells [18,19]. In addition, other study revealed that PI3K inhibitor LY294002 and MAPK inhibitor U-0126 suppressed aromatase activity in MCF-7aro cells, which suggest that aromatase activity could also be regulated by phosphorylation in breast cancer cells [20]. It is possible that compounds A and B regulate aromatase by interfering with the phosphoryla-

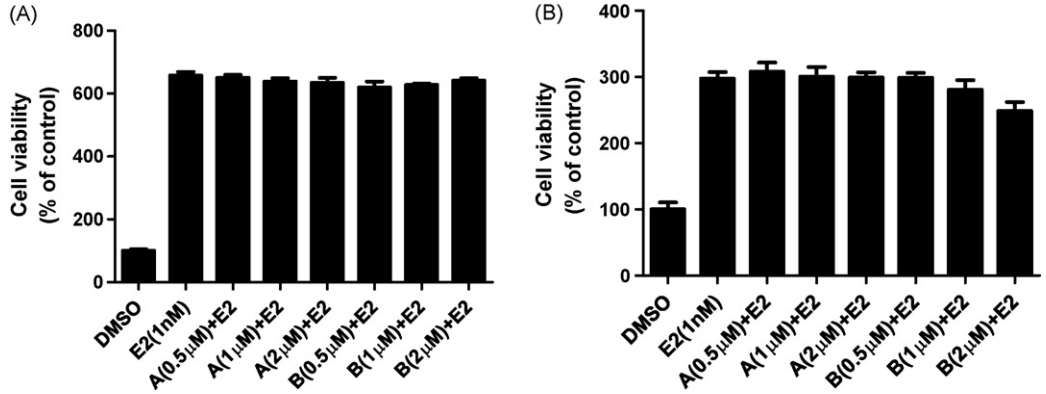

Fig. 6. Both compounds did not affect estradiol (E2) enhanced cell growth. Cells were hormone starved for three days before the assay, then exposed to E2 and various concentrations of the compounds for seven days, cell viability was measured by MTT assay as described in Section 2. The results were normalized against a control treatment with vehicle. Each data bar represents the mean results of six independent determinations ± SE. Similar results were obtained in at least two independent experiments. (A) Both compounds did not affect E2 enhanced MCF-7aro cell growth. (B) Both compounds did not affect E2 enhanced T47Daro cell growth.

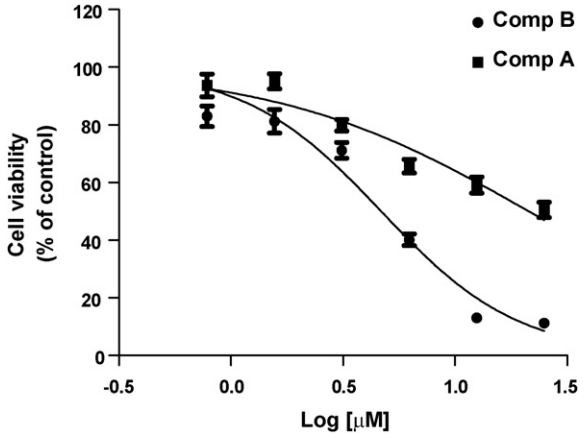

Fig. 7. Compound B inhibited LTED cell growth more effectively than Compound A. Compounds A and B dose-dependently suppressed LTED cell growth with IC50 of 21.35 and 4.68 μM respectively. Cells were treated with compounds as indicated for 72 h. Cell viability after drug treatment was determined by the MTT assay. Each data point represents the mean results of six independent determinations ± SE. Similar results were obtained in at least two independent experiments.

tion process, which so far is the only published mechanism of post-transcriptional modification of aromatase. Researchers found that c-Src kinase post-transcriptional regulates aromatase activity in breast cancer cells [21]. However, the c-Src kinase is too low to be detected in our cell lines. In addition, there is no commercially available antibody of phosphorylated aromatase currently which makes it difficult to further explore the possible molecular target for the compounds in our models. Both compounds also suppressed the cell proliferation stimulated by testosterone, but they did not affect the cell growth enhanced by estradiol. These results further underlined that these compounds inhibited cell proliferation by targeting aromatase, consequently decreased estrogen production in the cells. They are a new class of endocrine disrupt agents, different from AIs and SERMs (selective estrogen receptor modulators).

After the development of resistance to AIs, would compounds A and B be effective in inhibiting breast cancer cell growth? With a new mechanism to disrupt endocrine related breast cancer, aromatase suppressors A and B might be able to overcome AIs resistance. As a model for AIs resistance, LTED breast cancer cells are not sensitive to hormone stimulation and Her2 pathway is up-regulated in these cells [20,22–25]. The results demonstrate that only compound B significantly inhibited LTED cell growth, and compound A is much less potent than compound B. This is a very unique phenomenon because both compounds suppressed aromatase activity with similar potency. Aromatase suppression activity appears could not explain why compound B is superior to compound A in terms of inhibiting LTED cell growth. This suggests that other mechanisms are involved for the overcoming AI resistance by compound B, which is under investigation currently. These multiple anti-cancer properties of compound B make it a potential new therapeutic agent for hormone-dependent breast cancer as well as for overcoming AI resistance. Further optimization of this compound to generate more potent derivatives is undergoing.

Acknowledgments

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