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RESEARCH ARTICLE

Synthesis and Cytotoxic Activity of *N*-(4-bromo)-benzoyl-*N*'phenylthiourea and 4-(tert-butyl)-*N*-benzoylurea on Primary Cells of HER2-Positive Breast Cancer

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ABSTRACT:
Many of the breast cancer treatments use chemotherapeutic agents in the form of synthetic drugs. Some thiourea and benzoylurea derivatives have been proven to inhibit the proliferation of breast cancer cells. Up until now, the derivation compounds are still being developed. In this study, we synthesized two compounds namely *N*-(4-bromo)-benzoyl-*N'*-phenylthiourea and 4-(tert-butyl)-*N*-benzoylurea. Before the two compounds were synthesized, the prediction of cytotoxic activity in silico was first performed by docking the synthesis compounds with the HER2 receptor (PDB code: 3PP0). The results of the in-silico test are Rerank Score (RS) value using the MVD program (Molegro Virtual Doker). Acyl nucleophilic substitution was carried out to synthesize the compounds. The structure of the synthesized compounds was identified using FTIR, ^{1}H-NMR, ^{13}C-NMR and Mass Spectrometry. In vitro cytotoxic activity was carried out on HER2-positive primary breast cancer cells and produced IC_{50} values. The results showed that the RS value of *N*-(4-bromo)-benzoyl-*N'*-phenylthiourea was smaller (-99.21kcal/mol) than 4-(tert-butyl)-*N*-benzoylurea (-88.86kcal/mol), meanwhile the RS value of hydroxyurea as a comparison compound which has a urea group and anticancer activity of -34.60kcal/mol. IC_{50} values of *N*-(4-bromo)-benzoyl-*N*'-phenylthiourea and 4-(tert-butyl)-*N*-benzoylurea were 0.54 mM and 0.61mM respectively. Hydroxyurea had an IC_{50} value of 11.61mM. It can be concluded that *N*-(4-bromo)-benzoyl-*N*'-phenylthiourea was more potent than 4-(tert-butyl)-*N*-benzoylurea so it can be developed further as an anticancer candidate for HER2-positive breast cancer.

KEYWORDS: Synthesis, Cytotoxic, Thiourea, Benzoylurea, Breast cancer, HER2.

INTRODUCTION:
Cancer is one of the major health burdens of disease in the world. Breast cancer ranks second in mortality after lung cancer and spreads fear to women around the world[1]. Cancer treatment options vary greatly depending on the stage of cancer at the time of diagnosis. Radiotherapy and surgery are commonly used in the early stages of breast cancer. Chemotherapy is cancer treatment options at an advanced stage of the disease[2,3].

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The use of chemotherapy is often limited predominantly due to unwanted side effects and limited choices of anticancer drugs[4]. Thus, the success of cancer treatment is a challenge in the 21st century. It underlines the need for developing new chemotherapy drugs with higher anticancer activity.

Many cancer drugs are developed through growth factor receptors (GFR) as targets of action. GFR kinases, known to play an important role in cancer are the epidermal growth factor receptor (EGFR or erbB-1) and human epidermal growth factor receptor-2 (erbB-2 or HER2). In the cases of breast cancer, 30% incidences are caused by overexpression of Human Epidermal Receptor 2 (HER2) with a poor prognosis[5].

The treatment that is currently widely used in handling breast cancer is using chemotherapy agents in the form of synthetic drugs. Thiourea derivative is an anticancer agent acting as an EGFR inhibitor. It inhibited Receptor Tyrosine Kinases (RTKs) in the intracellular region[6]. Li synthesized and looked at the structural relationship of the activities of the *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N'*-phenylureas and thioureas derivatives as anticancer. The results obtained that the compound derivatives proved to be as potential EGFR and HER2 kinase inhibitors and possessed high antiproliferative activity on MCF-7[5]. Another study by Huang has synthesized thioureas derivatives and then tested the cell growth inhibitory activity on NCl-H460, A549, HepG3, SKOV3 cell lines. The results showed that the derivatives of these compounds have greater anticancer activity compared to 5-fluorouracil[7]. Kesuma has docked phenyltiourea derivatives with EGFR receptors, synthesized them and tested cytotoxic activity on MCF-7 cells. The results showed that the phenyltiourea derivatives had better cytotoxic activities than hydroxyurea[8].

The benzoylurea derivative is also one of the promising anticancer agents for further development. Suhud has synthesized 1-benzyl-3-benzoylurea compound and tested its anticancer activity in MCF-7 cell culture. The results showed a better IC_{50} value obtained than hydroxyurea[9]. Diyah has docked benzoylurea derivatives with 3HNC receptors. Then the compounds were synthesized and tested for their cytotoxic activities using the Brine Shrimp Lethality Test (BST) method. All benzoylurea derivatives showed better LD_{50} values than hydroxyurea. Likewise, *N*, *N'*-dibenzoyl-*N*,*N'*-diethylurea and *N*-*N'*-carbonylbis-(*N*-ethylbenzamide) compounds have been synthesized and tested its cytotoxic activities on MCF-7 cells. IC_{50} values obtained from the cytotoxic activity test showed that all benzoylurea derivatives had better cytotoxic activities than hydroxyurea[10,11,12].

The use of hydroxyurea is reported to cause many side effects, especially to thrombocythemia patients[13]. The hydrophilic groups in hydroxyurea cause lower penetration ability resulting in less optimal biological activity. Therefore, it is necessary to develop new anticancer drugs from urea derivatives that provide better membrane penetration capacity.

In this study, two compounds were synthesized namely *N*-(4-bromo)-benzoyl-*N'*-phenylthiourea as a derivative of phenyltiourea and 4-(tert-butyl)-*N*-benzoylurea as a derivative of benzoylurea. This research began with the prediction of activity using in silico molecular modeling with the Molegro Virtual Docker 5.5 (MVD) program. In molecular modeling, docking is a method for predicting the affinity of a bond between a ligand and macromolecules such as proteins, lipids, carbohydrates, and nucleic acids that play an important role in signal transduction[14]. In silico technique is done by docking *N*-(4-bromo)-benzoyl-*N'*-phenylthiourea and 4-(tert-butyl)-*N*-benzoylurea compounds with HER-2 receptor (ID PDB: 3PP0)[15]. The *N*-(4-bromo)-benzoyl-*N'*-phenylthiourea (4Br-BPTU) compound was synthesized from *N*-phenylthiourea and 4-bromobenzoyl chloride while 4-(tert-butyl)-*N*-benzoylurea (4TBBU) was synthesized from urea and 4-(tert-butyl)benzoyl chloride using acyl nucleophilic substitution[16,17,18]. The two synthesized compounds were further identified using an IR spectrometer, ^{1}H-NMR, ^{13}C-NMR, and a mass spectrometer[18,19,20].

In vitro cytotoxic activity of the two test compounds was carried out using the MTT (Microculture tetrazolium) assay[21,22] in primary breast cancer cells. The MTT assay is based on metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using the mitochondrial dehydrogenase enzyme into water-insoluble formazan crystals, which provides direct correlation of viable cells[23]. In this study, primary cells were obtained by isolating the biopsy tissue of HER2-positive breast cancer patients. The results of the cytotoxic assay were in the form of IC_{50} values which were then compared to hydroxyurea (HU) as a comparative compound. This research provides potential new anticancer candidates on primary cells of HER2-positive breast cancer.

MATERIAL AND METHODS:

Chemicals and reagents:

The materials needed for synthesis are *N*-phenylthiourea, 4-bromobenzoyl chloride, urea, 4-(tert-butyl) benzoyl chloride (Sigma-Aldrich), tetrahydrofuran (THF), triethylamine (TEA), sodium bicarbonate, ethyl acetate, n-hexane, chloroform, methanol, and ethanol. The materials for primary breast cancer cell identification are CD133 biomarker, anti-HER2 monoclonal antibodies, PE and FITC anti-mouse IgG. Materials needed for in vitro cytotoxic activity test are Breast cancer cell cultures expressing HER2, culture media (Alpha MEM), Phosphate-buffered saline (PBS), trypsin, Penicillin-Streptomycin, fungizone, fetal bovine serum (FBS), DMSO, 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 10% SDS-0.01M HCl.

Instrumentation:

The instruments used were Fisher-John Electrothermal Mel-Temp, Bruker FT-IR spectrometer Alpha II, ^{1}H-NMR Spectroscopy and ^{13}C-NMR Jeol ECS-400, and Waters Xevo Q-ToF mass spectrometer. The instruments used for cytotoxic testing are 5% CO_2 incubator, LAF, blue and yellow micropipette, test tube, vortex, 96-well microplates, conical tube, inverted microscope, hemocytometer and ELISA reader. ChemBioDraw Ultra 15.0 and Molegro Virtual Docker (MVD) ver. 5.5 was used for molecular modeling.

Methods:

Molecular Modeling:

The synthesized compounds were predicted in advance for their cytotoxic activity using in silico molecular modeling. In silico technique was carried out through a simulation of drug-receptor interaction called docking using a computer. The two compounds were docked using the Molegro Virtual Docker (MVD) ver. 5.5 program with HER2 receptor (ID PDB: 3PP0). Hydroxyurea (HU) is used as a comparative compound.

4Br-BPTU and 4TBBU Synthesis:

N-Phenylthiourea was mixed with THF and TEA in a round flask and added a drop-by-drop solution of 4-bromo-benzoyl chloride to THF. Likewise, the same thing was done for mixing urea and 4-(tert-butyl)-benzoyl chloride. It was carried out in an ice bath and stirred with a magnetic stirrer. Furthermore, the mixture is refluxed in a water bath. The reaction is complete if a stain is produced on a paper thin-layer chromatography (TLC) using the solvent system of hexane: chloroform (1:2) for 4Br-BPTU, ethyl acetate: hexane (1:2) for 4TBBU and detected by UV chamber method[18]. Next, THF was evaporated in a rotary evaporator. After that add saturated sodium bicarbonate to the residue, filter the solid product on a Buchner funnel and wash it with a little cold water. Finally, recrystallize it with hot ethanol, filter off the crystals and dry them upon filter paper at temperature 50°C[9,24]. The structure of new compounds was identified using IR spectroscopy, ^{1}H-NMR, ^{13}C-NMR, and HRMS[18,19].

Identification of Primary Breast Cancer Cells:

Breast tissue obtained from the biopsy was isolated to obtain primary cells[25,26]. Then the isolated cells were identified using CD133 biomarkers by immunofluorescence to find out that the isolated cells were cancer cells. Furthermore, HER2 monoclonal antibodies are used to identify primary cells of breast cancer through immunofluorescence.

Cytotoxicity Test:

Primary breast cancer cell cultures were planted in 96 wells plate and incubated for 24 hours in a CO_2 incubator. Then various concentrations of the two test compounds and HU were added. Each concentration was replicated three times. The controlled media used were those that do not contain cells but only culture media. It was incubated again for 24 hours and then each well was added 100µL of MTT reagent 0.5mg/mL followed by incubation for 4 hours[27]. After 4 hours of incubation, the MTT reaction was stopped by adding 100µL 10% SDS 0.01N HCl into each well to solubilize the formazan crystals. The microplate was wrapped in paper and incubated at 37ºC for 24 hours, then its absorbance was read using ELISA reader at λ = 595nm and surviving cell fraction was calculated[22,28,29]. The IC_{50} values of both test compounds and HU as a comparison were obtained using probit analysis from SPSS version 25.0[21].

RESULTS:

The cytotoxic activity of 4Br-BPTU, 4TBBU and HU were predicted using in silico molecular modeling with a rerank score (RS) as the indicator. Compounds that have small RS values are predicted to have a large cytotoxic activity[30,31]. RS values of the test compounds and comparative compound can be seen in Figure 1. The interaction of the compounds with amino acids on the active site of the HER2 receptor is shown in Table 1 and Figure 2.

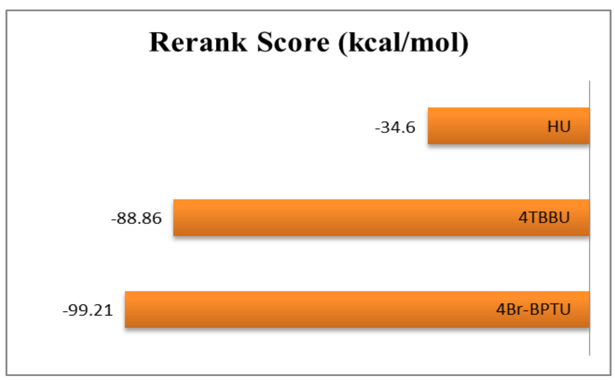


Fig. 1: Rerank Score (RS) of 4Br-BPTU, 4TBBU and HU

Table 1: The Interactions of 4Br-BPTU, 4TBBU, and HU with Amino Acids on the HER2 Receptor (3PP0)

Compound	Amino Acids Interaction													
	Cys 805	Pro 802	Met 801	Leu 800	Ala 751	Thr 798	Gln 799	Val 734	Leu 852	Gly 804	Leu 726	Thr 862	Asp 863	Asn 850
4Br-BPTU	2S	3S	7S	5S	1S	1S	1S	1S	4S	1S	-	-	-	-
4TBBU	1S		1S		1S	1S	1S		5S		1S	1H 1S	-	-
HU	-	-	-	-	-	-	-	-	-	-	-	-	1H	1S

H: hydrogen bond; S: Steric Interactions (Van der Waals and Hydrophobic Bonds)

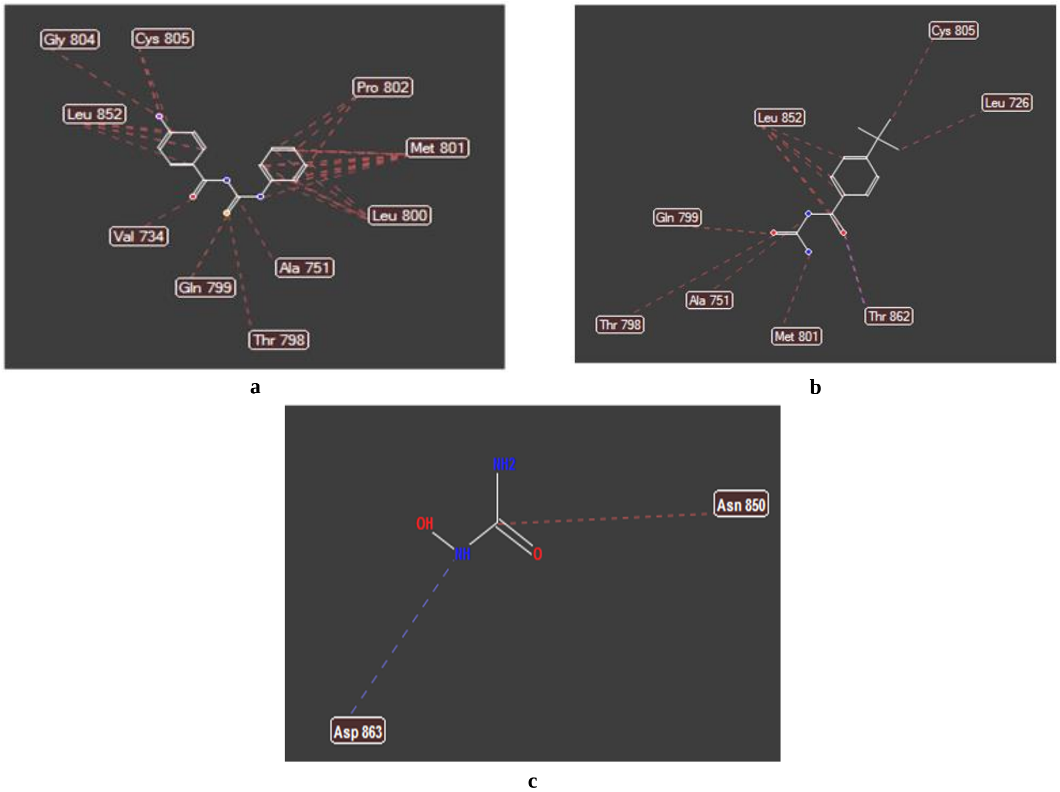
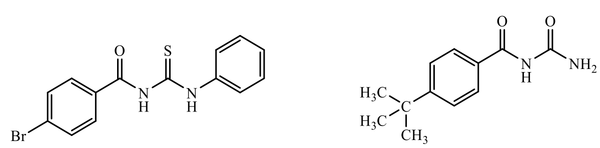


Fig. 2: Hydrogen bonding (blue dashed line) and steric interactions (red dashed line) between the compounds and amino acids on active site HER2 receptor: 4Br-BPTU (a), 4TBBU (b) and HU (c)

After predicting cytotoxic activity through in silico, 4Br-BPTU and 4TBBU compounds were synthesized. The 4Br-BPTU compound was synthesized from 4-bromo-benzoyl chloride with *N*-phenylthiourea and the 4TBBU compound was synthesized from 4-(tert-butyl)-benzoyl chloride with urea. The structure of the synthesis compounds is shown in Figure 3.



N-(4-bromo)-benzoyl-*N*'-phenylthiourea (a) 4-(tert-butyl)-*N*-benzoylurea (b)

Fig. 3: The structure of 4Br-BPTU (a) and 4TBBU (b)

The structure of the synthesized compound identified using the IR, ^{1}H-NMR, ^{13}C-NMR and HRMS spectrometers is as follows:

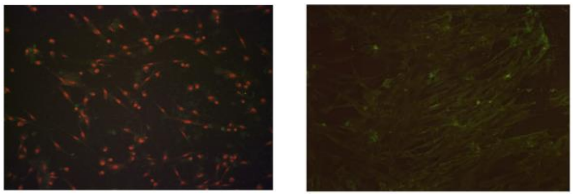
N-(4-bromo)-benzoyl-*N*'-phenylthiourea:

The compound was in the form of yellowish white crystal with a yield of 56 %, m.p 180-182°C. ^{1}H-NMR (DMSO-d_6, 400MHz) δ: 7.28 (t, *J* 7.2 Hz, 1H, Ar-H), 7.43 (t, *J* 7.2 Hz, 2H, Ar-H), 7.69 (d, *J* 8.0 Hz, 2H, Ar-H), 7.76 (d, *J* 9.2 Hz, 2H, Ar-H), 7.92 (d, *J* 9.2 Hz, 2H, Ar-H), 11.67 (s, 1H, O=C-NH-C=S), 12.50 (s, 1H, S=C-NH-Ar). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 124.30 (1C, Ar), 126.33 (2C, Ar), 127.05 (1C, Ar), 128.66 (2C, Ar), 130.75 (2C, Ar), 131.37 (2C, Ar), 131.43 (1C, Ar), 137.94 (1C, Ar), 167.37 (1C, C=O), 179.95 (1C, C=S). IR, υ_{max} (cm^{-1}): 1660 (C=O amide), 1067 and 837 (C=S), 1588 and 1469 (C=C aromatic), 3309 and 1588 (NH strecth secondary amide). HRMS (*m/z*): $C_{14}H_{10}N_2OSBr$ $(M-H)^-$ = 334.9842 and Calc. Mass = 334.9809.

4-(tert-butyl)-*N*-benzoylurea:

This yellowish white crystal compounds has a yield of 29 %, m.p. 168 - 170°C. ^{1}H-NMR (DMSO-d_6, 400MHz) δ: 7.40 (s, 1H, O=C-NH_2(1)), 8.09 (s, 1H, O=C-NH_2(2)), 7.52 (App.d, *J* 8.4 Hz, 2H, Ar-H), 7.92 (App.d, *J* 8.4 Hz, 2H, Ar-H), 10.48 (s, 1H, O=C-NH-C=O), 1.30 (s, 9H, -C-$(CH_3)_3$). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 30.84 (3C, C-(**C**H_3)$_3$), 34.79 (1C, -**C**-$(CH_3)_3$), 155.74 (1C, Ar), 125.32 (2C, Ar), 128.08 (2C, Ar), 129.88 (1C, Ar), 167.88 (1C, O=C-NH), 154.30 (1C, HN-C=O-NH_2). IR, υ_{max} (cm^{-1}): 1692 and 1655 (pair C=O amide), 1605 and 1468 (C=C aromatic), 3321 (NH strecth secondary amide), 3214 and 3148 (NH_2 stretch primary amide). HRMS (*m/z*): $C_{12}H_{17}N_2O_2$ $(M+H)^+$ = 221.1255 and Calc. Mass = 221.1245.

In vitro cytotoxic activity of the test compounds and comparative compound were carried out on primary breast cancer cells. Primary breast cancer cells were identified using CD133 biomarker (Figure 4a) and anti-HER2 monoclonal antibodies using immunofluorescence technique (Figure 4b).



a b

Fig. 4: The identification of CD133 and HER2 in Breast Cancer Cells through the Immunofluorescence Technique. Cells that Express CD133 is Red Fluorescent and cells that express HER2 is green fluorescent: CD133 in breast cancer cells (a), HER2 in breast cancer cells (b)

Figure 5 displays the results of a cytotoxic test in the form of IC_{50} values of 4Br-BPTU, 4TBBU, and HU compounds.

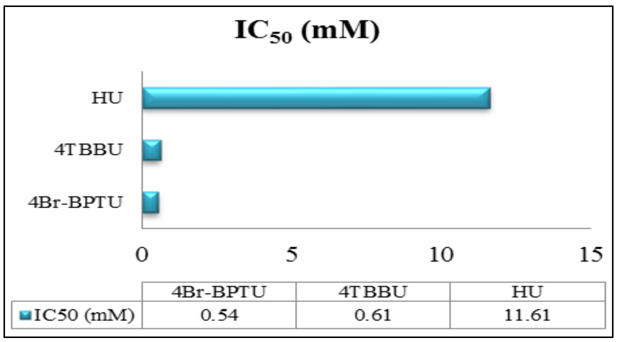


Fig. 5: IC_{50} Values of 4Br-BPTU, 4TBBU, and HU Compounds

DISCUSSION:

Based on Figure 1, the RS values of 4Br-BPTU and 4TBBU were smaller than those of HU. Thus, they were predicted to have better cytotoxic activities than HU as a comparison compound. Meanwhile, the RS value of 4Br-BPTU was smaller than 4TBBU, it means that the cytotoxic activity of 4Br-BPTU was better than 4TBBU. The number of hydrogen bonds and steric interactions illustrates the strength of ligand-receptor bonds. The more number of chemical bonds causes the ligand-receptor bond to be more strength and then affects the biological activity of the compound[18]. Table 1 and Figure 2 depict that 4Br-BPTU had more steric interactions than 4TBBU even without hydrogen bonds. 4TBBU compounds have one hydrogen bond and fewer steric interactions with amino acids at HER2 receptors. Therefore, the RS value of 4Br-BPTU to be smaller than the 4TBBU compound. HU compound had one hydrogen bond and one steric bond so the RS value of hydroxyurea was much higher.

The synthesized compound is purely based on IR spectroscopy,[1] H-NMR,[13] C-NMR and mass spectrum as stated in the results section of this study. Then the synthesis compound was tested in vitro cytotoxic activity on primary breast cancer cells. The primary breast cancer cells were isolated from breast tissue of breast cancer patients. Isolated breast cancer cells were identified using CD133 biomarkers. CD133 or called prominin-1 is a single-chain transmembrane glycoprotein, mainly placed protruding inward from the plasma membrane cell and associated with cholesterol. Cells that express high CD133 show high proliferation ability and differentiation and increase the expression of proteins involved in metastasis and resistance to anticancer drugs. CD133 is expressed in solid tumors so that it is known as an important biomarker to identify and isolate specific cellular subpopulations, which are cancer stem cells (CSC) from several types of cancer including breast cancer[32,33]. Based on Figure 4a can be seen that positive cells express CD133 (red fluorescent) showing that the isolated cells are cancer cells. Thus, this study is focused on breast cancer cells that express HER2. The cells that have been identified as positive with CD133 biomarkers must be tested positive for expressing HER2 by immunofluorescence using HER2 monoclonal antibodies. The human epidermal growth factor receptor (HER) family of receptors plays a central role in the pathogenesis of several human cancers. They regulate cell growth, survival, and differentiation via multiple signal transduction pathways and participate in cellular proliferation and differentiation. Breast cancers can have up to 25 – 50 copies of the HER2 gene, and up to a 40-100-fold increase in HER2 protein resulting in 2 million receptors expressed at the tumor cell surface[34]. Figure 4b shows that cells with green fluorescence are cells that express HER2.

Based on Figure 5, the 4Br-BPTU compound had the smallest IC_{50} value among the three compounds tested. Therefore 4Br-BPTU had the best cytotoxic activity. This is consistent with the RS value and the number of chemical bonds with amino acids on the HER2 receptor. The RS value of 4Br-BPTU was smallest and its number of chemical bonds was much more.

CONCLUSIONS:

N-(4-bromo)-benzoyl-*N*'-phenylthiourea (4Br-BPTU) and 4-(tert-butyl)-*N*-benzoylurea (4TBBU) which have been synthesized had higher cytotoxic activity against primary breast cancer cells than hydroxyurea. *N*-(4-bromo)-benzoyl-*N*'-phenylthiourea was more potent than 4-(tert-butyl)-*N*-benzoylurea. It is highly recommended that *N*-(4-bromo)-benzoyl-*N*'-phenylthiourea be developed further as an anticancer candidate for HER2-positive breast cancer.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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