Current Trends in Biotechnology and Pharmacy
Vol. 17 (4A) 91 - 97, October 2023, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2023.4s.95

2-Benzoxazolinone as Breast Cancer Cells Inhibitor via Estrogen Receptor

Binar Asrining Dhiani*[1,2], Ghani Ismail[1], Fariz Subhan Ma'ruf[1], Rochmadi Budi Setiyanto[1], Shintia Lintang Charisma[1], and Fitriyani[3]

[1] Department of Pharmaceutical Biology, Faculty of Pharmacy, University Muhammadiyah of Purwokerto
[2] Cancer and Stem Cell Research Centre, University Muhammadiyah of Purwokerto
[3] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University Muhammadiyah of Purwokerto, Indonesia
*Corresponding author email: binar_dhiani@ump.ac.id

Abstract

Estrogen receptors alpha (ERα) and beta (ERβ) is highly expressed in different cancer cells. Inhibition of ERs by small molecules is a promising approach to developing novel anticancer agents amidst increased endocrine therapy resistance. A heterocyclic molecule, 2-benzoxazolinone (2-BOA) and its derivatives, found in *Acanthus ilicifolius* leaves, possesses anticancer on varied cancer cell types such as HeLa, MCF-7, A-549, and SW-480. However, the mechanism of its cancer cell growth inhibition is an enigma. This study aimed to unmask the activity of 2-BOA to inhibit the growth of the MCF-7 breast cancer cell line via estrogen receptors. The modulation mechanism was predicted by docking molecular of 2-BOA toward ERα (PDB ID: 2JF9) and ERβ (PDB ID: 5TOA). Subsequently, the MCF-7 cell viability assay was performed to validate the *in-silico* prediction. We preliminary identified the presence of 2-BOA in *Acanthus ilicifolius* leaves using high-performance liquid chromatography (HPLC). The binding energy of 2-BOA on ERα and ERβ exhibited a similar score (-6.3 kcal/mol). 2-BOA showed inhibition toward the MCF-7 breast cancer cell line with IC50 value 35.4 µM. 2-BOA may be a potent small molecule inhibitor of the MCF-7 breast cancer cell growth via estrogen receptors

Keywords: 2-Benzoxazolinone, breast cancer, estrogen receptor, ERα, ERβ.

Introduction

Breast cancer is the cause of the highest mortality among 2 million women worldwide (1–3). In 2020, breast cancer prevalence reached 8 million cases (3). ER is the crucial target for treating and preventing breast, prostate, colon, and cervical cancer (4–6). ERα and ERβ are distributed in different tissues in which 70% of breast cancer cases are ERα positive (7). The increasing rate of breast cancer drug resistance, tamoxifen, increases the urge to discover novel anti-cancer agents targeting ER (8).

A heterocyclic molecule, 2-BOA, and its derivatives possess anticancer on varied cancer cell types such as HeLa, MCF-7, A-549, SW-480, and other cancer cell lines (9). The 2-BOA ring is the key feature of the structure for its activity (10). *Acanthus ilicifolius* is a mangrove plant species native to the coastal area stretching from southeast Asia to upper south Asia (11). The leaves of *Acanthus ilicifolius* contains steroid, triterpenoid, flavonoid, tannin, saponin, and alkaloid (12), including α- Amyrin, Acanthicifoline, quercetin, and 2-BOA (13). *Acanthus ilicifolius* extracts and fractions are reported to have activity to inhibit the proliferation of various cancer cell lines (11,13–17). However, none of the reports discover the mechanism of inhibition related to ER. Thus, we reported our initial study to unmask the activity of 2-BOA to inhibit the growth of the MCF-7 breast cancer cell line via ERs.

Material and Methods

Materials

Materials for molecular docking: The 3D structure of ERα (PDB:2JF9) and ERβ (PDB:5TOA) were retrieved from Protein Data Bank (18). The compound 2-BOA (ID: 6043) and estradiol (ID: 5757) structures were downloaded from Pubchem (10,19).

Plant materials: *Acanthus ilicifolius* leaves used in this experiment were collected from Tritih Kulon village, Cilacap Regency, Central Java, Indonesia. The leaves were cleaned and dried and the specimen was stored in the Pharmaceutical Biology Laboratory University Muhammadiyah of Purwokerto.

Materials for MCF-7 cell viability assay: MCF-7 cells (ECCAC), DMEM (Gibco), Fetal Bovine Serum (FBS) (Gibco) and 1% of PenStrep (Gibco), MTT (thiazolyl blue tetrazolium bromide) (Sigma-Aldrich), Phosphate Buffered Saline (PBS) (in-house stock), stop solution (10% SDS in HCl O,1N) (in-house stock), 2-BOA (157058, Sigma-Aldrich), tamoxifen (T2859, Sigma-Aldrich).

Molecular Docking of 2-BOA to ERα and ERβ:

Protein and ligand preparation

The crystal 3D structures were removed from ligands and water associated. The polar hydrogen atom and Kollman Charges were also added to the protein structure. The structure was then saved as .pdbqt file and input as a macromolecule input in PyRx-Vina for molecular docking.

The ligands were prepared by adding the hydrogen atom and Gasteiger partial charges on the structure using AutodockTools 1.5.7. The ligand structure was then also saved in .pdbqt file.

Molecular docking and visualization.

Molecular docking of 2-BOA to ERα (PDB:2JF9) and ERβ (PDB:5TOA) was performed in PyRx-Vina 0.8 tools. The ligand optimization was performed in the Open Babel, a built-in program in PyRx-Vina. Molecular docking method validation was performed by redocking the native ligand estradiol by gridbox setting for ERα docking is: center; X: 69.4570, Y: 39.0027, Z: 16.8174; the dimension (Å); X: 19.4816, Y: 25.1583, Z: 22.0685, and for ERβ docking is set on: center; X: 13.8898, Y: 42.7567, Z: 16.8528; the dimension (Å); X: 51.7320, Y: 26.6124, Z: 30.1712. Analysis of the amino acid residue of ERα and ERβ, which interact with 2-BOA was performed by PLIP tools.

MCF-7 Cell Viability Assay

MCF-7 cell was cultured in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% of PenStrep (Gibco). The MCF-7 cell was cultured at 37°C in the incubator with 5% CO2 to reach 70-80% confluency to be used in the experiment. The MTT assay was performed according to previous standard protocols reported elsewhere with some modifications (20). The 2-BOA was prepared to reach a series of final concentrations ranging from 100 µM to 0.10 nM, and all compounds were prepared in triplicate. After incubation of the compounds to the 5000 cells on each well in a 96-well plate, incubation was performed at 37°C in the incubator with 5% CO2 for 24 hours before the MTT solution was added. After adding the stop solution, the plates were left overnight at room temperature before the absorbance was read on the microplate reader (Epoch-Biotek) at 595 nm wavelength. DMSO was included in the experiments as a control, with 1% DMSO as the highest final concentration. The same set of plates without cells (as a blank) was also measured its absorbance to correct the sample absorbance. Finally, the % cell availability was calculated using the formula:

$$\%\ \text{cell viability} = \frac{\text{Absorbance sample} - \text{Absorbance blank}}{\text{Absorbance control DMSO}} \text{ x } 100\%$$

Identification of 2-BOA

Acanthus ilicifolius leaves subfraction was prepared from column fractionation of its ethyl acetate fraction of *Acanthus ilicifolius* leaves ethanolic extract using a gradient elution of solvent mixture n-hexane: ethyl acetate: ethanol with different ratios. Each

band appeared on the column was collected, and the solvent evaporated under vacuum.

Identification of 2-BOA on subfraction number 3 (SF-3) was initially checked using Thin Layer Chromatography (TLC) and further analyzed by High-Performance Liquid Chromatography (HPLC). The TLC system HPLC system used in the experiment was: Shimadzu Prominence-i LC-2030C 3D chromatograph equipped with a PDA detector, a column oven and an autosampler (Shimadzu, Kyoto, Japan), Column: Shimadzu, shim-pack VP-ODS (250mm x 4,6mm i.d), Software: LabSolutions (Shimadzu, Kyoto, Japan) using C-18 column, mobile phase MeOH/EtOAc: 8/2, temperature 30°C, and flow rate 1 mL/min.

Results and Discussion

2-BOA binding to ERα and ERβ

Molecular docking analysis showed that 2-BOA binds to ERα and ERβ (Figure: 1). All models generated by Autodock Vina tools built in PyRx 0.8 showed the binding affinity ranging from -6.3 to -5.3 kcal/mol. This binding affinity was higher than estradiol (-11.0 kcal/mol). This was supported by the analysis using PLIP tools, which showed the amino acid residue interaction of ERα and ERβ with 2-BOA (Table: 1). There are more hydrophobic interactions occurred between estradiol to ERα and ERβ than 2-BOA. Phi-stacking interaction was likely to increase the energy binding affinity between estradiol to ERα and ERβ, which was absent in 2-BOA. Interestingly, 2-BOA showed interaction with ERα and ERβ in their homolog sequences of amino acid residue, except for isoleucine (ILE386) in ERα and valine (VAL338) in ERβ (see Figure: 2 for ERα and ERβ sequence alignment). It means that 2-BOA interacted with ERα and ERβ, even though the interaction was weaker than the native ligand (estradiol).

2-BOA inhibit the MCF-7 cells growth

The breast cancer cell line MCF-7 viability assay of 2-BOA exhibited that 2-BOA reduces the cell viability started from concentration 10^{-3} M (Figure: 3). Non-linear regression analysis based on log (inhibitor)

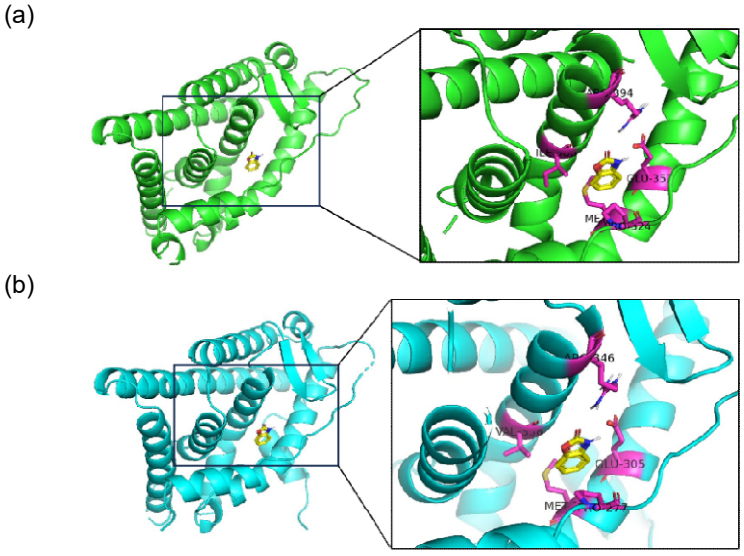

Figure: 1. The three-dimensional structure visualization of 2-BOA binds to ERα (a) and ERβ (b). ERα is displayed in green, ERβ in tosca, and 2-BOA in yellow. The binding site of 2-BOA is zoomed in 12 Å and showed the amino acid residue involved in the interaction.

Table: 1. Amino acid residue interaction of ERα and ERβ with 2-BOA based on PLIP analysis

Compound name	Type of interaction	ERα		ERβ	
		Amino acid residue	Distance (Å)	Amino acid residue	Distance (Å)
Estradiol (Native ligand)	Hydrophobic Interactions	LEU346 LEU349 ALA350 TRP383 LEU384 LEU387 PHE404 LEU428 HIS524 LEU525 LEU525	3.96 3.79 3.68 3.73 3.61 3.92 3.54 3.60 3.90 3.59 3.82	LEU298 LEU339 LEU343 PHE356 ILE373 ILE376 LEU380	3.64 3.98 3.86 3.81 3.48 3.76 3.84
	Hydrogens Bonds	GLU353 ARG394	2.98 2.84	GLU305 ARG346 HIS475	2.98 3.71 3.24
	π-Stacking Interactions	PHE404	4.94	PHE356	5.09
2-Benzoxazolinone	Hydrophobic Interactions	PRO324 GLU353 MET357 ILE386	3.73 3.73 3.68 3.54	PRO277 GLU305 MET309 VAL338	3.60 3.85 3.72 3.50
	Hydrogens Bonds	GLU353 ARG394	3.24 2.36	GLU305 ARG346	3.61 3.10

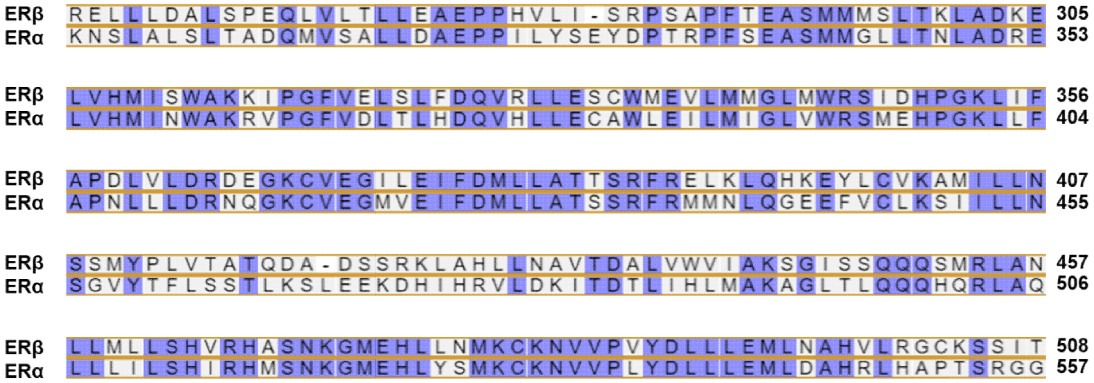

Figure: 2. Sequence alignment ERα and ERβ retrieved from uniprot.org. The blue color displays the amino acid sequence homology between ERα and ERβ.

versus dose-response curve using GraphPad Prism 9.5 resulted that 2-BOA IC50 is 35.4μM. The hormonal breast cancer drug tamoxifen used as a positive control, showed the activity to decrease the MCF-cell viability with IC50 8.1 μM.

2-BOA identified in Acanthus ilicifolius leaves subfraction

It is reported that BOA is one of the compounds found in *Acanthus ilicifolius*. Thus, we identified the presence of 2-BOA in *Acanthus ilicifolius* subfraction by TLC and HPLC identification. Ethyl acetate fraction was separated by solvent mixtures with polarity variation by applying several ratios to achieve a gradient concentration of n-hexane: ethyl acetate: ethanol. Subfraction number 3 (SF-3) had the same spot (spot number 5), which showed the same Rf as synthetic 2-BOA by TLC. This evidence was supported by the HPLC identification, in which the peak of 2-BOA was also exhibited in the SF-3 (Figure: 4.). This indicated that *Acanthus ilicifolius* could be a potential source of compound which shows activity to be developed as an anti-breast cancer.

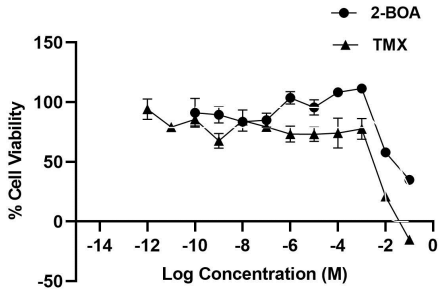

Figure: 3. The log concentration (M) versus dose-response (%) curve of the 2-BOA activity toward MCF-7 cell viability. Experiments were performed in triplicate, and data are presented as mean ± SEM. Tamoxifen was used as a positive control.

Discussion

2-BOA interaction with ERα and ERβ leads to the inhibition of breast cancer cell growth. 2-BOA may promote ER-mediated signaling by binding to ERα or ERβ, activating the protein downstream involved in the RAS/RAF, PI3K, and AKT signaling pathway. The activation of these signaling pathways modulates the cell cycle, cell growth, apoptosis, DAN repair, or angiogenesis, which is responsible for cancer progression (4,21–24). 2-BOA may also promote ER dimerization, which affects the transcriptional regulation of cancer-related target genes (24,25).

Conclusion

Based on the molecular docking analysis, 2-BOA established interaction with ERα and ERβ even though the binding affinity is weaker than the estradiol (native ligand). MCF-7 breast cancer cell viability assay revealed that 2-BOA inhibits the MCF-7 cell

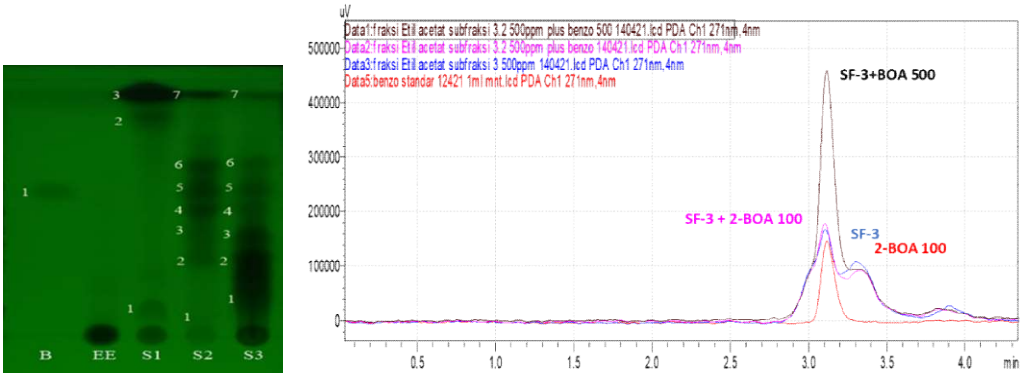

Figure: 4. TLC and HPLC profile of SF-3 which was obtained from the separation of ethyl acetate fraction of *Acanthus ilicifolius* leaves. TLC condition; static phase: silica gel F254 TLC plate, mobile phase: n-hexane:EtOAc (6:4), detected under UV 254nm.

growth with IC50 35.4 µM. 2-BOA was successfully identified in the subfraction of *Acanthus ilicifolius* leaves derived from its ethyl acetate fraction. 2-Benzoxazolinone may be a potent small molecule inhibitor of the MCF-7 breast cancer cell growth via estrogen receptors.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

The author would like to thank Ms. Popi Meri Andini and Ms. Alicia Asa Nurulita for helping prepare *Acanthus ilicifolius* leaves subfractions and cell culture maintenance. This research was partially funded by the National Research and Innovation (BRIN) Republic of Indonesia and The Indonesia Endowment Funds for Education (LPDP) under the scheme Riset dan Inovasi untuk Indonesia Maju (RIIM) year 2022. Author Contribution: BAD: conceptualization, conducting research, analyze data, supervision, writing article, proofreading; GI: conducting research, analyze data; FSM: conducting research, analyze data, RBS: conducting research, analyze data; SLC: co-supervision, analyze data, proofread article; F: co-supervision, analyze data, proofread article.

References

1. WHO. Age standardized (World) incidence rates, breast, all ages. (2020). Available from: http://gco.iarc.fr/today.

2. WHO. WHO | Breast cancer. (2020). Available from: http://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/.

3. WHO I. Breast Cancer Fact Sheets. (2020). Available from: https://gco.iarc.fr/today/data/factsheets/cancers/20-Breast-fact-sheet.pdf

4. Huang B, Warner M, Gustafsson JÅ. (2015). Estrogen receptors in breast carcinogenesis and endocrine therapy. Mol Cell Endocrinol. 418: 240–4.

5. FEB. (2022). Novel drugs that target the estrogen-related receptor alpha: their therapeutic potential in breast cancer. Cancer Manag Res. 6(1): 225–52.

6. Nilsson S, Gustafsson JÅ. (2011). Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther. 89(1): 44–55.

7. Tecalco-Cruz AC, Ramírez-Jarquín JO, Cruz-Ramos E. (2018). Estrogen Receptor Alpha and its Ubiquitination in Breast Cancer Cells. Curr Drug Targets. 20(6): 690-704

8. Viedma-RodríGuez R, Baiza-Gutman L, Salamanca-Gómez F, Diaz-Zaragoza M, Martínez-Hernández G, Esparza-Garrido RR, et al. (2014). Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (review). Oncol Rep. 32(1):3–15.

9. Verma H, Silakari O. (2018). Benzoxazolinone. In: Key Heterocycle Cores for Designing Multitargeting Molecules. Elsevier. p. 343–67.

10. https://pubchem.ncbi.nlm.nih.gov. 2022 [cited 2022 Feb 15].

11. Bora R, Adhikari PP, Das AK, Raaman N, Sharma GD. (2017). Ethnomedicinal, Phytochemical, and Pharmacological Aspects of Genus Acanthus. Int J Pharm Pharm Sci. 9(12): 18.

12. Singh D, Aeri V. (2013). Phytochemical and pharmacological potential of Acanthus ilicifolius. J Pharm Bioallied Sci. 5(1): 17–20.

13. Saranya AR, Ramanathan T KK and AA. (2015). Traditional Medicinal Uses, Chemical Constituents and Biological Activities of a Mangrove Plant, Acanthus ilicifolius Linn. : A Brief Review. Am J Agric Environ Sci. 15(2): 243–50.

14. Khajure P V, Rathod JL. (2011). Potential anticancer activity of Acanthus ilicifolius extracted from the mangroves forest of Karwar, West coast of India. 1 (1): 1-6

15. Singh D, Aeri V. (2013). Phytochemical and pharmacological potential of Acanthus ilicifolius. J Pharm Bioallied Sci. 5(1): 17–20.

16. Paul T, Ramasubbu S. (2017). The antioxidant, anticancer and anticoagulant activities of Acanthus ilicifolius L. roots and

Lumnitzera racemosa Willd. leaves, from southeast coast of India. 7(03): 81–7.

17. Tian XQ, Ke LN, Chen QH, Zeng XH, Wang HM, Li P. (2021). Synthesis of Acanthus ilicifolius Linn alkaloid 2-Benzoxazolinone Derivative and its effect on cervical cancer C-33A cells. IOP Conf Ser Earth Environ Sci. 858(1): 12014.

18. https://www.rcsb.org/. RCSB PDB (2022) [cited 2022 Feb 5]. Available from: https://www.rcsb.org/

19. https://pubchem.ncbi.nlm.nih.gov. (2022). [cited 2022 Feb 15]. 7

20. Miccoli A, Dhiani BA, Mehellou Y. (2019). Phosphotyrosine prodrugs: design, synthesis and anti-STAT3 activity of ISS-610 aryloxy triester phosphoramidate prodrugs. Med Chem Comm. 10(2). 202-208

21. Zhou Y, Liu X. (2020). The role of estrogen receptor beta in breast cancer. Biomark Res. 8(1): 1–12.

22. Lindberg K, Helguero LA, Omoto Y, Gustafsson J-Å, Haldosén L-A. (2011). Estrogen receptor β represses Akt signaling in breast cancer cells via downregulation of HER2/HER3 and upregulation of PTEN: implications for tamoxifen sensitivity. Breast cancer Res. 13(2): 1–13.

23. Alanazi IO, Khan Z, Alanazi IO, Khan Z.(2018). Endocrine and Cell Surface Receptor Signaling in Breast Carcinogenesis. Breast Cancer Surg. IntechOpen.

24. Feng Y, Spezia M, Huang S, Yuan C, Zeng Z, Zhang L, et al. (2018). Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes Dis.5(2): 77–106.

25. Zheng Y, Shao X, Huang Y, Shi L, Chen B, Wang X, et al. (2016). Role of estrogen receptor in breast cancer cell gene expression. Mol Med Rep. 13(5): 4046–50.