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Benzimidazoles diminish ERE transcriptional activity and cell growth in breast cancer cells

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Abstract

Estrogen receptors (ER α and ER β) are members of the nuclear receptor superfamily. They regulate the transcription of estrogen-responsive genes and mediate numerous estrogen related diseases (i.e., fertility, osteoporosis, cancer, etc.). As such, ERs are potentially useful targets for developing therapies and diagnostic tools for hormonally responsive human breast cancers. In this work, two benzimidazole-based sulphonamides originally designed to reduce proliferation in prostate cancer, have been evaluated for their ability to modulate growth in estrogen dependent and independent cell lines (MCF-7 and MDA-MB 231) using cell viability assays. The molecules reduced growth in MCF-7 cells, but differed in their impact on the growth of MDA-MB 231 cells. Although both molecules reduced estrogen response element (ERE) transcriptional activity in a dose dependent manner, the contrasting activity in the MDA-MB-231 cells seems to suggest that the molecules may act through alternate ER-mediated pathways. Further, the methyl analog showed modest selectivity for the ER β receptor in an ER gene expression array panel. However, the naphthyl analog diminished gene expression. The molecules were docked in the ligand binding domains of the ER α -antagonist and ER β -agonist crystal structures to evaluate the potential of the molecules to interact with the receptors. The computational analysis complimented the results obtained in the assay of transcriptional activity and gene expression suggesting that the molecules upregulate ER β activity while down regulating that of ER α .

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Keywords

Breast cancer; estrogen receptors; docking; ERE; MCF-7; MDA-PCa 2b; benzimidazole; celecoxib

Introduction

The development and progression of breast cancer is a multi-step biological process that is largely hormone dependent, primarily facilitated through estrogen-related pathways. It has been predicted that in 2014, there will be 232,670 new incidences of the disease in women and, although not as common, 2,360 new incidences in men [1]. The disease is responsible for one in 36 deaths that occur in all women [1]. Luminal A and B breast cancers account for approximately 60% of all subtypes diagnosed in the United States [2,3]. Both subtypes are characterized as being estrogen (ER) and/or Progesterone (PgR) receptor-positive. As a result, there is significant interest in the role of the ER in breast tumorigenesis. In most cases, the development and progression of breast cancers are governed by the activity of ER α and ER β . The receptors regulate the transcription of estrogen-responsive genes and mediate numerous estrogen-related conditions (i.e., fertility, osteoporosis, cancer, etc.) [4,5]. Acting in concert, the receptors have opposite functions where ER α triggers the induction of carcinogenic pathways, while ER β prevents the development and progression of the disease. This parallel activity provides further evidence regarding the potential utility of the receptors as drug targets for developing therapies and diagnostic tools for hormonally responsive human breast cancers.

ER α and ER β , members of the nuclear receptor superfamily, are structurally similar with slight differences in their ligand binding domains. Because of this, the receptors can be modulated by ligands that are structurally similar to the endogenous ligand 17 β -estradiol (E₂) [6,7]. Molecules such as tamoxifen are similar in size to estrogen and bind competitively to the receptor leading to partial estrogen antagonism. Other anti-estrogen molecules include fulvestrant, which completely diminishes estrogenic activity through the degradation of ER α .

Similar to the known activity of tamoxifen and fulvestrant, researchers have reported the ability of celecoxib analogs to inhibit growth in breast cancer cells associated with the decreased expression of ER α and activation of ER β [8,9]. The molecules described herein are considered celecoxib analogs given their tricyclic structure including a toluene group and a para-substituted benzsulphonamide. A number of benzimidazole-based molecules similar in size, shape, and polarity to that of compounds 1 and 2 have demonstrated inhibitory activity in the life cycle of both ER-negative and ER-positive breast cancer cells [10,11,12]. The molecules were previously reported to reduce growth in prostate cancer cells [13]. Because of this, the central benzimidazole ring found in compounds 1 and 2 is believed to be a biologically relevant feature of the molecule. In unreported studies, the molecules showed favorable activity in NCI's Human Tumor Cell Line Screen, particularly in estrogen related cells such as MCF-7, T-47D, and OVCAR-4. Therefore, the present study was designed to further evaluate the biological impact of the molecules on the growth of

estrogen dependent and independent cell lines MCF-7 and MDA-MB 231, respectively. The potential of the molecules to modulate ERE transcriptional activity and gene expression in breast cancer cells was evaluated to complement the growth assays. Computational analysis was conducted to illustrate potential binding modes of the molecule in ER α and ER β .

Materials and Methods

Cell Culture

Human cancer cell lines derived from breast (MCF-7, ER-positive cells) and (MDA-MB 231, ER-negative cells) were cultured in 75-cm² culture flasks in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), basic minimum MEM essential (50x, Invitrogen, Grand Island, NY) and MEM non-essential (100x, Invitrogen, Grand Island, NY) amino acids, sodium pyruvate (100x Invitrogen, Grand Island, NY), antimycotic-antibiotic (10,000-U/mL penicillin G sodium; 10,000- μ g/mL streptomycin sulphate; 25- μ g/mL amphotericin B as Fungizone®), and human recombinant insulin (4-mg/mL Invitrogen, Grand Island, NY). The culture flasks were maintained in a tissue culture incubator in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For estrogen studies, MCF-7 cells were washed with PBS 3 times and grown in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS) for 72 hours before plating for each particular experiment.

Cell viability and proliferation assay

MCF-7 and MDA-MB 231 cells were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% CS-FBS) for 72 hours before plating. 1×10^3 MCF-7 cells/well were plated in 6-well plates containing phenol red-free DMEM media supplemented with 10% charcoal-stripped FBS. Forty-eight hours later, cells were treated with vehicle (DMSO), 17 β -estradiol (0.001- μ M), fulvestrant (0.1- μ M), compound 1 (0.1–10- μ M), and compound 2 (0.1–10- μ M). Cells were incubated at 37 °C, 5% CO₂ in a humidified incubator for 10 to 14 days. 10 to 14 days later, cells were fixed with glutaraldehyde for 30 minutes and then stained with 0.1% crystal violet in 20% methanol for 30 minutes. The colony was quantified using an automatic colony counter.

ERE-Luciferase assay

The cells were plated in 24-well plates at a density of 5×10^5 cells/well in the same media and allowed to attach overnight [14,15]. After 18 hours, cells were transfected for 5 hours in serum-free DMEM with 300- μ g pGL2-ERE2X-TK-luciferase plasmid, using 6- μ l of Effectene (Qiagen, Valencia, CA) per μ g of DNA. After 5 hours the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% CS-FBS containing either DMSO (vehicle), 17 β -estradiol (1.0×10^{-5} - μ M), fulvestrant (0.1- μ M), tamoxifen (TAM, 0.1- μ M), compound 1 (0.1–10- μ M) or compound 2 (0.1–10- μ M). The cells were incubated at 37 °C after treatment. After 18 hours the medium was removed, and 100- μ l of lysis buffer was added per well and then incubated for 15 minutes at room temperature. Cell debris was pelleted by centrifugation at $15,000 \times g$ for 5 minutes. Cell extracts were normalized for protein concentration using reagent according to the protocol supplied by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Luciferase activity for

the cell extracts was determined using Luciferase substrate (Promega, Madison, WI) in an Autoluminat Plus luminometer.

Gene Expression SuperArray Analysis

MCF-7 cells were seeded into 25-cm² flasks in phenol red free DMEM media supplemented with 5% charcoal stripped fetal bovine serum. On the following day, the media was changed. The cells were treated with DMSO (vehicle), 17 β -estradiol (0.001- μ M), fulvestrant (0.1- μ M), compound 1 (0.1- μ M), and compound 2 (0.1- μ M) for 18 hours. Total RNA was extracted. Each array profiles the expression of a panel of 84 genes. For each array, 1- μ g RNA was reverse transcribed into cDNA in the presence of gene-specific oligonucleotide primers as described in the manufacturer's protocol. cDNA template was mixed with the appropriate ready-to-use PCR master mix, equal volumes were aliquoted to each well of the same plate, and then the real-time PCR cycling program was run. Quantitative RT-PCR was performed using manufacturer's protocols for the RT² ProfilerTM PCR Array (Human Breast Cancer and Estrogen Receptor Signaling Superarray, Gaithersburg, MD). Relative gene expressions were calculated by using the 2^{-Ct} method, in which Ct indicates the fractional cycle number where the fluorescent signal reaches detection threshold. The 'delta-delta' method (which is described by Pfaffl et al.,) uses the normalized Ct value of each sample, calculated using a total of five endogenous control genes (18S rRNA, HPRT1, RPL13A, GAPDH, and ACTB). Fold change values are then presented as average fold change = 2^{-(average Ct)} for genes in treated relative to control samples. Clinical variables were characterized using descriptive statistics, and the statistical significance of differences in gene expression between groups was calculated using the student's t-test.

Docking Analysis

ER α antagonist (pdb codes: 2QE4) and ER β agonist (pdb codes: 2JJ3) structural files were obtained from the RCSB Protein Data Bank. The pdb structures contained a co-crystallized benzopyrene ligand, (3a,4r,9br)-4-(4-hydroxyphenyl)-6-(methoxymethyl)-1,2,3,3a,4,9b-hexahydro-cyclopenta[c]chromen-8-ol. Docking models were generated using the MOE software. With the exception of adding protons and optimizing the orientation of groups using the LigX function, all computational analyses are based on the imported data [16]. Various conformations of the molecules were examined in the E2 binding pocket of the receptors. The best scored conformation was retained, and the ligand-receptor complex was optimized. Standard force field parameters defined by Merck Molecular Force Field (MMFF94) were used to calculate the binding energies (reported as affinity by the software) of each complex.

Results and Discussion

Novel Benzimidazole Analogs Differentially Alter the Viability of ER Positive and ER Negative Breast Cancer Cells

The effects of the benzimidazole-based compounds were evaluated using colony formation assays in ER-positive MCF-7 and ER-negative MDA-MB 231 cells (Fig. 1A). Compared to fulvestrant, compound 1 exhibited an overall decrease in colony formation (Fig. 1A). Interestingly, compound 1 inhibited colony formation most effectively at the lowest dose.

The molecule, however, had no effect in MDA-MB 231 cells, suggesting that antiproliferative activity of compound 1 is dependent on the presence of ER α . Compound 2 demonstrated stimulatory activity in MCF-7 cells while dramatically inhibiting the colony formation in MDA-MB 231 cells at doses of 0.1- and 10- μ M (Fig. 1B).

Novel Benzimidazole Analogs Inhibit ER Transcriptional Activity

Based on the compound's antiproliferative effects, it is hypothesized that this decreased proliferation may be mediated through ER interaction. Therefore, ERE transcriptional assays were performed to examine the potential of the benzimidazoles to alter ER transactivation. In this study, both molecules caused a dose-dependent decrease in the transcriptional activity. Their impact on ERE activity was comparable to tamoxifen (TAM) and fulvestrant at the 10- μ M dose (Fig. 2).

Novel Benzimidazole Analogs Differentially Regulate the Expression of Genes Involved in Breast Cancer and Estrogen Receptor Signaling

The expression of genes commonly altered in breast cancer and estrogen signaling was evaluated in the presence of each molecule. The results demonstrated that while compound 1 causes a 3.05-fold decrease in aromatase gene expression, compound 2 causes a 2.61-fold increase in this gene (Table 1). Interestingly, compounds 1 and 2 differentially regulated pS2; compound 1 caused a 3.19-fold increase in pS2 expression, while compound 2 did not significantly alter the expression. Compound 1 had no effect on the expression of either nuclear receptor. However, compound 2 caused a modest decrease in ER α (-2.39-fold) with a robust increase in ER β expression (7.69-fold), suggesting that the benzimidazole analogs selectively and differentially alter the two ER isoforms.

Computational Analysis

The biological analysis of benzimidazoles suggests that the molecules' interactions with the estrogen receptors should be taken into account. Docking models were created to examine the mode by which compounds 1 and 2 potentially bind to the ER β agonist and the ER α antagonist conformations (Fig. 3). The benzopyrene ligand found in the crystal structures has a reported selectivity for the ER β agonist conformation [17]. Overall, the calculated binding energies for compounds 1 and 2 were comparable to that of the co-crystallized ligand (Figures 6 and 7). The benzopyrene has hydroxyl groups at either end of the molecule which form hydrogen bonds to Glu 305 and His475 in ER α , and Glu353 and His524 in ER β . The naphthyl group of compound 1 occupies the same hydrophobic region of the ER α structure as that occupied by the cyclohexyl ring of the benzopyrene ligand (Fig. 3). However, the calculated binding energy of compound 1 in the ER α structure is less favorable than that of the co-crystallized ligand. During the computational analysis, it was observed that the structure of compound 1 was significantly distorted to avoid unfavorable binding interactions in the pocket of ER α . This is likely the reason for the decreased stability of the ER α -compound 1 complex in comparison to that of the ER α -benzopyrene complex. In the ER α receptor, the naphthyl derivative lies in a different binding orientation than that of the methyl derivative (Fig. 3A). The molecules have similar binding orientations in ER β (Fig. 3B). Compound 2 has a more favorable binding energy than does compound 1 (Fig. 4) in both receptors. However, compound 2 appears to be selective for ER β . Based on

the calculated binding energies, the compound 2 could mimic the binding activity of the co-crystallized ligand which is reported to have selectivity for ER β .

In this study, the activity of benzimidazole analogs, compounds 1 and 2, in ER positive and ER-negative breast cancer cells has been demonstrated for the first time. The results of this study demonstrate the benefits of the benzimidazole molecules towards the selective modulation of the estrogen receptors and the reduction of ERE transcriptional activity. In addition, the results suggest that molecules similar to those described herein could have value in the development of hormonally responsive human breast cancer therapies.

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Abbreviations

DMEM	Dulbecco's Modified Eagle's Media
E2	17 β -estradiol
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
MOE	Molecular Operating Environment
MEM	Minimum Essential Medium
PgR	Progesterone receptor

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Highlights

- The methyl-substituted benzimidazole inhibited growth in MDA-MB 231 cells
- The naphthyl-substituted benzimidazole was more effective at inhibited growth in MCF-7 cells than ICI.
- The benzimidazole molecules demonstrated a dose-dependent reduction in ERE transcriptional activity.
- The benzimidazole molecules had binding mode in ER α and ER β comparable to that of the co-crystallized ligand.

Fig. 1. Differential treatment effects on ER+ and ER- breast cancer cell viability. Colonies of MCF-7 (ER+) and MDA-MB 231 (ER-) cells were grown for 14 days in the absence and presence of E2 (0.001-μM), ICI (0.1-μM), compound 1 (0.1–10-μM), or compound 2 (0.1–10-μM). (A) Differential growth effects of compound 1. (B) Differential growth effects of compound 2.

Fig. 2. ERE activity in MCF-7 cells in the presence of DMSO (control), E2 (1.0×10^{-5} -μM), ICI (0.1-μM), or TAM (0.1-μM), compound 1 (0.1–10-μM), and compound 2 (0.1–10-μM).

Fig. 3. Structures of estrogen receptors (grey ribbon) co-crystallized with a benzopyrene ligand (orange tube structure). Compound 1 (pink) and compound 2 (green) are shown as tube structures (A) Binding orientation of compounds 1 and 2 ERβ (pdb code: 2QE4). Helix 12 shown in purple. (B) Binding orientation of compounds 1 and 2 ERα (pdb code: 2JJ3).

Fig. 4. Binding interactions of the benzopyrene ligand, compound 1, and compound 2 in the ER α antagonist (top row) and ER β agonist (bottom row) conformations. Binding energies and RMSD are values calculated using the MOE software.

Table 1

E2, ICI, and Benzimidazole compounds differentially regulate gene expression in MCF-7 cells. Numbers in bold indicate significant fold changes in gene expression greater than 2.

Gene Name (Gene Symbol)	E2	ICI	Compound 1	Compound 2
BCL2-antagonist of cell death (BCL2)	4.65	-1.05	2.39	4.68
Aromatase (CYP19A1)	-1.62	-1.36	-3.05	2.61
Estrogen Receptor α (ESR1)	-1.48	-1.31	-1.54	-2.39
Estrogen Receptor β (ESR2)	1.11	1.58	-1.33	7.69
Progesterone Receptor (PGR)	66.29	1.20	9.78	26.63
Trefoil Factor 1 (pS2, TFF1)	9.40	-2.08	3.19	-1.42