RESEARCH ARTICLE

Characterization of Potential Target Genes of Borneol in Increasing Trastuzumab Sensitivity in HER2+ Trastuzumab-Resistant Breast Cancer: Bioinformatics and *In Vitro* Studies

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Abstract

   Objective: The long-term use of trastuzumab (TRZ), a therapeutic agent for human epidermal growth factor receptor 2 (HER2)+ breast cancer subtype (HER2+ BC), induces resistance. Borneol (BOR) exerts anticancer effects on various types of cancer. However, its anticancer effect on HER2+ BC remains unknown. This study aimed to determine the potential target genes of BOR and its effect on overcoming the resistance of HER2+ BC to TRZ. **Methods:** The hub gene of BOR's potential target on HER2+ BC cells was determined via a bioinformatics approach. Resistant HCC1954 cells (HCC1954-TR) were obtained through repeated inducement of HCC1954 cancer cells with TRZ. The cells were then subjected to cytotoxic tests involving single compounds and their combinations. Then, the hub gene expression was determined using quantitative reverse-transcription polymerase chain reaction. The interaction between BOR and selected proteins was measured through molecular docking. **Results:** Hub genes *IL6, TNF, ESR1, IL1B, CYP19A1, AR, NR3C1, RELA, CYP17A1*, and *GPT* were obtained via a bioinformatics approach. HCC1954-TR cells were successfully established. The TRZ–BOR combination treatment of parental HCC1954 (400 μg/mL and 25 μM) and HCC1954-TR (800 μg/mL and 100 μM) yielded considerably better results compared with BOR or TRZ alone. The expressions of *AR, GPT,* and *ESR1* under the TRZ–BOR combination were notably different compared with those under single exposure. The molecular docking study of CYP19A1, CYP17A1, NR3C1, and IL-1β highlighted the potential interaction between BOR and such proteins. **Conclusion:** BOR improved the cytotoxic effects of TRZ on HCC1954 and HCC1954-TR cell lines, where it specifically targets *AR, ESR1*, and *GPT* genes. In addition, the BOR effect, which counteracted the resistance of HCC1954-TR cells to TRZ, was mediated by genes *CYP19A1, CYP17A1, NR3C1, IL-1*, and *RELA*. However, additional research is required to validate their role in BOR activity to circumvent the resistance of HER2+ BC to TRZ.

Keywords: HER2+- Breast Cancer- Trastuzumab-Resistant- Borneol- targeted therapy

Introduction

   Breast cancer (BC) refers to the most diagnosed cancer in women worldwide [1]; it has surpassed lung cancer as the most frequently diagnosed cancer [2]. The human epidermal growth factor receptor 2 (HER2)+ BC subtype (HER2+ BC) shows a poor prognosis, such as high aggressiveness, recurrence, and low survival [3]. Trastuzumab (TRZ), which is used in HER2+ BC therapy, binds to the extracellular domain of HER2 and suppresses the intracellular HER2 signaling pathway [4].

TRZ provides important clinical benefits in the treatment of HER2+ BC. However, some patients suffered from disease recurrence, and metastatic patients showed no response to therapy within 1 year of treatment [5]. TRZ resistance results from to the dysregulation of the phosphatidylinositol-3 kinase (PI3K) pathway, which results in reduced levels of phosphatase and tensin homolog, an enzyme that acts as a tumor suppressor and crucially participates in the dephosphorylation of PIP3 to PIP2 in the PI3K/AKT signaling pathway [6].

   In this study, borneol (BOR) was selected to overcome

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the problem of TRZ resistance. BOR is a bicyclic monoterpene compound [7]. This substance can be developed to overcome the problem of TRZ resistance because of its anticancer activity. The combination treatment of BOR with cisplatin and paclitaxel induced apoptosis in glioma cells [8] and esophageal squamous carcinoma cells [9]. Meanwhile, combination BOR with curcumin and selenocysteine can induce apoptosis in melanoma cells [10] and hepatocellular carcinoma cells [11]. BOR increases the efficacy of doxorubicin in glioma cells [12]. BOR increased the anticancer efficiency of temozolomide in glioma cells [13], the efficacy of cisplatin in glioma cells [8], and the efficacy of paclitaxel in esophageal squamous carcinoma cells [9]. BOR can also reverse the resistance to mitoxantrone [14] and increase the sensitivity of cisplatin with a resistance experience [15]. A recent study revealed the importance of the regulation of receptor tyrosine kinase-RAS-PI3K/AKT by BOR in overcoming BC resistance to tamoxifen through bioinformatics analysis [16]. We speculate that BOR can overcome the resistance of human BC cells to TRZ. To date, no studies have been conducted on TRZ therapy with the addition of BOR in BC. This study aimed to investigate the effect of BOR on the improving the responsiveness of TRZ in HER2+ BC cells. Moreover, it aimed to identify the primary target of BOR in HER2+ BC to overcome TRZ resistance in HER2+ BC.

Materials and Methods

Data Mining

Various databases served as sources of the BOR target genes: SwissTargetPrediction (http://www.swisstargetprediction.ch/) [17], TargetNet (http://targetnet.scbdd.com/) [18], PubChem (https://pubchem.ncbi.nlm.nih.gov/) [19], BindingDB (https://www.bindingdb.org/) [20], and Similarity Ensemble Approach (SEA) (https://sea.bkslab.org/) [21] with SMILES code of BOR. Genes associated with HER2+ BC were acquired other databases using certain keywords: from"HER2-positive carcinoma of breast" from DisGeNET database (http://www.disgenet.org/) [22], "breast cancer" from Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/) [23], with the keyword and "HER2 overexpression breast cancer" from GeneCards (https://www.genecards.org/) [24]. The intersecting genes of BOR and HER2+ BC-related gene clusters were constructed using a Venn diagram via InteractiVenn (http://www.interactivenn.net/) [25].

Functional, disease–gene, and drug–gene association annotations

WebGestalt was used in the analysis of functional, disease–gene, and drug–gene association annotations (http://www.webgestalt.org/) [26]. For functional annotation, symbols of intersecting genes of BOR and HER2+ BC-related gene clusters were submitted to WebGestalt and analyzed using the "over-representation analysis (ORA)" default settings. Disease–gene and drug–gene associations were analyzed using WebGestalt using several selected parameters, such as the following: set "method of interest" to "over-representation," "functional database" to "disease," and "Select a function database name" with "DisGeNet" for the analysis of disease–gene associations. The "functional database" was changed to "drug" and "Select a function database name" to "DrugBank" for drug–gene association analysis. A p-value of less than 0.05 was selected as the selection criterion [26].

Protein–protein interaction (PPI) and hub gene selection

PPI analysis involved the use of STRING-DB v11.5 (https://string-db.org/) [27]. The symbols of intersecting genes of BOR and HER2+ BC-related gene clusters were submitted to STRING database, and several parameters, such as "multiple proteins," and set "organisms" to "*Homo sapiens*" were selected. PPIs obtained from STRING-DB were exported to Cytoscape v3.9.1 (https://cytoscape.org/) to determine the hub gene (top gene) and ranked by degree score using the Cytohubba plugin [28] in Cytoscape.

Genetic Alteration and Prognostic Analysis

cBioportal (https://www.cbioportal.org/) [29] was used to determine genetic alteration and survival of hub genes. Hub genes from previous experiments were submitted to cBioportal via studies related to "BC." OncoPrint was used in viewing genetic alterations [30]. The survival of each hub gene was compared with the data on patients with unaltered genes, and the results were combined in the form of Kaplan–Meier curves using Graphpad Prism [31].

Cell Culture

HCC1954, which comprised HER2+ BC cell lines, was collected from the APS Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada. HCC1954 cells were cultured in Roswell Park Memorial Institute 1640 media (Sigma, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany) with 1% penicillin–streptomycin (USA) and subjected a 5% CO_2 humidified atmosphere at 37 °C until the 70%–80% confluency [32]. HCC1954 TRZ-resistant (HCC1954-TR) cells were collected from the APS Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada and induced with a low concentration of TRZ (Roche, Germany) for 72 h. The media were replaced with a new one and then induced again [33, 34]. This process was repeated to obtain TRZ-resistant HCC1954 cells (HCC1954-TR).

Cytotoxicity test

Parental HCC1954 and HCC1954-TR cells were plated in 96-well plates (3000 cells/well) and subjected to for 24 h incubation [35]. Parental HCC1954 and HCC1954-TR cells were treated with BOR (Sigma, Germany) at concentrations of 10, 20, 40, 80, 160, and 320 μM for 72 h, in accordance with previous studies [7, 36]. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/mL) was added to each well after treatment, and incubation was extended for another 4 h, followed by the addition of 10% sodium dodecyl sulfate in 0.1 N HCl to dissolve the formazan crystal. Absorbance was measured at 570 nm [37], and cell viability was calculated in accordance with a previous study [38].

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

HCC1954-TR cells were grown, incubated, and treated with the combination of 50 μM BOR and 800 μg/mL TRZ for 72 h. RNA was isolated in accordance with the instructions of RNeasy Kits (Qiagen). Total RNA was obtained and used for cDNA synthesis. cDNA synthesis was accomplished following the instructions of SensiFAST cDNA Synthesis Kit (Meridian Bioscience). qRT-PCR was performed in accordance with the instructions of SensiFAST™ SYBR® No-ROX Kit with the synthesized cDNA as the sample [39] and using the selected primers (Supplementary Table 1). Gene expression measurement accordance with the comparative threshold cycle (ΔΔCT) method. Statistical analysis of the combination treatments included one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. The significance levels are as follows: $*p < 0.05$, $***p < 0.001$.

Molecular Docking

The three-dimensional (3D) structures of cytochrome P450 family 19 subfamily (CYP19A1) (PDB ID: 3S7S), cytochrome the P450 family 17 subfamily A member 1 (CYP17A1) (PDB ID: 6CHI), RelA (PDB ID: 1NFI), NR3C1 (PDB ID: 7PRX), and interleukin (IL)-1β (PDB ID: 5R8O) were downloaded from the RCSB protein database (https://www.rcsb.org/). Docking protocol was performed in accordance with a previous study [40, 41] and using the Molecular Operating Environment v2020. Briefly, proteins were prepared with "Protonate 3D" at 310 K and pH 7.4, and the forcefield was assigned under default settings. BOR, the test ligand, was also prepared using the MOPAC system with default settings. Each protein was redocked with its corresponding native ligand, and the root mean square deviation (RMSD) was calculated. The RMSD <2.0 Å corresponds to valid docking protocol and was thus applied to dock all proteins with BOR. The docking results were analyzed based on the best pose and score. The 2D and 3D interactions were visualized in accordance with previous studies [42, 43].

Results

Data Mining

A total of 187, 30, 5, 18, and 7 BOR genes were secured from various databases, namely, TargetNet, SwissTargetPrediction, SEA, PubChem, and BindingDB, respectively (Supplementary Table 2). In addition, 288, 22, and 2629 HER2+ BC-related genes were obtained from DisGeNET, OMIM, and GeneCards, respectively (Supplementary Table 3). Next, the removal of duplicated genes yielded 224 BOR genes and 2682 HER2+ BC-related genes (Supplementary Tables 2 and 3, respectively). A total of 43 genes intersected, which indicates the association of BOR genes with HER2+ BC (BGBC; Figure 1A, Supplementary Table 4).

Functional, disease–gene, and drug–gene associations

Gene Ontology (GO) revealed the role of BGBC in biological process (BP), cellular component (CC), and molecular function (MF). Under BP, 41 genes played a role in metabolic processes and 38 genes in biological regulation. CC comprised 28 and 22 genes whose products exist in the membrane and cytosol, respectively. Finally, MF included 35 and 27 genes for protein and ion binding, respectively (Figure 1B). Disease–gene association analysis showed the significant association of BGBC (false discovery rate (FDR) < 0.05) with mammary neoplasms, prostatic intraepithelial neoplasia, prostate cancer, adenocarcinoma, and hyperalgesia (Figure 1C). Drug–gene association analysis revealed the significant association of BGBC was (FDR < 0.05) with several drugs, such as binimetinib (a mitogen-activated protein kinase 1/2 inhibitor), tamoxifen (selective estrogen receptor (ER) modulators), stanolone, gestrinone, and levonogestrel (Figure 1D).

Protein–protein interaction (PPI) and hub gene selection

PPI was searched using STRING database, and 43 nodes with 176 edges were obtained, with an average node degree of 8.19, an average local clustering coefficient of 0.579, and a PPI enrichment p-value of <1.0e-16 (Figure 1E). The hub gene selection revealed the following top 10 genes: *IL6, TNF, ESR1, IL1B, CYP19A1, AR, NR3C1, RELA, CYP17A1,* and *GPT* (Figure 1F, Supplementary Table 5).

Genetic alterations and survival analysis

A study named Metastatic BC (INSERM, Plos Med 2016) was selected from cBioportal (Figure 1G). The selection was based on because TRZ resistance, results in the metastasis of BC. OncoPrint, which provides a graphical summary of genomic alterations [44], revealed changes in *ESR1* (7%), *AR* (1.5%), *NR3C1* (1.1%), *TNF* (2.1%), *IL6* (2%), *IL1B* (0.8%), *RELA* (2.9%), *GPT* (16%), *CYP19A1* (1.4%), and *CYP17A1* (1%) in BC patients enrolled in the selected study (INSERM, Plos Med 2016) (Figure 1H). The survival curves of 5698 BC patients in TCGA [45] were presented as Kaplan–Meier curves of the hub gene compared with the data of patients with unaltered genes (Figure 1I). Patients with BC with altered genes in the *ESR1, GPT,* and *IL6* groups significantly differed from those without alterations (p < 0.05). This finding indicates the poor survival of those genes in patients with BC from the TCGA study.

Cytotoxicity assay

Bioinformatics findings were validated through *in vitro* methods, starting with the cytotoxicity assay. The cytotoxicity of TRZ in HCC1954 parental and HCC1954-TR cells were initially checked, and statistically significant variations (p < 0.05) were observed across all concentrations of TRZ except at 40 μg/mL (Figure 2A). Thus, HCC1954 cells with TRZ resistance were successfully generated. A single treatment of BOR was applied to parental HCC1954 and HCC1954-TR cells. The subjects were exposed to various doses of BOR from 10 μM to 320 μM (Figure 2B). The half maximal inhibitory concentration (IC_{50}) of BOR against the parental HCC1954 cell line was 130 μM, and that against HCC1954-TR was above 320 μM.
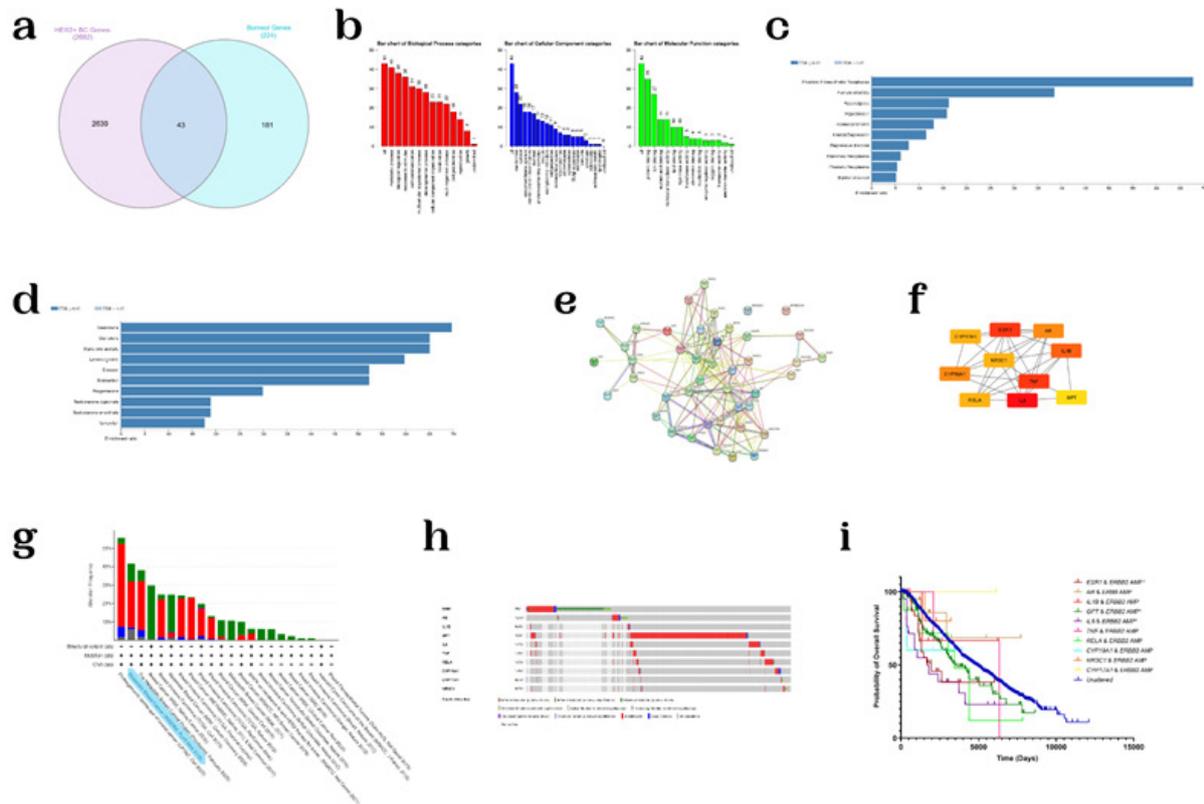
Figure 1. *In silico* Test via a bioinformatics approach. (A) Gene slice showing the BOR gene targets and gene associated with HER2+ BC (BGBC). (B) GO, (C) gene–disease association analysis, and (D) gene-drug association analysis of BGBC, as analyzed using the ORA of WebGestalt. (E) Protein–protein interaction of BGBC, as analyzed by STRING. (F) Top ten hub genes, as analyzed by the CytoHubba plugin of Cytoscape based on degree score. (G) Breast cancer studies found in cBioportal server. (H) OncoPrint analysis of genetic alterations of hub genes using the Metastatic Breast Cancer (INSERM, Plos Med 2016) study in cBioportal server. (I) Survival curve related to genetic alterations of hub genes determined using the TCGA study in cBioportal server. The results are shown as a Kaplan–Meier curves generated using GraphPad Prism 9, with * indicating statistical significance with a p-value of less than 0.05.
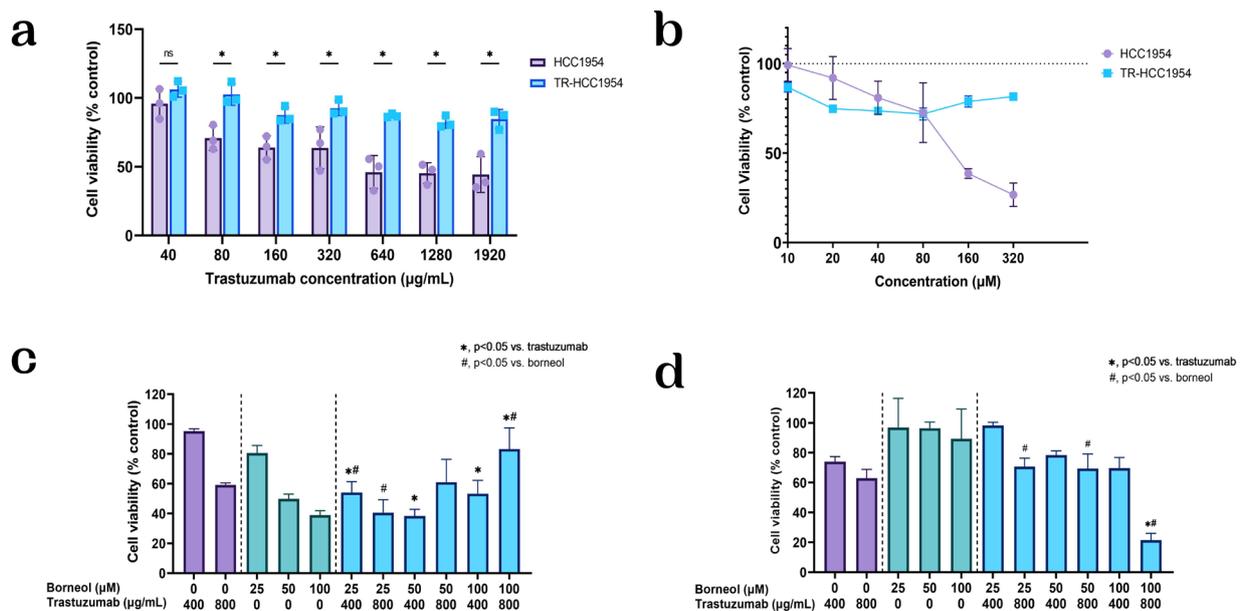

Figure 2. (A) Cytotoxicity of TRZ toward HCC1954 and HCC1954-TR cells in the MTT Assay. (B) BOR cytotoxicity toward parental HCC1954 and HCC1954-TR cells. Cytotoxicity of combined TRZ–BOR treatment toward (C) parental HCC1954 or (D) HCC1954-TR cells. Cells were treated with TRZ or BOR for 72 h. Results are displayed as the mean percentage and standard deviation (SD) of cell viability for each concentration group. (n = 3, statistical analysis using one-way ANOVA with Tukey's multiple comparison; significant: $p < 0.05$).

Table 1. Docking Scores of Native Ligands and BOR

Protein (PDB ID)	RMSD	Native Ligand	Interacted Amino Acid	BOR	Interacted Amino Acid
CYP19A1 (3S7S)	0.2487	−9.8590	MetA374 MetB374	−6.4238	MetA374 MetB374
CYP17A1 (6CHI)	0.3178	−10.9010	AsnB202 AsnA202 ArgB239 ArgA239	−5.8945	ValA482 ValB482
RELA (1NFI)	-	-		−4.7940	GlnB220
NR3C1/GR (7PRX)	1.5807	−12.5608	GlnB570 GlnA570 PheB623 MetA604 GlnB642 GlnA542 AsnA564 AsnB584	−4.7125	GlnB570 GlnA570
IL-1β (5R8O)	0.373	−6.3107	GluB25 GluA25 TyrA24 TyrB24 LeuB26 LeuA26	−4.0235	TyrA24 TyrB24

After determining the effect of BOR treatment alone, a combination test was performed to determine the capability of the compound to kill parental HCC1954 cells in combination with TRZ, and the results revealed a significant difference ($p < 0.05$) when using 25 μM BOR and 400 μg/mL TRZ compared with TRZ (*) or BOR (#) alone but not when using BOR 50 μM and TRZ 400 μg/mL (Figure 2C). The combination test was also
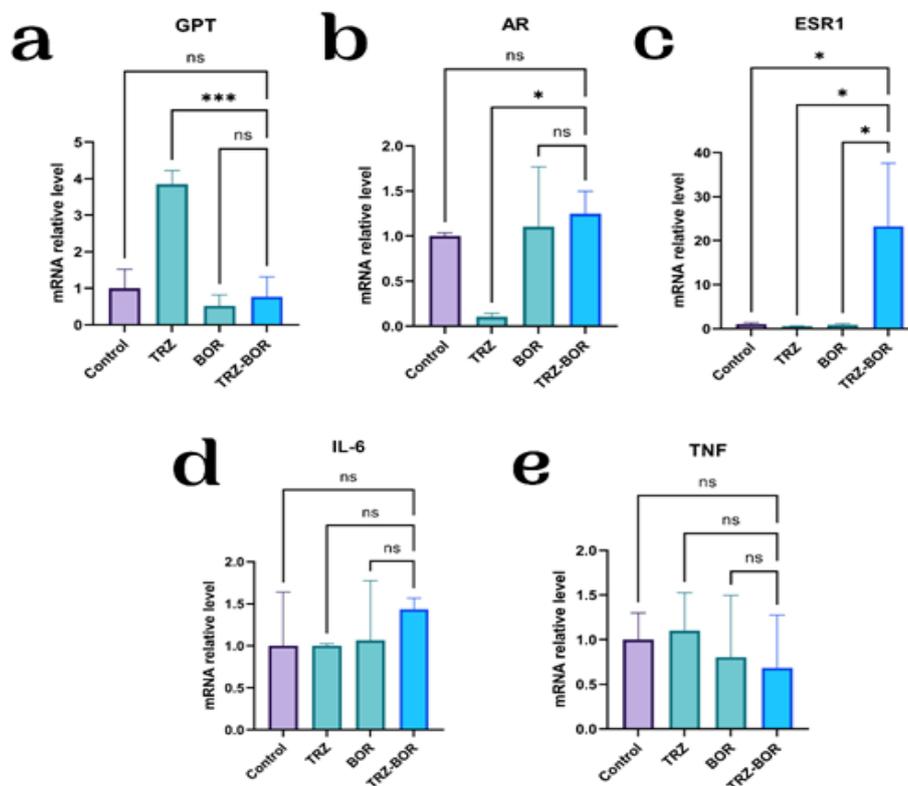

Figure 3. Effects of TRZ, BOR, and their combination on the mRNA Levels of (A) *GPT*, (B) *AR*, (C) *ESR1*, (D) *IL-6*, and (E) *TNFA*. Gene expression was quantified via q-RT PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control in this study. The data were evaluated via a ΔΔCT method and presented as a fold change compared with the control. The results are presented as the arithmetic mean ± SD of three separate and distinct experiments. (n = 3; statistical analysis was performed via one-way ANOVA with Tukey's multiple comparison test; *$p < 0.05$; ***$p < 0.001$; ns = not significant).
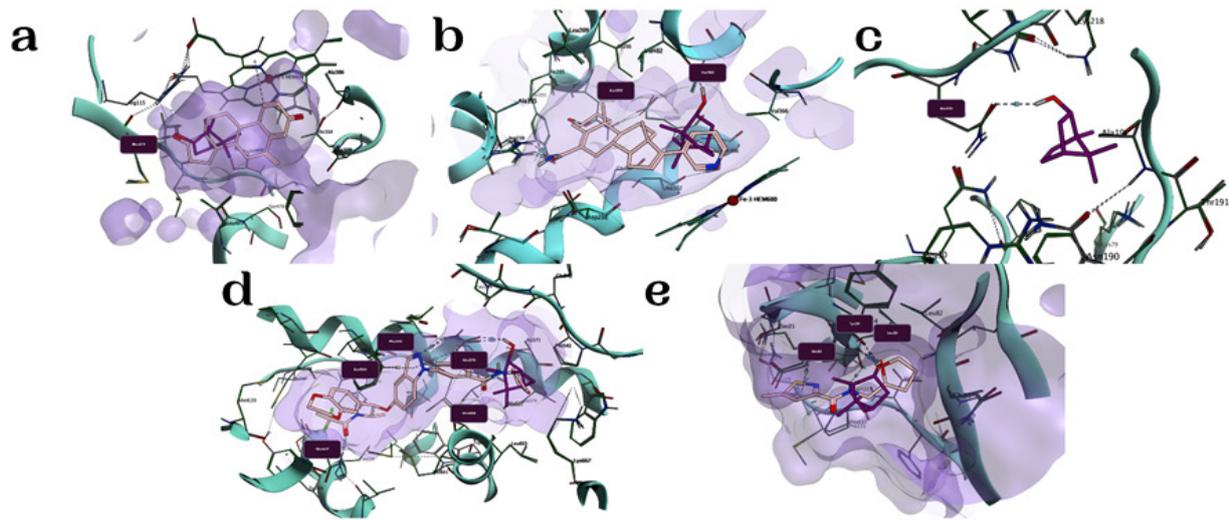
Figure 4. 3D Visualization of the (a) native ligand and BOR interaction with CYP19A1, (b) native ligand and BOR interaction with CYP17A1 (c) BOR interaction with RelA/p65, (d) native ligand and BOR interaction with NR3C1/ GR and (e) native ligand and BOR interaction with IL-1β. (light pink: 3D native ligand; purple: 3D BOR)

performed on HCC1954-TR cells (Figure 2D), with 25 or 50 µM BOR and 800 µg/mL TRZ exhibiting significant differences compared with TRZ alone (#). Moreover, the combination with the highest concentration, namely, 100 µM BOR and 800 µg/mL TRZ, effectively eradicated HCC1954-TR cells.

qRT-PCR

To verify the findings of bioinformatics analysis, we conducted a comprehensive test and assessed the expression of the central gene via quantitative qRT-PCR. *GPT, AR*, and *ESR1* under combined BOR and TRZ treatment exhibited significant mRNA expression differences ($p < 0.05$) (Figure 3A–C) compared with those treated with TRZ alone. These results indicate the importance of *ESR, AR*, and *GPT* in the effect of BOR on overcoming the resistance of BC cell toward TRZ. *IL6* and *TNFA* gene expressions under combined TRZ–BOR treatment showed no significant difference ($p > 0.05$) compared with those under TRZ treatment alone (Figures 3D–3E).

Molecular Docking

Molecular docking was performed to study the interaction between BOR and CYP19A1 (PDB ID: 3S7S), CYP17A1 (PDB ID: 6CHI), RelA (PDB ID: 1NFI),
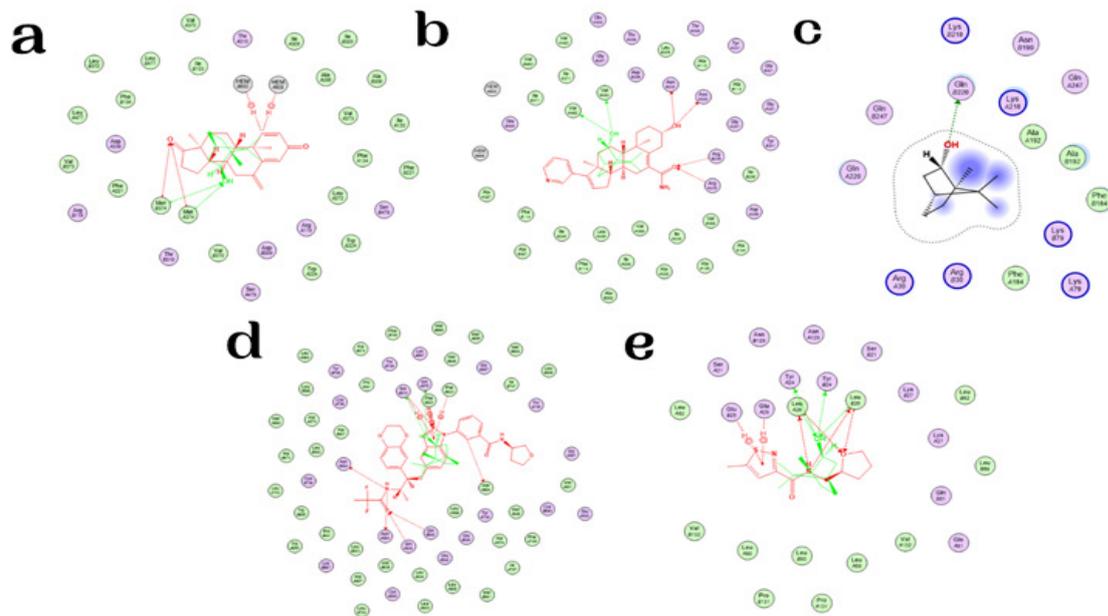

Figure 5. 2D Visualization of (a) native ligand and BOR interaction with CYP19A1, (b) native ligand and BOR interaction with CYP17A1, (c) BOR interaction with RelA/p65, (d) native ligand and BOR interaction with NR3C1/ GR, (e) native ligand and BOR interaction with IL-1β. (red: 2D native ligand; green: 2D BOR).
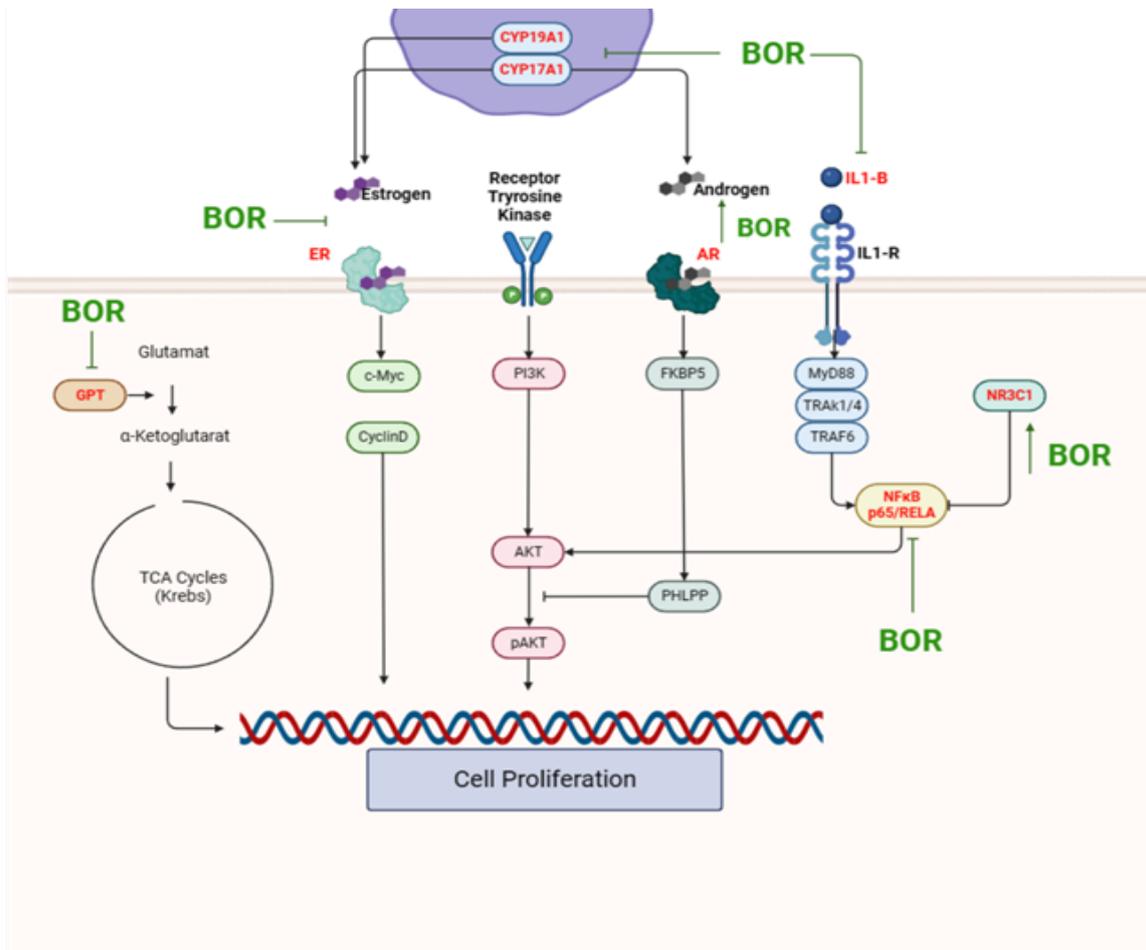
Figure 6. Proposed Mechanism of BOR in Overcoming BC Resistance to TRZ.

NR3C1 (PDB ID: 7PRX), and IL-1β (PDB ID: 5R8O). These proteins were part of the results obtained for the hub genes. The redocking findings on four proteins, namely, CYP19A1, CYP17A1, NR3Cl, and IL-1, and their respective ligands showed RMSD values of 0.2487, 0.3178, 1.5807, and 0.3730, respectively (Figure 4A-E Table 1). An RMSD less than 2.0 Å indicates the feasibility of the docking protocol [46]. BOR exhibited a consistently greater docking score than the native ligands (Table 1). Moreover, fewer amino acids interacted with BOR than native ligands (Figures 5A–E). The key residues Met 374, Arg 239, Gln570, and Tyr-24 were displayed after docking BOR with CYP19A1, CYP17A1, NR3C, and l IL-1β (Figures 5A–E, Table 1).

Discussion

This study aimed to determine the effect of BOR on the increased sensitivity of TRZ and its potential target genes in overcoming the resistance of HER2+ BC toward TRZ. The results of this study demonstrate the top 10 hub genes, including *IL6, TNF, ESR1, IL1B, CYP19A1, AR, NR3C1, RELA, CYP17A1,* and *GPT. IL6, TNF, ESR1,* and *IL1B*, which encode *IL-6*, tumor necrosis factor (*TNF*), ER-α, and *IL-β1*, respectively. *IL-1β* induces the expression of hypoxia-inducible factor 1 (HIF-1α) or nuclear factor (NF)-κB, which are major hypoxia-

sensitive pathways that participate in tumor progression by promoting angiogenesis, cell migration, and invasiveness of cancer cells [47]. Glutamate-pyruvate transaminase 1 (GPT) (also called ALT1 or ALAT1) contributes to glutamine catabolism in tumor cells and the generation of reactive oxygen species necessary for Kras-induced anchorage-independent growth through regulation of the signal-regulated kinase/mitogen-activated protein kinase signaling pathway [48]. *NR3C1* encodes the glucocorticoid receptor (GR), which can act as a tumor suppressor in breast tissue [49].

The gene *CYP19A1* encodes CYP19A1, an enzyme that belongs to the cytochrome P450 monooxygenase family and contributes to the conversion of C19 androgens, specifically androst-4-ene-3,17-dione (androstenedione) and testosterone, into C18 estrogens, namely, estrone and estradiol, respectively [50]. *AR* encodes the androgen receptor (AR), which is a ligand-activated transcription component that regulates gene expression in eukaryotic cells and takes part in the control of cellular proliferation and differentiation in specific tissues [51]. *RELA* encodes the RELA proto-oncogene, an NF-kB subunit and a family of transcription factors that is activated and enters the nucleus during cell stimulation involving extracellular signals, such as TNFα and IL1 [52]. *CYP17A1* encodes CYP17A1, an enzyme that performs a crucial function in the steroidogenic pathway via 17-alpha-hydroxylase

and 17,20-lyase activities [53]. CYP17A1 accounts for the production of progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens [53].

The cytotoxicity of TRZ against parental HCC1954 cells compared with HCC1954-TR cells showed significant differences (p < 0.05) at all concentrations, except 40 µg/mL. Exposure to various concentrations of TRZ yielded significant differences. In addition, an extremely high IC_{50} indicates that TRZ induction produced TRZ-resistant HCC1954 cells (HCC1954-TR). HCC1954 exhibited resistance to TRZ as a result of PIK3CA mutations [54].

Parental HCC1954 and HCC1954-TR cells also received single BOR treatment, which influenced parental HCC1954 cells with an IC_{50} of 130 µM. The treatment with an unquantified IC_{50} value was unable to kill HCC1954-TR cells (more than 320 µM). A significant difference was observed with the combination effect on parental HCC1954 cells (p < 0.05), in particular when 25 µM BOR/400 µg/mL TRZ and 100 µM BOR/800 µg/mL TRZ were used. Single BOR caused no significant effect on C6 glioma cells compared with the control at concentrations of 12.5–100 µg/mL [55], on A549 lung cancer cells at concentrations of 0–160 µg/mL [56] and on glioblastoma U251 cells at concentrations of 0–80 µM [13]. However, BOR showed a better inhibitory effect on C6 glioma cells when combined with 2.5–40 µM piperlongumine [55], on A549 lung cancer cells when combined with 62.5–250 µM doxorubicin [56], and on U251 glioblastoma cells when combined with 20–40 µM temozolomide [13].

Glutaminolysis provide cancer cells with energy [57, 58]. GPT/ALT1 converts glutamate (from glutamine) to α-ketoglutarate, which then enters the tricarboxylic acid/Krebs and produces energy to facilitate tumor growth [59]. The high expression of GPT is directly proportional to cancer cell survival and growth [60]. The results of this study revealed significantly higher GPT expression in cells treated with TRZ alone under compared with the TRZ–BOR combination treatment. This finding indicates the more effective treatment effect of TRZ–BOR exposure than TRZ alone. Other studies also mentioned the high expression of other glutaminolysis agents in BC [61]. However, to date, no research related to GPT expression in BC and relationship with BOR compounds has been conducted, which will be an interesting topic for future studies.

ERα, which is encoded by *ESR1*, is an alternative pathway to the HER2 pathway for BC cell growth and survival [62]. HER2 blockade in HER2+ BC upregulates or reactivates a ER-regulated gene transcription, called a compensatory mechanism [63]. The results of this study revealed the significantly higher *ESR1* expression under TRZ–BOR exposure than that treated with TRZ or BOR alone. This finding indicates the capability of TRZ–BOR to suppress HER2 better than TRZ alone. Currently, studies on the effect of BOR on ER signaling are lacking, which necessitate further studies in the future.

AR signaling pathways are in crosstalk with PI3K/AKT [64]. The PI3K/AKT signaling pathway serves as a contributor to cancer growth, cell proliferation,

and survival [65]. Another study showed activated AR signaling due to PI3K inhibition in prostate cancer [64]. The results of this work indicate a significantly increased *AR* expression (*p < 0.05) during exposure to TRZ–BOR compared with single exposure. We speculated that BOR exerts a synergistic effect on TRZ by inhibiting the PI3K/AKT pathway. However, in this study, the expressions of *IL6* and *TNF* genes in the TRZ–BOR treatment showed no significant differences compared with those exposed to TRZ or BOR alone. Further studies are needed to determine the protein levels of IL6, AR, and TNFα and PI3K/AKT regulatory proteins in cells treated with the combination of BOR and TRZ.

The crystal structure of CYP19A1 used in this study bound to the ligand EXM (exemestane; aromasin) as an inhibitor. EXM is an aromatase inhibitor and applied in BC therapy [66]. The results of docking study demonstrate the hydrogen bonds formed by the carbonyl group of EXM and hydroxyl group of BOR with the same amino acid residue (Met374) on CYP19A1. However, BOR exhibited various interactions with abiraterone, a CYP17A1 inhibitor [67]. The interaction of the hydroxyl group of BOR with Val482 transpired through hydrogen bond formation. Meanwhile, the carbonyl and hydroxyl groups of abiraterone showed interaction with Asn202 and Arg 239. The NF-κB transcription factor RelA/p65 shows activity in the canonical NF-κB pathway, which is responsible for survival, proliferation, tumor development, and inflammation. Therefore, cancer drug development can target the selective inhibition of the canonical NF-κB pathway [68]. Another study revealed the capability of BOR to reduce p65 levels. This finding correlates with the inhibition of the NF-κB pathway, which can suppress neuroinflammation in mice [69].

The results of docking study indicate that the hydroxyl group of BOR bound to Gln220 through hydrogen binding. NR3C1/GR performs two roles during activation, namely, transactivation and transrepression. A transrepression mechanism enables GR to inhibit transcription factors [70]. The capability of GR to inhibit transcription factors (proinflammatory) is applied in the therapy of several cancers, such as lymphoid cancer, leukemia, and prostate cancer [71]. Velsecorat, as the native ligand of the GR crystal structure, provides selective support to the role of GR in the transrepression pathway [72]. Although more amino acid residues bound to velsecorat, the hydroxyl group of BOR showed interaction with same amino acid residue, Gln570. IL-1β alleviates the aggressive nature of BC through the increased levels of NF-kβ. In addition, IL-1β plays contributes to the initiation of metastasis and promotion of BC metastasis in bones [73]. BOR can also inhibit IL-1β signaling [74]. S8P, the native ligand of IL-1β, interacts with three different amino acid residues. Among these residues, Tyr24 bound to the hydroxyl group of BOR via hydrogen binding. Overall, the docking results demonstrate that BOR occupied all protein binding sites with similar orientations. In addition, the hydroxyl group of BOR presented a predominant interaction with amino acid residues. Therefore, expectedly BOR exhibits a similar bioactivity. BOR achieved lower docking scores. However, native ligands are not endogenous ligands.

Thus, we assumed that BOR can still bind to the proteins regardless of the negative scores.

We proposed the mechanism of genes involved in the action of BOR when overcoming the TRZ resistance of HER2+ BC (Figure 6). The results of this study unveiled the involvement of eight genes, namely, *AR, ESR1, GPT, CYP19A1, CYP17A1, NR3C1, IL1B,* and *RELA*. A reciprocal mechanism regulates the AR and PI3K pathways, given that the inhibition of one inactivates the other, which allows cancer cell survival and progression. The inactivation of AR leads to the overregulation of PI3K/AKT. AR inhibition reduces FKBP5 levels and impairs the function of PHLPP [75], which dephosphorylates and inactivates AKT [76]. CYP19A1 contributes to estrogen synthesis. The ligand-dependent transcription factor ERα regulates the expressions of various genes, such as Bcl-2, which can act as a barrier to apoptosis and facilitate tumor progression and resistance to cancer therapy [62]. BOR can also downregulate Bcl-2 expression [15]. IL-1β activates various proinflammatory pathways, including that of NF-κB. IL-1β induces the expression of HIF-1α and NF-κB, which are major hypoxia-sensitive pathways and participate in tumor progression by promoting angiogenesis, cell migration, and invasiveness [47]. In addition to inhibiting IL-1β signaling [15], BOR may support transrepression work on GR/NR3C1, which can suppress proinflammatory transcription factors, such as NF-κB. BOR can suppress GPT given the greatly decreased gene expression during exposure to combined TRZ–BOR treatment compared with that during exposure to TRZ alone. However, this mechanism needs to be further investigated.

In conclusion, the combination of 100 μM BOR and 800 μg/mL TRZ provided significant results on HCC1954-TR and showed that BOR exerted a synergistic effect that increased the TRZ sensitivity of HCC1954-TR cells. In addition, this study revealed the ten genes that are most likely to be the target hub genes of BOR: *GPT, IL6, IL1B, ESR1, AR, NR3C1, TNF, RELA, CYP19A1,* and *CYP17A1*. Among the hub genes, *AR, ESR1,* and *GPT* are most likely to play significant roles in the BOR's effect on overcoming TRZ resistance. CYP19A1, CYP17A1, NR3C1, IL-1β, and RELA genes may be possibly involved, but further *in vitro* and *in vivo* research are needed to confirm their role in BOR's activity in overcoming HER2+ BC resistance toward TRZ.

Author Contribution Statement

A portion of the data, incorporating notable enhancements, was utilized for the undergraduate thesis of IAL. IAL: performed the experiments, analyzed, interpreted, and visualized data, and wrote the original draft of the paper. IMRP and NF: performed experiments and analyzed data. NSOU: analyzed data, provided supervision, and edited the paper. DDPP: contributed reagents and materials, analyzed data, and provided supervision. AH: conceptualized and designed the study, provided supervision, secured funding acquisition, analyzed data, and participated in writing (reviewing and editing) the paper.

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Availability of data and materials

All data produced by this study are disclosed in the manuscript. Supplementary files are available in https://ugm.id/RTABOR

Compliance with ethical standards

This study was approved by the Medical and health Research Ethics Committee (MHREC) of Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, and Dr. Sardjito General Hospital (Ref. No. KE/FK/1364/EC/2023).

Competing interests

The authors declare no competing interests.

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