RESEARCH ARTICLE

Editorial Process: Submission:01/08/2022 Acceptance:08/01/2022

Synergistic Cytotoxic and Antimigratory Effect of Brazilein and Doxorubicin on HER2-Overexpressing Cells

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Abstract

Objective: The present research aims to report cytotoxic and antimigratory activities of the oxidized form of brazilin, i.e., brazilein, and the effects of the combination of brazilein-doxorubicin on MCF-7/HER2 cells. **Methods:** The MTT assay was conducted to test the cytotoxic activity, while flow cytometry with PI and PI-annexin V staining were respectively performed for cell cycle and apoptosis analyses. Migration and invasion analyses were assessed via Boyden chamber assay, while HER2, Rac1, p120, MMP2, and MMP9 protein levels were determined by immunoblotting and gelatin zymography. Molecular docking of ligands with HER2, Src, PI3Kα, PI3KΔ, and PI3Kγ proteins was evaluated using MOE 2010. **Results:** The MTT assay showed that the IC_{50} value of brazilein against MCF-7/HER2 cells was 51 ± 2.1 μM. Moreover, brazilein and its combination with doxorubicin-induced G2/M accumulation and apoptosis. Combination of brazilein-doxorubicin inhibited cell migration and tended to decrease HER2, Rac1, p120, MMP2, and MMP9 protein expression levels. Based on our molecular docking study, the docking score of brazilein with PI3Kγ is comparable to that of the native ligand. **Conclusion:** Taken together, a combination of brazilein-doxorubicin exhibited synergistic cytotoxic and antimigratory effects on MCF-7/HER2 cells.

Keywords: Combination therapy- *Caesalpinia sappan L*- matrix metalloproteinase- PI3K- molecular docking

Asian Pac J Cancer Prev, **23 (8)**, 2623-2632

Introduction

HER2 protein overexpression in breast cancer cells is mainly responsible for the aggressiveness, invasiveness, and drug resistance of the disease. The Vav2 substrate, i.e., p120 catenin (p120), is required to activate the HER2 signaling pathway. HER2 overexpression modulates cell migration by increasing Src synthesis and activating Vav2-p120, which, in turn, activates of Rho-GTPases, such as Rac1, cdc42, and RhoA. HER2 overexpression also activates matrix metalloproteinases (MMPs), including MMP9 and MMP2 (Johnson et al., 2010). MMP family proteins play a strategic role in tumor growth and metastasis by degrading various components of the basal matrix and extracellular matrix (ECM) (Brooks et al., 2010). Trastuzumab, an antineoplastic agent against the HER2 receptor, binds to the extracellular domain of HER2 receptor and inactivates HER2 signaling (Ahmad et al., 2014). Another anti-HER2 agent, lapatinib, inhibits HER2 activation via a mechanism similar to that of a tyrosine kinase inhibitor (Chandrika et al., 2016). Unfortunately, several studies have reported drug resistance to HER2-targeting agents in breast cancer cells (Liu et al., 2009; Ahmad et al., 2014). HER2 overexpression in breast cancer cells triggers the phosphoinositide-3 kinase (PI3K)-dependent activation of Akt and NF-κB. This process is related to an increase in cell resistance to various chemotherapeutic drugs, including doxorubicin (Zhang et al., 2011; Kang et al., 2014). Thus, an agent that sensitizes HER2-positive breast cancer cells to chemotherapeutic agents may present a suitable solution to overcome this problem.

Caesalpinia sappan L. is traditionally used as a food ingredient and medicine (Nirmal et al., 2015) but its anticancer effects have also been reported (Rachmady et al., 2016; Haryanti et al., 2017; Jenie et al., 2017). *C. sappan* L. contains numerous phenolic and flavonoid compounds, such as sappanin, protosappanin, brazilide, brazilin, and brazilein. Brazilein and brazilin are the major homoisoflavones of this plant (Cuong et al., 2012; Nirmal et al., 2015), and the former is an oxidized form of the latter (Figure 1A). The hydroxyl group of brazilin at the C9 position can be oxidized to form brazilein (Nirmal et al., 2015). Brazilin has been extensively studied for its anticancer activity (Lee et al., 2012; Handayani et al., 2017; Jenie et al., 2018). Brazilein has also been reported

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to exhibit anticancer activity by altering the expression of several MAP kinase signal transduction pathways (Tao et al., 2013; Handayani et al., 2017). Breast cancer therapy using synergistic drug combinations is generally more efficient compared with therapies using single treatments (Jafari-Gharabaghlou et al., 2018). Our previous study revealed that co-treatment of brazilein or brazilin with anticancer agents (cisplatin or doxorubicin) (Handayani et al., 2016, 2017; Jenie et al., 2018). Rac1, MMP2, and MMP9 proteins are involved in the inhibition of migration and invasion of triple-negative breast cancer cells via co-treatment of brazilein–cisplatin (Handayani et al., 2016). Our previous study also reported that the combination of brazilin with doxorubicin exerts cytotoxic and antimigratory activities on HER-2 positive breast cancer cells (Jenie et al., 2018). Because brazilin and brazilein feature similar cytotoxic mechanisms (Handayani et al., 2017), we propose that brazilein can increase the response of HER2-breast cancer cells to doxorubicin by influencing HER2 signaling with the same potency as brazilin. Thus, the present study aimed to observe the potency of brazilein alone or in combination with doxorubicin against HER2-positive breast cancer cells (MCF-7/HER2); specifically, the cytotoxicity and cell migration inhibitory activity of the target compound was assessed.

Materials and Methods

Preparation of samples

Doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Brazilein was isolated from the dried heartwood powder of *C. sappan* L. by using a previously reported method (Handayani et al., 2016).

Cytotoxicity assay

MCF-7/HER2 cells (1×10^4 cells/well) were grown and treated with 10 - 75 μM of brazilein alone or in combination with doxorubicin at 1/10-1/2 of their IC_{50}. The IC_{50} of doxorubicin is 3 μM (Jenie et al. 2018). After incubation for 24 h, the cells were washed with phosphate buffer saline and added with 0.5-mg/mL MTT reagent (Sigma-Aldrich). After 4 h, the reaction was stopped by the addition of 0.01-M HCl containing 10% of sodium dodecyl sulphate (SDS), and the plate was incubated overnight in the dark. The absorbance of the solution in the wells was measured by an ELISA reader (Bio-Rad) at 595 nm. The absorbance was converted to cell viability, and the values obtained were used to calculate the IC_{50} and combination index (CI).

Cell cycle distribution

Cells were plated at a density of 5×10^4 cells/well in 24-well plates and then treated with the ½ IC_{50} of brazilein (25 μM) alone or in combination with doxorubicin (1.5 μM). Thereafter, the cells were placed in a CO_2 incubator for 24 h, collected, stained with 2-μg/mL PI/RNase (BD Biosciences), and then incubated in a dark room for 10 min. The DNA content of the cells was examined using flow cytometry (BD Biosciences) with Flowing software (version 2.5.1; Cell Imaging Core, Turku Center for Biotechnology, Turku, Finland).

Apoptosis detection

PI-annexin V assay was performed using a kit from Roche Mannheim (Germany). The ½ IC_{50} concentration of brazilein (25 μM) alone or in combination with doxorubicin (1.5 μM) was added to 24-well plates containing 5×10^4 cells/well. The plates were then placed in a CO_2 incubator for 24 h. The cells were collected, added with 1× binding buffer, stained with PI and annexin V, and then incubated once more for 5 min in the dark. The number of apoptotic cells was examined using flow cytometry (BD Biosciences).

Migration and invasion assay

Cells were serum-starved for 24 h, collected, and suspended in 0.5% FBS/DMEM. Cells (3×10^5 cells/well) were distributed in the upper part of an insert chamber for migration and invasion assays. The 10% FBS/DMEM medium was added to the lower chamber. After 24 h of incubation, non-migrating cells in the upper part of the membrane were removed and migrating cells in the lower part of the membrane were stained using CytoSelectTM kit for 10 min at room temperature (RT). Extraction solution was used to wash and dissolve the cells carefully. The absorbance of staining cells was read at 560 nm (Hitachinaka, Japan).

Gelatin zymography

Gelatin zymography was used to measure the expression of MMP9 and MMP2, which were secreted into the medium. Cells were plated at a density of 1×10^6 cells/well into six-well plates and grown in a CO2 incubator at 37°C for 24 h. The cells were treated with 1/4 IC_{50} of brazilein, doxorubicin, or their combination in serum-free medium for 24 h. The medium containing the secreted MMPs was collected, and gelatin zymography was performed by loading the samples onto 10% SDS-PAGE gel containing 0.1% gelatin and then running it with SDS buffer. Next, the gel was washed with a renaturing solution containing 2.5% Triton X-100 for 30 min. Thereafter, the gel was incubated with incubation buffer (50-mM Tris-HCl, 150-mM NaCl, 10-mM $CaCl_2$) for 20 h at 37°C, stained with 0.5% CBB stain, incubated for 30 min at RT, and destained with destaining solution (10% [v/v] methanol and 5% [v/v] acetic acid). The results were documented by scanning the gel.

Immunoblotting assay

Cells (1×10^6) were grown in a 100-mm culture dish at 37°C in a CO_2 incubator for 24 h, incubated with 1/4 the IC_{50} of the samples for 24 h, and then lysed in RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail. The protein lysates were loaded into 7%–15% SDS-PAGE gels and blotted onto PVDF membranes (Immobilon; Millipore Sigma). 1× NET gelatin buffer was used to block the membranes. Antibodies for Rac1 (ab33186; Abcam, Cambridge, UK), HER2 (sc-52439), p120 (sc-13957), and β-actin (sc-47778; Santa Cruz Biotechnology, TX, USA) were used to probe the membrane. The cells were then treated with anti-mouse

(sc-2031; Santa Cruz Biotechnology) or anti-rabbit (7074P2, Cell Signaling Technology, Danvers, MA, USA) horseradish peroxidase-conjugated secondary antibodies. Protein band visualization was performed using an Amersham enhanced chemiluminescence system (GE Healthcare Life Sciences, Marlborough, MA, USA).

Molecular docking studies

All computational simulations were conducted on a computer running on Windows 10 with an Intel Core i5-7th Gen processor and 4 GB of RAM. We chose the PDB IDs 2BDF, 3T8M, 4GB9, and 3L54 as the crystal structural models of Src, PI3Kα, PI3KΔ, and PI3Kγ, respectively, because of the occupancy of the known inhibitors in the proteins. Docking simulation, RMSD calculation, and binding interaction visualization were performed using MOE 2010 (licensed from the Faculty of Pharmacy UGM). The default settings were applied if no detailed explanation. The structures of brazilein and brazilin were drawn using Chemdraw software, and conformational searches and energy minimization were then performed. Docking simulation was conducted on the basis of the flexible structures of the rigid receptors and ligands at the native ligand binding site. The docking simulation settings used for score functions and placement settings were London dG and Triangle matcher, respectively. The docking results from 30 retained settings were refined using the force field method. The analysis results could describe the binding affinity and interaction of each compound with the protein target.

Statistical analysis

The data were statistically analyzed using Student's t test (Microsoft Excel 2013). $P < 0.05$ was considered significant. CI was determined using CompuSyn software (version 1.0; ComboSyn; Zhang et al. 2015). The data scanned from gelatin zymography were quantified using ImageJ (National Institutes of Health).

Results

Cytotoxic activity of brazilein and its combination with doxorubicin

MTT assay was used to verify the cytotoxic activity of brazilein and its co-chemotherapeutic potency with doxorubicin in MCF-7/HER2 cells. The potency of doxorubicin against MCF-7/HER2 has been previously reported by Jenie et al., (2018), with an IC_{50} value is 3 μM. As shown in Figure 1B, brazilein suppressed MCF-7/HER2 cell proliferation, with an IC_{50} value of 51 ± 2.1 μM, in a dose-dependent manner. The cytotoxic activity of brazilein could be categorized as mild (Larsson et al. 2006).

We have previously reported the potency of a combination of brazilein with cisplatin in 4T1, triple-negative breast cancer cell line (Handayani et al. 2016). Therefore, the effect of the combination of brazilein and doxorubicin were analyzed to confirm whether brazilein gives a beneficial effect on doxorubicin cytotoxicity on MCF-7/HER2 cells. CIs were determined to evaluate the synergistic potency of the combination of these two compounds. Combinations of 1/10, 1/8, 1/4, and 1/2 the IC_{50} of brazilein and doxorubicin yielded CI values less than 1 (Figure 1C-D), thereby indicating synergistic inhibitory effects on cell growth. Inhibition of cell cycle progression and induction of cell apoptosis are two common types of cytotoxic mechanisms of anticancer agents (Hanahan and Weinberg, 2011). The cytotoxic activity of the combination of brazilein and doxorubicin may be due to either of these mechanisms. Thus, we observed the occurrence of cell cycle inhibition and apoptosis in MCF-7/HER2 cells following treatment with brazilein and doxorubicin.

Modulation of the cell cycle and apoptosis

Flow cytometry revealed G2/M-phase accumulation after a single treatment of half the IC_{50} (sub IC_{50}) of either brazilein or doxorubicin (Figure 2B-C; Table 1). G2/M-phase accumulation was also observed after treatment with the combination of brazilein and doxorubicin at half the IC_{50} (Figure 2D; Table 1). Flow

Table 1. Effect (%) of Co-Treatment with Brazilein (Be) and Doxorubicin (Dox) on the Cell Cycle Distribution of MCF-7/HER2 Cells

Phase	Untreated	½ IC_{50} (1.5 μM) Dox	½ IC_{50} (25 μM) Be	Be-Dox
G0/G1	54.7 ± 1.01	46.6 ± 0.22***	32.4 ± 0.08***	41.2 ± 0.12***
S	15.3 ± 0.28	13.5 ± 0.08	24.8 ± 0.06	16.4 ± 0.04
G2/M	30.0 ± 0.58	39.9 ± 0.22***	42.8 ± 0.11***	42.4 ± 0.12***

***p<0.001

Table 2. Effect (%) of Co-Treatment with Brazilein (Be) and Doxorubicin (Dox) on the Apoptosis of MCF-7/HER2 Cells

	Untreated	½ IC_{50} (1.5 μM) Dox	½ IC_{50} (25 μM) Be	Be-Dox
viable cell	87.54±1.28	79.15±0.67***	66.38±0.47***	53.40±0.53***
early apoptosis	6.74±1.10	9.71±0.28***	1.01±0.15***	1.32±0.03***
late apoptosis	3.95±0.29	3.62±0.15	0.53±0.04***	0.81±0.02***
necrosis	1.80±0.11	7.53±0.33***	32.08±0.60***	44.47±0.56***

***p<0.001

Table 3. Binding Interactions of the Native Ligand, Brazilein, and Brazilin on Src Protein

Compound	Parameter	Src (PDB ID: 2BDF)		
Native ligand (AP23451)	Docking Score (kcal/mol)		-11.28	
	RMSD (Å)		2.042	
	Ligand Atom	H	C	C
	Amino Acid	Met341	Leu273	Leu393
	Binding Type	H-bond	Arene-H	Arene-H
	Distance	1.76		
Brazilein	Docking Score (kcal/mol)		-10.79	
	RMSD (Å)		1.767	
	Ligand Atom	C		
	Amino Acid	Leu393		
	Binding Type	Arene-H		
	Distance			
Brazilin	Docking Score (kcal/mol)		-12.42	
	RMSD (Å)		0.889	
	Ligand Atom	H	O	
	Amino Acid	Asp404	Lys295	
	Binding Type	H-bond	H-bond	
	Distance	1.68	1.84	

cytometry with PI-annexin V staining was used to observe apoptosis induction after brazilein and doxorubicin treatment. The results revealed that treatment with doxorubicin alone led to apoptosis whereas treatment with brazilein and its combination with doxorubicin led to necrosis rather than apoptosis (Table 2). In the current study, we treated the cells with a combination of ½ IC_{50} of each compound, these concentrations may be too high to observe the apoptosis induction of the combination. Due to this high concentration treatment, the apoptotic cells may rapidly progress into late apoptosis (secondary necrosis), with a more permeable cell membrane. We discuss this in more detail in the discussion section.

Inhibition of cell migration and invasion

We performed migration and invasion assays to observe the antimetastatic activity of brazilein and doxorubicin alone and in combination in MCF-7/HER2 cells. We observed that 12.5 µM brazilein alone could inhibit migration by up to 15% but did not affect cell invasion compared with untreated cells. Doxorubicin increases the migration and invasion of MCF-7/HER2 cells (Jenie et al., 2018). In this study we detected that cell migration and invasion were inhibited by 50% and 15%, respectively, following treatment with 12.5 µM brazilein in combination with 0.75 µM doxorubicin compared doxorubicin alone (Figure 3A-B).

Several proteins in the HER2 signaling pathway are involved in cell migration and invasion (Johnson et al., 2010). Thus, we attempted to elucidate this pathway in our next experiment. We confirmed that the inhibition of cell migration and invasion afforded by brazilein and doxorubicin alone and combination is correlated with the HER2 signaling pathway. Treatment with brazilein and doxorubicin alone slightly decreased HER2 protein expression (Figure 3E), but the combination of brazilein and doxorubicin resulted in a clear decrease in HER2 protein expression (Figure 3E). Moreover, we found that this combination modulated proteins involved in migration and invasion in the HER2 signaling pathway, such as p120, Rac1, MMP2, and MMP9 (Figure 3). The combination of brazilein and doxorubicin decreased the protein expression of p120 and Rac1 compared with that in untreated cells (Figure 3E). MMPs are ECM-degrading proteases that have a strategic function on migration and invasion–metastasis (Hua et al., 2011). The results

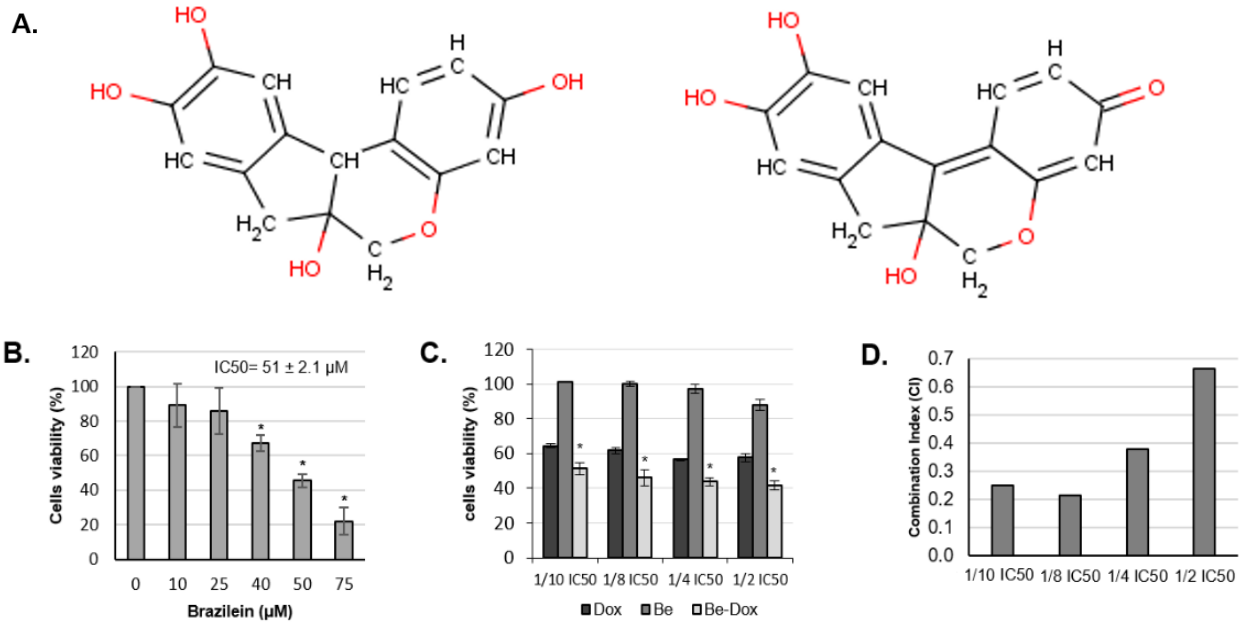

Figure 1. Cytotoxic Activity of Brazilein (Be) Alone and in Combination with Doxorubicin (Dox) in MCF-7/HER2 Cells. Structure of brazilin (1A, left) and brazilein (1A, right). Cytotoxic activity was measured by MTT assay after 24 h of treatment. (B) Effects of Be on the viability of MCF-7/HER2 cells. (C) The combination of Be and Dox at sub IC_{50} as indicated in the graph. (D) Combination indices (CIs) between Be and Dox in MCF-7/HER2 cells. Data are expressed as the mean ± SD of three independent experiments. *P < 0.05.

Table 4. Interactions of the Native Ligand, Brazilein, and Brazilin on PI3Kα Protein

Compound	Parameter	PI3Kα (PDB ID: 3T8M)					
Native ligand (3T8)	Docking Score (kcal/mol)	−14.69					
	RMSD (Å)	1.315					
	Ligand Atom	O	O	H	H	C	C
	Amino Acid	Ser806	Val882	Val882	Tyr867	Ile963	Met963
	Binding Type	H-bond	H-bond	H-bond	Arene-H	Arene-H	Arene-H
	Distance	1.52	2.04	1.69			
Brazilein	Docking Score (kcal/mol)	−9.78					
	RMSD (Å)	0.879					
	Ligand Atom	H	C				
	Amino Acid	Glu880	Ile963				
	Binding Type	H-bond	Arene-H				
	Distance	1.8					
Brazilin	Docking Score (kcal/mol)	−10.02					
	RMSD (Å)	1.07					
	Ligand Atom	H					
	Amino Acid	Tyr867					
	Binding Type	Arene-H					
	Distance						

indicate that, compared with untreated cells, doxorubicin inactivates MMP9 but not MMP2 in MCF-7/HER2 cells. By contrast, brazilein and its combination with doxorubicin inactivate both MMP2 and MMP9 (Figure 3C-D). The combination of brazilein and doxorubicin downregulated the protein expression of HER2, p120, and Rac1 and inactivated MMP2 and MMP9.

Molecular docking studies

We confirmed the association of brazilein with the NFκB-HER2 signaling pathway via in silico docking assay. Because the cytotoxic and antimigratory effects of brazilein are nearly identical to those of with brazilin (Handayani et al., 2016, 2017, 2020; Jenie et al., 2018), we tested both compounds in our in silico study. Here, our molecular docking studies focused on Src and PI3K proteins. The native ligands representing the selective

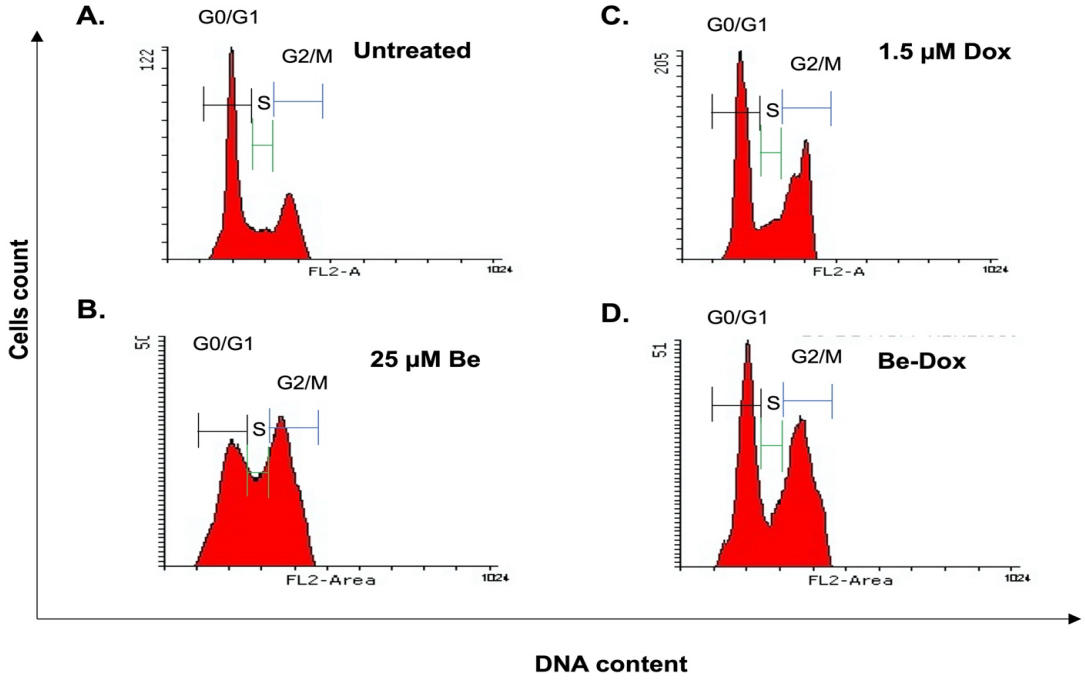

Figure 2. Alterations in MCF-7/HER2 Cell Cycle Profiles after Single and Combination Treatment with Brazilein (Be) and Doxorubicin (Dox). (A) Control cells (treated with vehicle) or cells treated with ½ IC_{50} Be (B), ½ IC_{50} Dox (C), or the combination of ½ IC_{50} Be or ½ IC_{50} Dox (D) for 24 h were subjected to cell cycle analysis by flow cytometry with PI/RNase staining as described in the Methods section.

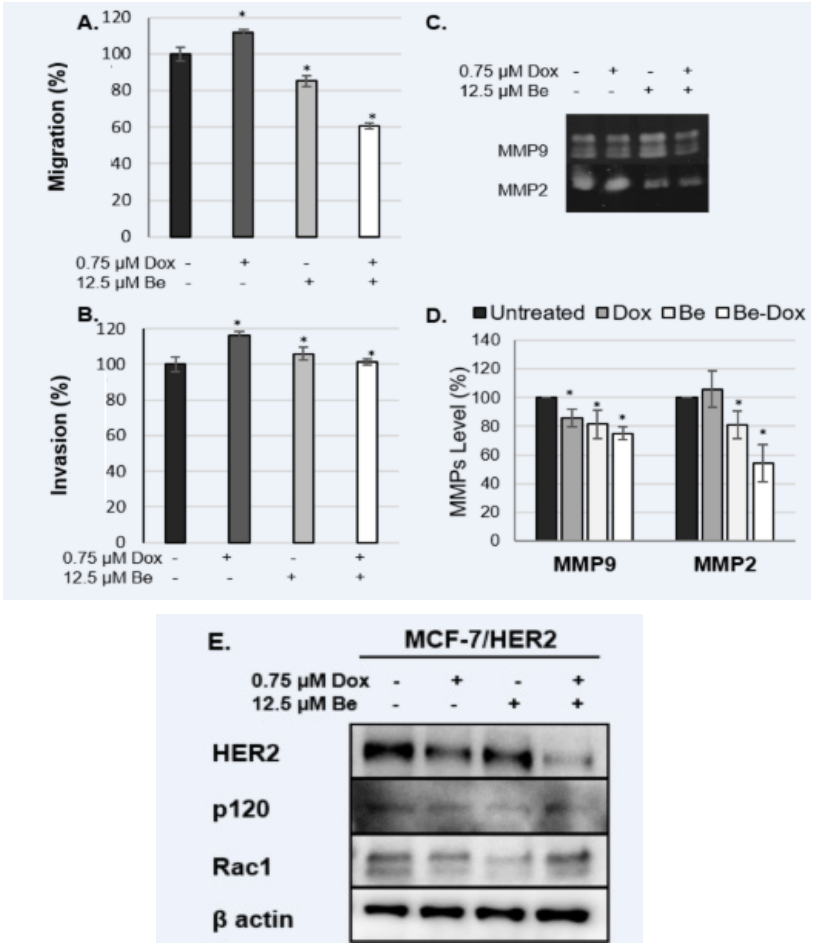

Figure 3. Effects of Co-Treatment of Brazilein (Be) and Doxorubicin (Dox) on MCF-7/HER2 Cell Migration and Invasion. Cells were treated with ¼ IC_{50} Be alone or in combination with Dox for 24 h in serum-starved medium. Cell (A) migration and (B) invasion below the kit chamber were observed using the protocol described in the Methods section. (C) Detection of MMP protein bands by using gelatin zymography. (D) Quantification of the gelatinase activity of the MMPs. (E) Expression levels of migration- and invasion-regulatory proteins detected by using Western blot. Data are expressed as the mean ± SD of three independent experiments. *P < 0.05.

Table 5. Interactions of the Native Ligand, Brazilein, and Brazilin on PI3KΔ Protein

Compound	Parameter	PI3KΔ (PDB ID: 4GB9)				
Native ligand (benzimidazole)	Docking Score (kcal/mol)			-14.21		
	RMSD (Å)			1.066		
	Ligand Atom	N	O	C	C	H
	Amino Acid	Lys830	Ile881	Ile963	Ile879	Trp812
	Binding Type	H-bond	H-bond	Arene-H	Arene-H	Arene-H
	Distance	1.77	2.79			
Brazilein	Docking Score (kcal/mol)			-9.76		
	RMSD (Å)			2.01		
	Ligand Atom	H	C	C	H	
	Amino Acid	Ile963	Ile963	Ile963	Tyr867	
	Binding Type	H-bond	H-bond	Arene-Arene	Arene-H	
	Distance	1.97	3.2			
Brazilin	Docking Score (kcal/mol)			-11.16		
	RMSD (Å)			2		
	Ligand Atom	H	C			
	Amino Acid	Lys830	Ile963			
	Binding Type	H-bond	Arene-H			
	Distance	2.05				

Table 6. Interactions of the Native Ligand, Brazilein, and Brazilin on PI3Kγ Protein

Compound	Parameter	PI3Kγ (PDB ID: 3L54)						
Native ligand (GSK2126458)	Docking Score (kcal/mol)				-13.22			
	RMSD (Å)				0.623			
	Ligand Atom	N	N	H	H	C	C	C
	Amino Acid	Lys890	Val882	Lys833	Tyr867	Ile879	Ile963	Trp812
	Binding Type	H-bond	H-bond	H-bond	Arene-H	Arene-H	Arene-H	Arene-H
	Distance	1.54	1.91	1.7				
Brazilein	Docking Score (kcal/mol)				-13.39			
	RMSD (Å)				1.49			
	Ligand Atom	O	O	H	C			
	Amino Acid	Lys890	Lys890	Tyr867	Ile963			
	Binding Type	H-bond	H-bond	Arene-H	Arene-H			
	Distance	2.21	1.95					
Brazilin	Docking Score (kcal/mol)				-10.14			
	RMSD (Å)				1.096			
	Ligand Atom	O	H-bond	C				
	Amino Acid	Thr887	Tyr867	Ile963				
	Binding Type	H-bond	Arene-H	Arene-H				
	Distance	2.11						

inhibitors of the Src, PI3Kα, PI3KΔ, and PI3Kγ models were AP23451, 3T8, benzimidazole inhibitor, and GSK2126458, respectively. Our studies revealed that the interaction of brazilein–Src protein yields the highest docking score, whereas that of brazilin-Src yields the lowest docking score (Figure 4; Table 3). Docking

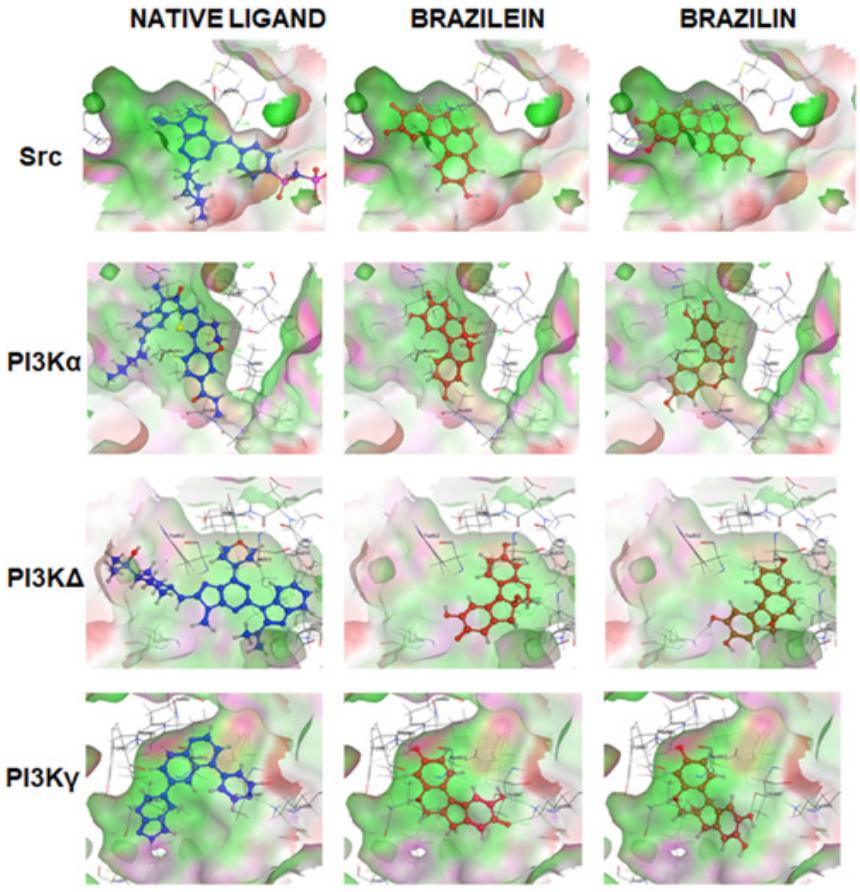

Figure 4. Docking Visualization of the Ligand–Protein Interactions of Brazilein and Brazilin with Src, PI3Kα, PI3KΔ, and PI3Kγ Proteins.

interactions with PI3Kα and PI3KΔ further demonstrated higher docking scores for brazilein compared with brazilin (Tables 4-5). However, the docking score of brazilein-PI3Kγ was lower than that of brazilin and comparable with that of GSK2126458 (Table 6). Brazilein also showed the best interactions with PI3Kγ protein among the other proteins in this in silico study.

Discussion

Both brazilin and brazilein are found in *Caesalpinia sappan*, L. Brazilein is the oxidized form of brazilin and it is more stable than brazilin (Rondão et al., 2013). When brazilin is exposed to light or air, it often turns into brazilein. Therefore, in this study, considering the structural similarities between brazilin and brazilein, we wanted to determine whether brazilein exhibits similar activity against HER2 overexpressed breast cancer cells. Furthermore, we would like to know whether the mechanism of action between the two compounds in the regulation of HER2 signaling is the same.

HER2 protein overexpression in breast cancer is well correlated with poor prognosis (Rouanet et al., 2014). Our group previously reported the synergism between brazilin and doxorubicin on MCF-7/HER2 breast cancer cells (Jenie et al., 2018). In the present study, we observed that the cytotoxic potency of brazilein against MCF-7/HER2 breast cancer cells is similar to that of brazilin, with an IC_{50} value 51 ± 2.1 μM. Brazilein also showed synergistic effects with doxorubicin (Fig 1). We elucidated the mechanisms of the synergistic cytotoxic effect of brazilein by using the in vitro and in silico approaches. We observed that brazilein, doxorubicin, and their combination induce G2/M accumulation (Figure 2). While doxorubicin accumulates cells in the G2/M phase through DNA intercalation and topoisomerase II inhibition (Carvalho et al., 2009), brazilein appears to implement a different mechanism of action. The isoflavone genistein induces G2/M accumulation via its ability to inhibit p53 and p21 protein expression, binds to tubulin, and leads to microtubule depolymerization (Mukherjee et al., 2010; Ono et al., 2018), while the isoflavone analog RY-2f induces G2/M arrest by repressing the PI3K/AKT/mTOR pathway (Liu et al., 2015). Given the isoflavone-type structure of brazilein, this compound may possess the same mechanism of action as other isoflavones. Our current findings demonstrate that the combination of brazilein with doxorubicin synergistically induces G2/M accumulation through different pathways.

Brazilein and its combination with doxorubicin-induced necrosis rather than apoptosis (Figure 2). We believe that the phenomenon of necrosis observed in this study is actually secondary necrosis, which involves the autolytic process of cell disintegration following completed apoptosis when scavenger intervention is absent (Silva, 2010). Brazilin inactivates NF-κB and downregulates the expression of HER2 and the antiapoptotic protein Bcl2 (Jeon et al., 2014; Jenie et al., 2018). HER2 overexpression activates the NFκB transcription factor and leads to the transcription of antiapoptotic genes (Merkhofer et al., 2010; Qu and Xiao, 2015). Brazilein-induced apoptosis may be associated with the ability of the compound to inactivate NFκB via the HER2 signaling pathway.

A distinct characteristic of HER2-positive breast cancer is its highly metastatic nature. We previously revealed the potency of brazilin in inhibiting HER2-positive breast cancer cell migration and invasion (Jenie et al., 2018). In the present study, we observed that brazilein also impedes the MCF-7/HER2 cell migration and invasion induced by doxorubicin (Figure 3A-B). The related mechanism may involve the partial suppression of MMP2 and MMP9 upon treatment with brazilein or its combination with doxorubicin (Figure 3C-D). MMP proteins degrade the ECM, leading to cell migration and invasion (Hua et al., 2011). ECM degradation is a process related to migration and invasion. This finding is in line with the results of a previous study on brazilin (Jenie et al., 2018). The homoisoflavone protosappanin could inactivate NFκB, leading to low levels of MMP9 in rabbit serum (Huang et al., 2018). Similar to brazilin (Jenie et al., 2018), brazilein and its combination with doxorubicin downregulated the protein expression of HER2, Rac1, and p120 (Figure 3F). Rac1 and p120 play important roles in migration and invasion through the HER2 pathway (Johnson et al., 2010). Because NFκB is a transcription factor for MMPs and involved in HER2 overexpression (Johnson et al., 2010; Chiu et al., 2015), the present study demonstrated that the inhibitory effect of brazilein and its combination with doxorubicin on the proliferation and migration-invasion of HER2-positive breast cancer cells is closely related to the HER2 signaling pathway. Thus, brazilin and brazilein, the two major compounds of *C. sappan*, have similar antimigratory effects by regulating the HER2 signaling pathway.

In vitro experiments indicated that the cytotoxic and antimigratory effects of brazilein, especially its effect on the downstream pathway of NFκB, are nearly similar to that of brazilin (Handayani et al., 2016, 2017; Jenie et al., 2018). Molecular docking studies with Src and PI3K protein, two proteins crucial to the upstream pathways of NFκB protein, especially the HER2 signaling pathway, were conducted to discover the specific mechanisms of brazilein and brazilin. Here PI3Kα, PI3KΔ, and PI3Kγ were selected as known isoforms of PI3K contributing diverse pharmacological effects (Ameriks and Venable, 2009). Differences in functional groups at the C9 position between brazilein and brazilin are believed to contribute to the distinct affinity and mechanism of these molecules. Molecular docking studies of the Src protein revealed that brazilin has a fairly low docking score, which indicates greater binding affinity compared with brazilein and the native ligand (Table 3). The hydroxyl group at the C9 position facilitates suitable fitting through H-bonds with Asp 404 (Figure 4; Table 3). Docking interactions with PI3Kα also showed that brazilin possesses fairly high affinity; however, the affinity of this compound remained lower than that of 3T8 (Table 4). The binding visualization revealed that even though the hydroxyl group at C6a position of brazilein forms an H-bond with Glu880, the lower affinity of brazilein- PI3Kα cannot be avoided because arene-H binding between the hydroxyl group at the C9 position and Tyr867 as represented on brazilin-

PI3Kα interaction, did not present on brazilein- PI3Kα (Figure 4; Table 4). The important role of the hydroxyl group at the C9 position was confirmed on PI3KΔ via the formation of an H-bond with Lys890, resulting in the greater affinity of brazilin compared with that of brazilein but not that of the benzimidazole inhibitor (Table 5). Interestingly, the hydroxyl group at C9 position did not show affinity equivalent to that offered by the carbonyl group to PI3Kγ. Brazilein bearing a carbonyl group at the C9 position demonstrated greater affinity than brazilin to PI3Kγ but not to PI3Kα, PI3KΔ, or Src. Nevertheless, the docking score of brazilein was lower than that of brazilin and comparable with that of GSK2126458, a PI3K inhibitor (Table 6). The greater affinity of brazilein toward its target molecules was mediated by H-bonds between the carbonyl group at the C9 position and Lys890 and between the arene and Trp867 and Ile963 (Figure 4; Table 6).

Although we observed that the effect of brazilin and brazilein against HER2 overexpressed breast cancer cells are similar, based on our molecular docking study, they showed different binding preferences with HER2 regulator proteins. The docking score of brazilein with PI3Kγ was comparable to that of the native ligand (GSK2126458), whereas brazilin had more affinity to src, PI3Kα, PI3KΔ. It has been reported that overexpression of PI3Kγ caused non-metastatic breast cancer cells to become metastatic cells (Xie et al., 2013). Treatment with PI3Kγ inhibitor or with siRNA knockdown of PI3Kγ but not with inhibitors or siRNAs of PI3Kα or PI3Kβ rendered the migration and invasion of metastatic breast cancer cells (Xie et al., 2013). Moreover, PI3Kγ is an important regulator in both cardiac disease and tumor growth. The blockade of this protein in the in vivo study of mammary gland tumor protected doxorubicin-induced cardiotoxicity and synergized the antitumor activity of doxorubicin (Li et al, 2018). Even though we still need to determine whether brazilein functions as a PI3Kγ inhibitor but our findings suggested that brazilein may target PI3Kγ and inhibit its activation that leads to hampering the metastatic activity of HER2 overexpressed cells. A combination of doxorubicin and brazilein may give a beneficial effect to inhibit cancer cells metastasis and reduce cardiac side effects of doxorubicin. However, the concentration of each compound still needs to be optimized because too high concentrations may rapidly progress the apoptotic cells into necrosis (secondary necrotic cells) as we observed in our data (Table 2). Overall, our findings demonstrated that brazilein and its combination with doxorubicin show cytotoxic synergism and antimigratory activity toward MCF-7/HER2 cells via the HER2/PI3K signaling pathway.

In conclusion, brazilein and its combination with doxorubicin demonstrated cytotoxic and antimigratory activity against MCF-7/HER2 cells by downregulating HER2, p120, and Rac1 protein expression levels, suppressing MMP2 and MMP9 proteins, and interfering with the HER2/PI3K signaling pathway via PI3Kγ. Thus, brazilein may potentially be combined with other chemotherapeutic agents, particularly doxorubicin, to inhibit HER2-positive breast cancer cell proliferation, migration, and invasion.

Author Contribution Statement

SH: conceptualization, experimental design, searching the literature, preparation of the materials, performing in vitro experiment and the analysis, designing of the in vitro figures, drafting and editing of the manuscript. RAS: conceptualization, reviewing the manuscript draft. RYU: performing in silico experiment and the analysis, designing of in silico figures and tables. EM: conceptualization, planning of experimental designs, supervising, and giving the final approval for submission. RIJ: conceptualization, planning experimental designs, supervising, in vitro data curation, reviewing the manuscript draft and responsible for the submission. All authors discussed the results and commented on the manuscript.

Acknowledgements

We thank the National Research and Innovation Agency for funding this research through the Research Directorate of Universitas Gadjah Mada (Grant No. 6469/UN1/DITLIT/DIT-LIT/PT/2021). We also thank Ni Putu Linda Laksmiani for facilitating valuable discussions on the isolation of brazilein.

Ethical Issue

The study protocol has been approved by The Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Universitas Gadjah Mada-DR. Sardjito General Hospital.

Conflict of interest

We declare there is no conflict of interest.

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