Biochemical and Biophysical Research Communications 450 (2014) 1358–1362

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Florastina Payton-Stewart [a,1], Syreeta L. Tilghman [b,1], LaKeisha G. Williams [c], Leyte L. Winfield [d,*]

[a] Department of Chemistry, College of Arts and Sciences, Xavier University of Louisiana, New Orleans, LA, USA
[b] Division of Basic Pharmaceutical Sciences, College of Pharmacy, Xavier University of Louisiana, New Orleans, LA, USA
[c] Division of Clinical and Administrative Sciences, College of Pharmacy Xavier University of Louisiana, New Orleans, LA, USA
[d] Department of Chemistry, Spelman College, Atlanta, GA, USA

ARTICLE INFO

Article history:
Received 24 June 2014
Available online 2 July 2014

Keywords:
Breast cancer
Estrogen receptors
Docking
Benzimidazole
Celecoxib

ABSTRACT

Estrogen receptors (ERα and ERβ) 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 sulfonamides 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 ERβ receptor in an ER gene expression array panel, while the naphthyl analog did not significantly alter gene expression. The molecules were docked in the ligand binding domains of the ERα-antagonist and ERβ-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 ERβ activity while down regulating that of ERα.

1. 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, 2360 new incidences in men [1]. The disease is responsible for one in thirty-six 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 ERα and ERβ. 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 ERα triggers the induction of carcinogenic pathways, while ERβ prevents the development and progression of the disease. The paired activities of the two receptors is a target of interest in the development of therapies and diagnostic tools for hormonally responsive human breast cancers.

ERα and ERβ are members of the nuclear receptor superfamily. 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 ERα.

Abbreviations: DMEM, Dulbecco's modified Eagle's media; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen responsive element; MOE, molecular operating environment; MEM, minimum essential medium; PgR, progesterone receptor.

* Corresponding author. Address: Spelman College, Atlanta, GA 30314, USA.
E-mail address: lwinfield@spelman.edu (L.L. Winfield).

[1] Authors contributed equally to this work and share first-authorship.

http://dx.doi.org/10.1016/j.bbrc.2014.06.130
0006-291X/

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 ERα and activation of ERβ [8,9]. The molecules described herein are considered celecoxib analogs given their tricyclic structures which include an aromatic group (in this case para-tolyl and 2-napthyl), a para-substituted benzsulfonamide, and a benzimidazole. There is enthusiasm for the potential utility of the molecules as 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 [10–12]. Because of this, the central benzimidazole ring found in compounds 1 and 2 is believed to be a biologically relevant feature of the molecule. This is supported by the fact that compounds 1 and 2 were previously shown to reduce growth in prostate cancer cells [13]. 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 study also examines the potential of the molecules to modulate ERE transcriptional activity and gene expression in breast cancer cells. A computational analysis was conducted to illustrate plausible binding modes of the molecules in ERα and ERβ.

2. Materials and methods

2.1. 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-cm^2 culture flasks in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), basic minimum MEM essential (50×, Invitrogen, Grand Island, NY) and MEM non-essential (100×, Invitrogen, Grand Island, NY) amino acids, sodium pyruvate (100× Invitrogen, Grand Island, NY), antimycotic-antibiotic (10,000-U/mL penicillin G sodium; 10,000-μg/mL streptomycin sulfate; 25-μg/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% CO_2 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 h before plating for each particular experiment.

2.2. 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 h 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), 17β-estradiol (0.001-μM), fulvestrant (0.1-μM), compound 1 (0.1–10-μM), and compound 2 (0.1–10-μM). Cells were incubated at 37 °C, 5% CO_2 in a humidified incubator for 10–14 days. 10–14 days later, cells were fixed with glutaraldehyde for 30 min and then stained with 0.1% crystal violet in 20% methanol for 30 min. The colony was quantified using an automatic colony counter.

2.3. 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 h, cells were transfected for 5 h in serum-free DMEM with 300-μg pGL2-ERE2X-TK-luciferase plasmid, using 6-μl of Effectene (Qiagen, Valencia, CA) per μg of DNA. After 5 h the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% CS-FBS containing either DMSO (vehicle), 17β-estradiol (1.0×10^{-5}-μM), fulvestrant (0.1-μM), tamoxifen (TAM, 0.1-μM), compound 1 (0.1–10-μM) or compound 2 (0.1–10-μM). The cells were incubated at 37 °C after treatment. After 18 h the medium was removed, and 100-μl of lysis buffer was added per well and then incubated for 15 min at room temperature. Cell debris was pelleted by centrifugation at 15,000*g* for 5 min. 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.

2.4. Gene expression superarray analysis

MCF-7 cells were seeded into 25-cm^2 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), 17β-estradiol (0.001-μM), fulvestrant (0.1-μM), compound 1 (10-μM), and compound 2 (10-μM) for 18 h. Total RNA was extracted. Each array profiles the expression of a panel of 84 genes. For each array, 1-μg 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 RT^2 Profiler™ PCR Array (Human Breast Cancer and Estrogen Receptor Signaling Superarray, Gaithersburg, MD). Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ 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 ΔCt 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 = $2^{-(\text{average } \Delta\Delta Ct)}$ 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.

2.5. Docking analysis

ERα antagonist (pdb codes: 2QE4) and ERβ agonist (pdb codes: 2JJ3) structural files were obtained from the RCSB Protein Data Bank. The pdb structures contained a co-crystallized benzopyrene ligand, (3as,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.

3. Results and discussion

3.1. 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. 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 tested. The molecule, however, did not reduce growth in MDA-MB 231 cells, suggesting that antiproliferative activity of compound 1 is dependent on the presence of the estrogen receptor. 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-μM (Fig. 1B).

3.2. Novel benzimidazole analogs inhibit ER transcriptional activity

Based on the antiproliferative effects of compounds 1 and 2 in MCF-7 cells, it was hypothesized that this decreased proliferation may be mediated through their interactions with the ER. 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-μM dose (Fig. 2).

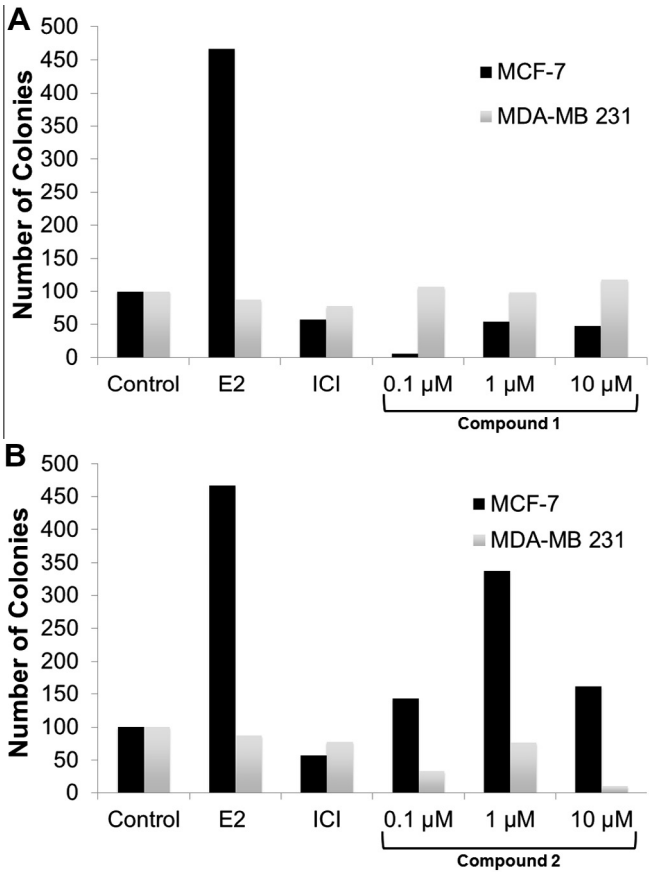

Fig. 1. Differential treatment effects on ER + and ER− breast cancer cell viability. Colonies of MCF-7 (ER+) and MDA-MB 231 (ER−) cells were grown for 14 days in the absence and presence of E2 (0.001-μM), ICI (0.1-μM), compound 1 (0.1–10-μM), or compound 2 (0.1–10-μM). (A) Differential growth effects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

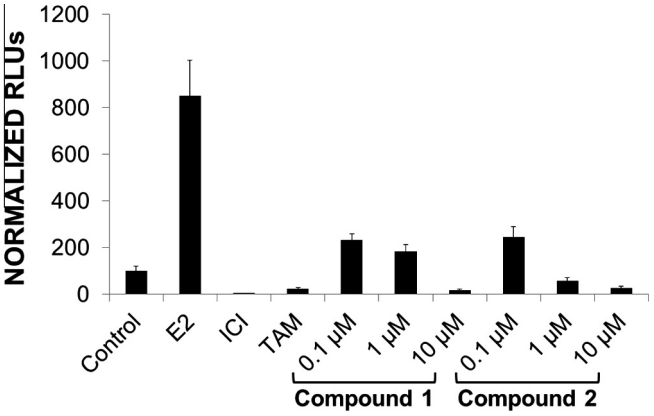

Fig. 2. ERE activity in MCF-7 cells in the presence of DMSO (control), E2 (1.0×10^{-5}-μM), ICI (0.1-μM), or TAM (0.1-μM), compound 1 (0.1–10-μM), and compound 2 (0.1–10-μM).

3.3. 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 of the estrogen nuclear receptor. However, compound 2 caused a modest decrease in ERα (−2.39-fold) with a robust increase in ERβ expression (7.69-fold), suggesting that the benzimidazole analogs selectively and differentially alter the two ER isoforms.

3.4. 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 ERβ agonist and the ERα antagonist conformations (Fig. 3). The benzopyrene ligand found in the crystal structures has a reported selectivity for the ERβ agonist conformation [17]. Overall, the calculated binding energies for compounds 1 and 2 were comparable to that of the co-crystallized ligand (Fig. 4). The benzopyrene has hydroxyl groups at either end of the molecule which form hydrogen bonds to Glu305 and His475 in ERα, and Glu353 and His524 in ERβ. The naphthyl group of compound 1 occupies the same hydrophobic region of the ERα structure as that occupied by the cyclohexyl ring of the benzopyrene ligand (Fig. 3). However, the calculated binding energy of compound 1 in the ERα structure is less favorable than that of the co-crystallized ligand. During the computational analysis, it was observed that the structure distorted significantly to avoid unfavorable binding interactions in the pocket of ERα. This is likely the reason for the decreased stability of the ERα-compound 1 complex in comparison to that of the ERα-benzopyrene complex. In the ERα 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 ERβ (Fig. 3B), although compound 2 has a more favorable binding energy than does compound 1 (Fig. 4) in both receptors. Based on the calculated binding energies, compound

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

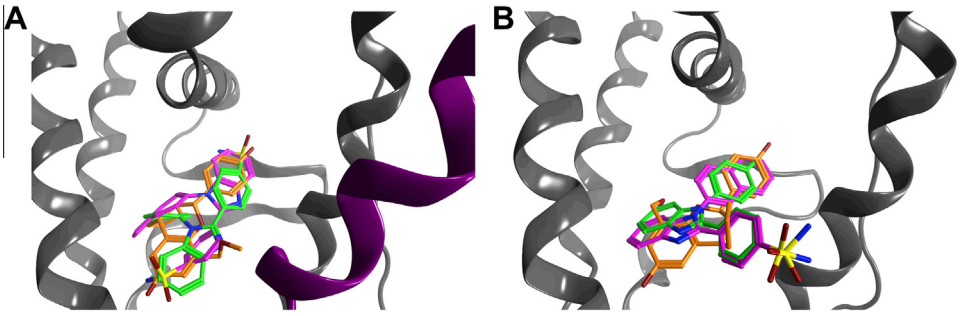

Fig. 3. Structures of estrogen receptors (gray ribbon) co-crystallized with a benzopyrene ligand (orange tube structure). Compound 1 (pink) and compound 2 (green) are shown as tube structures (A) Biding orientation of compounds 1 and 2 ERβ (pdb code: 2QE4). Helix 12 shown in purple. (B) Biding orientation of compounds 1 and 2 ERα (pdb code: 2JJ3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

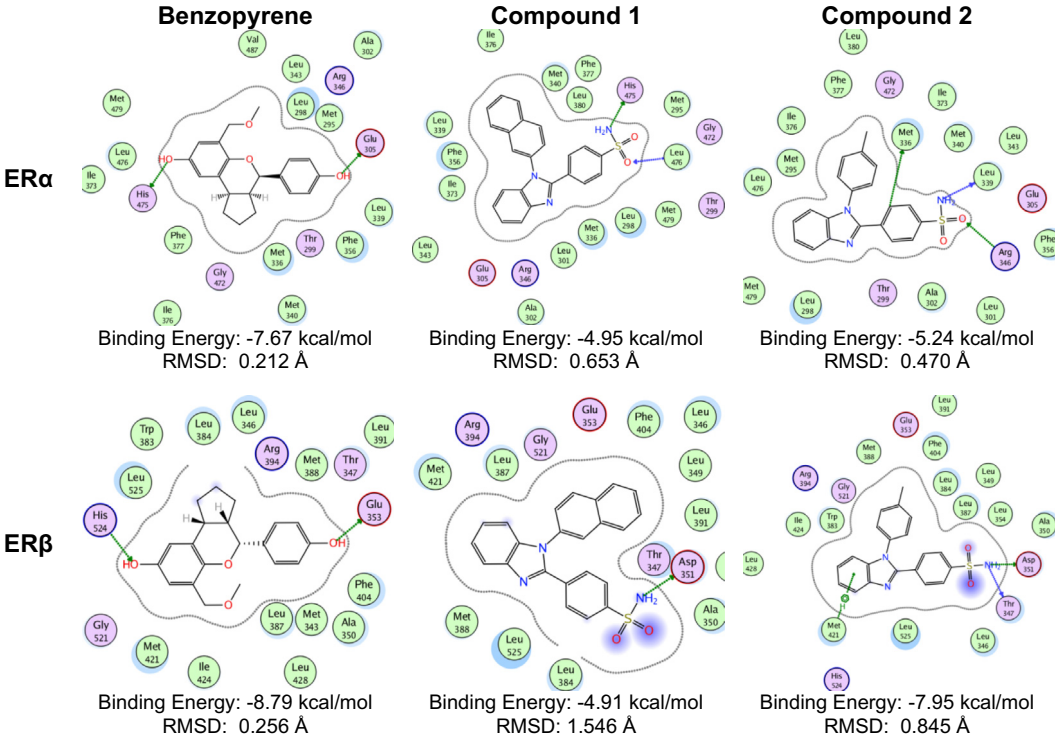

Fig. 4. Binding interactions of the benzopyrene ligand, compound 1, and compound 2 in the ERα antagonist (top row) and ERβ agonist (bottom row) conformations. Binding energies and RMSD are values calculated using the MOE software.

2 could mimic the binding activity of the co-crystallized ligand which is reported to have selectivity for ERβ.

In this study, the activity of benzimidazole analogs, compounds 1 and 2, in ER-positive and ER-negative breast cancer cells has been reported for the first time. The results 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 therapeutics for hormonally responsive human breast cancer.

Acknowledgments

This publication was made possible in part by funding from the Louisiana Cancer Research Consortium, NIH-National Institute on

Minority Health and Health Disparities RCMI Grant #8G12MD007595-04 and RIMI Grant #5P20MD000215-05, NIH-National Institute of General Medical Sciences SCORE Grant #1SC2GM099599-01A1, and the National Science Foundation Historically Black Colleges and Universities Undergraduate Program Grant #0714553. All funding agencies are located in the United States. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the Louisiana Cancer Research Consortium, National Institutes of Health, or the National Science Foundation.

References

[1] American Cancer Society, Cancer Facts & Figures 2014, Atlanta, Georgia, 2014. [Online]. Available: http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2014/. [Accessed 14 July 2014].
[2] C.M. Perou, A.L. Borresen-Dale, Systems biology and genomics of breast cancer, Cold Spring Harb. Perspect. Biol. 3 (2011).
[3] F.M. Blows, K.E. Driver, M.K. Schmidt, A. Broeks, F.E. van Leeuwen, J. Wesseling, M.C. Cheang, K. Gelmon, T.O. Nielsen, C. Blomqvist, P. Heikkila, T. Heikkinen, H. Nevanlinna, L.A. Akslen, L.R. Begin, W.D. Foulkes, F.J. Couch, X. Wang, V. Cafourek, J.E. Olson, L. Baglietto, G.G. Giles, G. Severi, C.A. McLean, M.C. Southey, E. Rakha, A.R. Green, I.O. Ellis, M.E. Sherman, J. Lissowska, W.F. Anderson, A. Cox, S.S. Cross, M.W.R. Reed, E. Provenzano, S.J. Dawson, A.M. Dunning, M. Humphreys, D.F. Easton, M. Garcia-Closas, C. Caldas, P.D. Pharoah, D. Huntsman, Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: A collaborative analysis of data for 10,159 cases from 12 studies, PLoS Med. 7 (2010).
[4] S. Nilsson, S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner, J.A. Gustafsson, Mechanisms of estrogen action, Physiol. Rev. 81 (2001) 1535–1565.
[5] J. Matthews, J.-Å. Gustafsson, Estrogen signaling: a subtle balance between ERα and ERβ, Mol. Interv. 3 (2003) 281–292.
[6] R.M. Evans, The steroid and thyroid-hormone receptor superfamily, Science 240 (1988) 889–895.
[7] M. Beato, P. Herrlich, G. Schutz, Steroid-hormone receptors – many actors in search of a plot, Cell 83 (1995) 851–857.
[8] L. Wang, L.H. Liu, B.E. Shan, C. Zhang, M.X. Sang, J. Li, Celecoxib promotes apoptosis of breast cancer cell line MDA-MB-231 through down-regulation of the NF-kappaB pathway, Ai Zheng 28 (2009) 569–574.
[9] C. Bocca, F. Bozzo, A. Bassignana, A. Miglietta, Antiproliferative effects of COX-2 inhibitor celecoxib on human breast cancer cell lines, Mol. Cell. Biochem. 350 (2011) 59–70.
[10] Y.K. Yoon, M.A. Ali, A.C. Wei, T.S. Choon, H. Osman, K. Parang, A.N. Shirazi, Synthesis and evaluation of novel benzimidazole derivatives as sirtuin inhibitors with antitumor activities, Bioorg. Med. Chem. 22 (2014) 703–710.
[11] S. Bonham, L. O'Donovan, M.P. Carty, F. Aldabbagh, First synthesis of an aziridinyl fused pyrrolo[1,2-a]benzimidazole and toxicity evaluation towards normal and breast cancer cell lines, Org. Biomol. Chem. 9 (2011) 6700–6706.
[12] A.S.A. Rahim, S.M. Salhimi, N. Arumugam, L.C. Pin, N.S. Yee, N.N. Muttiah, W.B. Keat, S. AbdHamid, H. Osman, I.B. Mat, Microwave-assisted synthesis of sec/tert-butyl 2-arylbenzimidazoles and their unexpected antiproliferative activity towards ER negative breast cancer cells, J. Enzyme Inhib. Med. Chem. 28 (2013) 1255–1260.
[13] L.L. Winfield, D.M. Smith, K. Halemano, C.S. Leggett, A preliminary assessment of the structure-activity relationship of benzimidazole-based anti-proliferative agents, Lett. Drug Des. Discov. 5 (2008) 369–376.
[14] M.E. Burow, Y. Tang, B.M. Collins-Burow, S. Krajewski, J.C. Reed, J.A. McLachlan, B.S. Beckman, Effects of environmental estrogens on tumor necrosis factor alpha-mediated apoptosis in MCF-7 cells, Carcinogenesis 20 (1999) 2057–2061.
[15] D.M. Klotz, B.S. Beckman, S.M. Hill, J.A. McLachlan, M.R. Walters, S.F. Arnold, Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays, Environ. Health Perspect. 104 (1996) 1084–1089.
[16] P. Labute, Protonate 3D: assignment of macromolecular protonation state and geometry [Online], 2007. Available from http://www.chemcomp.com/journal/proton.htm. [Accessed July 14, 2014].
[17] B.H. Norman, T.I. Richardson, J.A. Dodge, L.A. Pfeifer, G.L. Durst, Y. Wang, J.D. Durbin, V. Krishnan, S.R. Dinn, S. Liu, J.E. Reilly, K.T. Ryter, Benzopyrans as selective estrogen receptor beta agonists (SERBAs). Part 4: functionalization of the benzopyran A-ring, Bioorg. Med. Chem. Lett. 17 (2007) 5082–5085.