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Original Article

BRAZILEIN INCREASES THE SENSITIVITY OF DOXORUBICIN ON MCF-7 RESISTANT DOXORUBICIN (MCF-7/DOX) CELLS THROUGH INHIBITION OF HER-2 ACTIVATION

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ABSTRACT

Objective: Brazilein is a compound obtained in a large amount from the dried heartwood of Secang (*Caesalpinia sappan* L.). Brazilein has strong cytotoxic effect in several cancer cell lines. This study was designed to evaluate the cytotoxic effect of combination brazilein with a chemotherapy agent, doxorubicin on MCF-7/DOX breast cancer cell lines and evaluate the molecular mechanism of brazilein by in silico using molecular docking to HER-2.

Methods: In the cytotoxicity assay, MCF-7/DOX cells were cultured in the presence of brazilein and doxorubicin for 24 hours and cell viability was evaluated by using MTT assay. Interactions between brazilein and the target protein, HER-2 (3PP0) was evaluated and calculated *in silico* by molecular docking using PLANTS.

Results: Brazilein increased doxorubicin's cytotoxic activity on MCF-7/DOX cells. Both of single treatment with different concentration brazilein 12.5 and 25 μM or doxorubicin 0.8 and 1 μM gave cell viability percentage above 80%, but combination of them led to decrease the cell viability percentage significantly. The score docking of brazilein was -77.73 kcal/mol and lapatinib value -71.34 kcal/mol.

Conclusion: Interaction between brazilein and HER-2 (3PP0) as protein target in breast cancer higher affinity than lapatinib as HER-2 drug. Brazilein performed as a potent co-chemotherapy agent for breast cancer treatment.

Keywords: Brazilein, Doxorubicin, Cytotoxic, HER-2 (3PP0), MCF-7/DOX (Resistant MCF-7 Cells to Doxorubicin).

INTRODUCTION

Long term use of doxorubicin cause severe side effect such as toxic for normal cells and cancer resistance [1-5]. Breast cancer cell resistance to chemotherapeutic agents was caused by various factors, but predominantly due to increase of expression of Multi Drug Resistance 1 (MDR1) gene, the gene encoding P-glycoprotein (Pgp) after administration of doxorubicin [6]. One of the proteins that regulate expression of P-glycoprotein is HER-2 through NF-κB activation [7]. It is most desirable to have more effective new drug by finding co-chemotherapy drugs. Co-chemotherapy is a cancer therapy strategy, combine natural agent with chemotherapy drugs. This strategy can reduce the side effect, toxicity of drugs and increase the effectiveness of doxorubicin [1].

There are a lot of medicinal plants potentially to be used as co-chemotherapy agent. One of these plants is secang (*Caesalpinia sappan*). Secang has been traditionally used to be colorant agent in beverage and foods. Besides its function as colorant agent, secang have a lot pharmacological effects especially in cancer [8].

Phenolic compound are isolated from *Caesalpinia sappan* such as homoisoflavonoid protosappanin A, protosappanin B, 4-O-methylsappanol, caesalpin J, brazilin and brazilein [9]. Brazilein are the major compound of this plant proven responsible for the cytotoxic effect in cancer [10-13]. Brazilein have cytotoxicity effect in lung cancer, nasopharyngeal cancer and prostate cancer with IC_{50} 5-18 μM [12]. Brazilein inhibits survivin protein and induces apoptosis in hepatocellular carcinoma (HepG2 cell) [13].

This study was designed to evaluate the cytotoxic effect of combination brazilein with a chemotherapy agent, doxorubicin on MCF-7/DOX breast cancer cell lines by using MTT assay and evaluate the molecular mechanism of brazilein as co-chemotherapy agent by in silico using molecular docking to HER-2. Molecular docking is one of the approaches study to understand the molecular mechanism of anticancer associated with its docking protein and represent its potential biological activity [14;15]. This study will provide scientific data as the basic for brazilein, as co-chemotherapy agent to solve the problem of chemotherapy agent used related to it's toxicity and resistance effect.

MATERIALS AND METHODS

Sample

Brazilein was isolated from secang (*C. sappan*). Secang was obtained in the form of dried heartwood powder from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (BBPPTOOT) in Tawangmangu, Indonesia.

Cell culture

The MCF-7/DOX cells was breast cancer cell line resistant to agent chemotherapy doxorubicin that characterized by overexpression of HER-2 and Pgp. MCF-7/DOX was obtained from Cancer Chemoprevention Research Center (CCRC) Universitas Gadjah Mada, Yogyakarta. The cells were routinely cultured in MEM supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, USA) at 37°C in a 5% CO_2 atmosphere, 1% penicillin-streptomycin, and 0.5 % fungizone. Subcultures were obtained after treatment with 0.05% trypsin (Gibco, Auckland) in phosphate buffer saline (PBS).

Cell viability assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Exponentially growing cells were seeded on 96-well plates at 1×10^4 cells per well and incubated for 24 h prior to addition of drugs. Test compounds were initially dissolved in DMSO or H_2O to make stock solution and then diluted with medium. Following a 24 h incubation at 37 °C, 5% CO_2, 100 μL of various concentrations of brazilein were added in each well in triplicates and cells were further incubated for 24 h. After 24 hours of incubation at 37 °C, the medium was removed, and 100 μL of MTT reagent (1 mg/mL) in medium was added to each well. The plates were incubated at 37 °C for 4 h. At the end of the incubation period, the supernatant was removed, 10 % SDS 0.01N HCL (100 μL) was added to each well, and plates were shaken gently for 15 min. After an overnight incubation at 37 °C, the metabolized MTT product dissolved was quantified by reading the

absorbance at λ 595 nm using an ELISA reader (Bio-Rad). Absorbance was then calculated in order to get the number of viable cells. To determine cell viability, percentage of cell viability was calculated as [(absorbance of drug-treated) sample/(control absorbance)] × 100%. The IC_{50} value is defined as the drug concentration required to inhibit cells growth by 50% of the control value.

Molecular docking

Molecular docking was performed to get the drug-receptor binding energy. The PDB files obtained from Protein Data Bank (PDB) (www. rcsb. org) is a worldwide repository for processing and distribution of 3D biological macromolecular structure data [16]. The structure of HER-2 (3PP0) proteins were downloaded from PDB. The software used were as follows: PLANTS for molecular docking, YASARA for protein preparation, and MarvinSketch for ligand preparation. The downloaded PDB file of HER-2 were first read in YASARA, added waters removed, and polar hydrogens were added. Structure of brazilein and ATP were drawn by using Marvin Sketch. Next running docking protein target and ligand in file PLANTS configuration then the ligand's binding energy to target protein score was compared to native ligand's.

RESULT

Brazilein increased doxorubicin's cytotoxicity on MCF-7/DOX cells

In order to assess the increasing cytotoxic activity of doxorubicin by using brazilein as co-chemotherapy agent in MCF-7/DOX cells, the combinational cytotoxicity assay was conducted by using MTT assay. ANOVA test ($P > 0.05$) showed that the cytotoxic effect between brazilein and doxorubicin alone and in combination had significant difference. MTT assay result showed that brazilein increased the cytotoxic activity of doxorubicin on MCF-7/DOX cells (fig. 1) by inducing cell death (fig. 2).

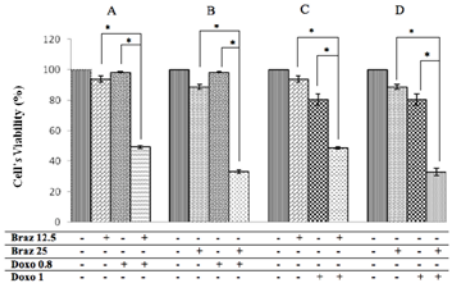

Fig. 1: Effect of Combination Treatment of Brazilein with Doxorubicin on the Cell's Viability of MCF-7/DOX Cells. MCF-7/DOX cells (1×10⁴ cells/well) were seeded on 96 wells plate. The cells were treated with various concentration of brazilein and Dox solley and in combination for 24 h and evaluate by MTT assay. Combinational treatment yielded less cell viability compared to single treatment (p 0.05)

(A) Combinational treatment of brazilein 12.5 μM and Doxorubicin 0.8 μM,
(B) Combinational treatment of brazilein 25 μM and Doxorubicin 0.8 μM,
(C) Combinational treatment of brazilein 12.5 μM and Doxorubicin 1 μM,
(D) Combinational treatment of brazilein 25 μM and Doxorubicin 1 μM

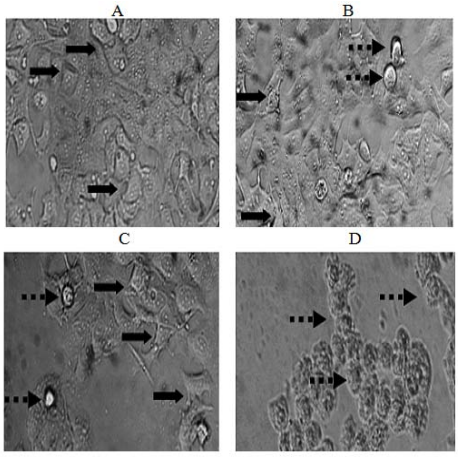

Fig. 2: MCF-7/DOX cells morphology after 24 hours brazilein treatment, control cell (A); brazilein 25 μM (B); Doxo 1 μM (C); Brazilein 25 μM + Doxo 1 μM (D) ➞ normal cell ■▶ morphologymorphology cell changing

Brazilein potentially inhibits the protein target HER-2

Molecular docking was performed to elucidate the molecular mechanism underlying doxorubicin's cytotoxic increased [14] by seeing the drug-receptor binding energy. Score docking shows the bond strength between the active compound and the target protein. Lower score docking show a stronger and more stable bond. When the bond grows stronger, the affinity between the compounds and the target protein increases. Higher its affinity will be caused higher its biological activity [17]. Validation result from HER-2 protein showed molecular docking protocol for that protein could be accepted with RMSD value < 2 Å (table 1) and molecular docking brazilein to HER-2 could be continued.

Many chemotherapy agents induces overexpression of HER-2. HER-2 is a key molecule in the regulation of apoptosis in breast cancer cells [7;18]. HER-2 inhibiton by brazilein downregulates the downstream proteins, such as survivin and Pgp, that have been proven to be responsible of cancer resistance. Based on previous studies, Lapatinib as chemotherapy agent was proven to be able to overcome trastuzumab resistance by binding to HER-2's ATP binding site, giving docking score value as much as -71.34, while the docking score of brazilein was -77.73 (Table 1). ATP as the native ligand has binding energy with HER-2 as much as -89.72 (Table 1). These data inform us that brazilein has higher affinity and more stable binding to HER-2 compared to Lapatinib. It means, brazilein might act as HER-2 inhibitor.

Table 1: Docking score obtained after docking of brazilein to HER-2

	Score Docking
	HER-2 (3PP0)
RMSD (Root Mean Square Deviation)	1.19 Å
ATP	-89.72
Brazilein	-77.73
Lapatinib	-71.34

Table 2: Interaction brazilein and ATP to HER-2 protein with amino acid residue in HER-2

Target proteins	Compound	Amino acid residue	Bonding formation	Bonding distance
HER-2 (3PP0)	ATP	Glu 43	Hydrogen Bond	2.53
		Tyr 91	Hydrogen Bond	1.54
		Thr 94	Hydrogen Bond	2.60
		Ser 44	Hydrogen Bond	2.31
		Asn 135	Hydrogen Bond	2.65
	Brazilein	Tyr 91	Hydrophobic Bond	1.65
	Lapatinib	Thr 94	Hydrogen Bond	1.72
		Asn 135	Hydrogen Bond	2.34

Molecular docking result performed there was a hydrophobic bonding between cyclic C_{11} atom in brazilein and benzene ring in amino acid residue Try 91. Lapatinib as chemotherapy agent that be proven as HER-2 inhibitor had a hydrogen bonding with amino acid residue Thr 94 and Asn 135. ATP interacted with HER-2 on ATP binding site using hydrogen bonding (Table 2). Interaction between brazilein and HER-2 or ATP and HER-2 as a native ligand showed in fig. 3.

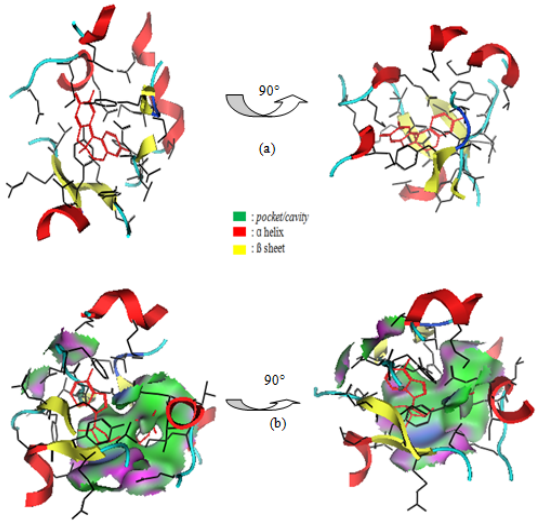

Fig. 3: Docking representations of brazilein and ATP interaction in ATP binding site to HER-2 target proteins using PLANTS. The 3D interaction between brazilein and ATP binding site of HER-2 (3PP0) (a); Interaction between ATP and ATP binding site of HER-2 (3PP0)

DISCUSSION

Brazilein demonstrated strong efficacy as co-chemotherapeutic agent when combined with doxorubicin. Lower doses of doxorubicin being used in combination with brazilein gave cytotoxic activity as potent as the doses used in single cytotoxicity assay. Different mechanism of doxorubicin and brazilein contributes to the combinational cytotoxic effect of both of them. Doxorubicin interacts with DNA by intercalation and inhibits DNA topoisomerase II. Based on previous study, brazilein was cytotoxic on MCF-7 cells by inducing apoptosis through suppression of survivin protein expression [19]. Survivin is the smallest member of the mammalian IAP (Inhibition of Apoptosis) family that regulates cell death and cell cycle arrest [20-21]. These molecular mechanism might be occurred by suppression of survivin protein expression through inhibition of its upstream protein, HER-2. Inhibition of HER-2 could suppress the Pgp expression that correlated to resistance breast cancer cell induced by chemotherapeutic agent, doxorubicin [7;19]. Therefore futher study had been done to investigate the molecular mechanism of brazilein increased doxorubicin's cytotoxicity on MCF-7/DOX cells using docking molecular. Molecular docking analysis demonstrated that brazilein potentially inhibits the target protein and disrupt the binding of ATP in ATP binding site of target proteins, such as HER-2. According to table 1, brazilein acts as HER-2 inhibitor that might inhibit signal transduction cascade of HER-2, such as phosphorilation of Grb 2 or Pgp. Pgp as transporter protein resulting in the increase of doxorubicin's as chemotherapy agent accumulation in the cell. Futher study must be done to investigate the accumulation of doxorubicin in cells when combined with brazilein. Another studies must be established to evaluate the ability of brazilein to inactivate NFκB through inhibition of HER-2 as its upstream and downregulation of the target genes of NFκB, such as Bcl-2, Bcl-X_L, survivin (antiapoptosis protein), and cyclin D.

CONCLUSION

Brazilein was potential as co-chemotherapeutic agent on MCF-7 breast cancer cells. The molecular docking demonstrate high affinity interaction between brazikein and HER-2. Futher study must be established to evaluate the molecular mechanism of co-chemotherapeutic effect of brazilein on MCF-7 cells in vitro.

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CONFLICT OF INTERESTS

Declared None

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