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Inhibition of Ras and STAT3 activity of 4-(*tert*-butyl)-*N*-carbamoylbenzamide as antiproliferative agent in HER2-expressing breast cancer cells

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Abstract

Objectives: Human epidermal growth factor receptor type 2 (HER2)-expressing breast cancer patients indicate poor prognosis in disease progression. HER2 overexpression can increase activities of Ras-mitogen activated protein kinase (Ras-MAPK) pathway and Janus Kinase (JAK)-STAT3, increasing breast cancer cell proliferation as demonstrated by marker Ki67. Therapeutic options for HER2-expressing breast cancer are limited and have major side effects, so anticancer development as an antiproliferative is needed. From previous research, synthetic chemical 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) compound has cytotoxic activity *in vitro* on HER2-expressing breast cancer cells. This study wanted to determine the mechanism 4TBCB compound in inhibiting HER2 signaling through Rat Sarcoma (Ras) and signal transducer and activator of transcription 3 (STAT3) pathway in HER2-expressing breast cancer cells.

Methods: Breast cancer cells were isolated from the biopsy tissue of breast cancer patients. The isolated cells were cultured and given 4TBCB test compound with three concentrations (0.305, 0.61, and 1.22 mM) and lapatinib 0.05 mM as a comparison compound. Cancer cell cultures were stained with monoclonal antibodies phosphorylated HER2 (pHER2), phosphorylated Ras (pRas), phosphorylated STAT3 (pSTAT3), and Ki67. The expression of pHER2, pRas, pSTAT3, and Ki67 proteins was observed using the immunofluorescence method and the results were compared with control cells, namely cancer cells that were not given 4TBCB and lapatinib but stained with monoclonal antibodies.

Results: 4TBCB compounds (0.61 and 1.22 mM) and lapatinib can reduce pHER2, pRas, pSTAT3, and Ki67 expressions compared to control cells.

Conclusions: 4TBCB compounds (0.61 and 1.22 mM) can reduce pHER2, pRas, pSTAT3, Ki67 expressions and predicted to inhibit HER2 signaling through the Ras and STAT3 pathways in HER2-expressing breast cancer cells.

Keywords: antiproliferative; breast cancer cells; HER2; Ras; STAT3; 4-(*tert*-butyl)-*N*-carbamoylbenzamide.

Introduction

Breast cancer is the second most malignant disease of all types of cancers in the world in 2018 and becomes an alarming disease for women around the world [1]. Twenty-five to thirty percent of patients with breast cancer are expressed with human epidermal growth factor receptor type 2 (HER2) excessively and this correlates with the increase of aggressiveness, poor prognosis, and shorter survival periods [2, 3]. HER2 is a family of human epidermal growth factor receptor (HER) that has a tyrosine kinase activity. HER2 receptor dimerization causes autophosphorylation of tyrosine residues in the cytoplasmic intracellular domain, thus, it activates HER2 signaling pathways, including Ras-mitogen activated protein kinase (Ras-MAPK) pathway, signal transducer and activator of transcription 3 (STAT3) and phosphoinositide-3-kinase-Akt (PI3K-Akt). Activating the HER2 signaling pathway leads to cell proliferation, cell differentiation, adhesion, migration, as well as cell survival [4–6]. HER2 activation enhances the level of Rat Sarcoma (Ras). In a study conducted by Eckert, five out of nine breast cancer cell lines exhibit an increase of the active Ras-GTP level due to HER-2 activation [7]. The active Ras phosphorylates and

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activates the next Raf, and subsequently activates Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase (MEK) and Erk1 and Erk2 [5, 7]. Ras-MAPK pathway involves a protein kinase cascade which contributes to regulate cell proliferation and cell survival [8, 9]. The activation of the STAT3 pathway on HER2 overexpression has been studied by Chung in breast cancer cell culture. His research stated that the STAT3 mRNA expression increased 3.62 times in Michigan Cancer Foundation (MCF)-7/HER2 compared to MCF-7-cell culture [10].

Ki67 is a marker related to cell proliferation assessing the growth of malignant cells. Ki67 is expressed throughout the cell cycle and the peaks during mitosis and at a lesser extent in normal breast tissue from estrogen receptor negative cells than in breast cancer tissue. Therefore, Ki67 can be used as an additional factor for decision making in developing treatment strategies for breast cancer patients [11].

HER2 positive breast cancer therapy is limited, with major side effects. One anticancer used for HER2 positive breast cancer is lapatinib, a tyrosine kinase inhibitor that inhibits the phosphorylation of tyrosine residues in the intracellular domain of the HER2 and EGFR cytoplasmic receptors, which causes inhibition of intracellular signaling pathways that reduce cell proliferation and induce apoptosis [12]. Currently, lapatinib is known to build resistance in a number of breast cancer patients [12, 13]. This is a significant underlying cause for developing new anticancer drugs with maximum therapeutic effect in inhibiting HER2 signaling in the Ras and STAT3 pathways.

Urea-derivative compounds have been extensively developed due to its cytotoxic activity against breast cancer cell lines. The synthesis of *N*-(phenylcarbamothioyl)-benzamide derivatives against MCF-7 cell lines has been reported to have better IC_{50} value compared to hydroxyurea, the least complex derivative of urea [14]. Li et al., synthesized and investigated the structure–activity relationship of *N*-benzyl-*N*-(X-2-hydroxybenzyl)-*N*′-phenylureas derivative compound and thiourea derivatives and found them to potentially inhibit EGFR and HER2 kinase, as well as inhibit MCF-7 cell proliferation [2]. The docking, synthesis, and cytotoxic test against T47D cell lines on *N*-(allylcarbamothioyl) benzamide compounds, yielded better outcome on IC_{50} (56.50 μg/mL) compared to 5-fluorouracil (5FU=132.37 μg/mL) [15].

Another way to develop anticancer drugs is by structure modification. The 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) compound is derived from structure modification of a parent compound *N*-carbamoylbenzamide aiming to enhance its cytotoxic activity. From previous research, it has known that 4TBCB cytotoxic activity of HER2-expressing breast cancer cells using the MTT (Microculture Tetrazolium) method gave an IC_{50} value of 0.61 mM and hydroxyurea with an IC_{50} value of 11.61 mM. This suggests that 4TBCB compounds have better cytotoxic activity to choose compared to hydroxyurea [16]. Therefore, 4TBCB can be used as anticancer candidates for HER2-expressing breast cancer. Meanwhile, this study aims to investigate the mechanism of the 4TBCB as an inhibitor of HER2 signaling on Ras and STAT3 pathway against HER2-expressing breast cancer cells.

Materials and methods

Isolation of breast cancer cells

This research is under the approval of the Health and Research, Ethical Commission of RSUD Dr. Soetomo Surabaya with ethical clearance number 1456/KEPK/VIII/2019. Breast cancer cells were isolated from the biopsy tissue of breast cancer patient, washed with NaCl solution, chopped and given 0.075% collagenase type 1 (Worthington, USA) for 45 min at 37 °C, then filtered with cell strainer. The supernatant obtained was centrifuged at 3,000 rpm for 5 min. Cell pellets were washed with PBS and centrifuged again for 5 min. Cell pellets were cultured in alpha Minimal Essential Medium (MEM) (Gibco, USA) with the addition of 10% FBS (Gibco, USA), 1% L-glutamine (Gibco, USA), 1% penicillin–streptomycin (Gibco, USA), and 1% amphotericin B (Sigma-Aldric, USA), then incubated in a CO_2 incubator at 37 °C. The medium was replaced every 3 days and after the cells reached 90%, confluent cell passage was carried out [17]. After isolation, cells were identified against cell surface markers (CD24, CD44, and CD90) by flow cytometry to ensure that the isolated cells were breast cancer cells. The cells were identified using HER2 immunofluorescence monoclonal antibody.

Expressions of pHER2, pRas, pSTAT3, and Ki67 protein using immunofluorescence assay

The 4TBCB was used as test compound and lapatinib as compared. The 4TBCB was made in three concentrations of 0.305 mM ($0.5 \times IC_{50}$), 0.61 mM ($1 \times IC_{50}$), and 1.22 mM ($2 \times IC_{50}$), while lapatinib 0.05 mM ($1 \times IC_{50}$) was based on *in vitro* cytotoxicity tests using the MTT method. From the cytotoxicity test, the IC_{50} values of 4TBCB and lapatinib were 0.61 mM ($1 \times IC_{50}$) and 0.05 mM ($1 \times IC_{50}$), respectively [16]. The protein expressions observed were pHER2, pRas , pSTAT3 and Ki67. Each protein expression was observed in five groups of cancer cells consisting of three groups of cancer cells given 4TBCB compounds with concentrations of 0.305, 0.61, and 1.22 mM. Meanwhile, one group of cancer cells was given 0.05 mM lapatinib, and one control group of cancer cells was not given 4TBCB and lapatinib, but only given monoclonal antibodies (pHER2, pRas, pSTAT3, and Ki67). Each group of cells was made on five replications.

Breast cancer cells were planted on 24 well plates totaling 10^5 cells/1,000 μL/well, then incubated in a CO_2 incubator at 37 °C until reaching 90% confluent. Cell cultures that have reached 90% confluent were given 4TBCB and lapatinib according to the prepared concentrations, then incubated again for 24 h, followed by cell culture fixation using methanol for 15 min, then washed twice with PBS (Sigma Aldrich, USA) – tween 0.2%. The cells were then given

TritonX100 (Sigma Aldrich, USA) 0.5% for 5 min to cell permeabilization and washed with 0.2% PBS tween three times for 1 min. Furthermore, the cells were given a blocking solution of 1% BSA (Sigma Aldrich, USA) for 30 min, then washed with 0.2% PBS tween and stained with monoclonal antibodies (Biolegend). The monoclonal antibodies were diluted in a ratio of 1:100 in BSA. Cells that have been stained with monoclonal antibodies were then incubated overnight in a container wrapped in aluminum foil. Furthermore, the cells were given secondary antibody labeled FITC and incubated for 1 h. The results were observed using a 100× fluorescence microscope (Automated Fluorescence Microscope, BX63, Olympus, USA) magnification [17]. If the cells express the pHER2, pRas, pSTAT3, and Ki67 proteins, the cells will fluoresce green. Fluorescence microscopy was observed over 10 large viewing areas for each cell replication. In the immunofluorescence method, negative control cells were needed to validate the method. Negative control cells were cancer cells that were not given 4TBCB, lapatinib, and monoclonal antibodies. Negative control cells must not provide fluorescence to certify that the immunofluorescence method is valid, these cells were not included in the study cell group, but only to validate the method.

Statistical analysis

Immunofluorescence visualization images were transferred into the ImageJ program to obtain quantitative data in numerical form, but there is no unit for data [18, 19]. The numerical data obtained were analyzed statistically using one way ANOVA or Kruskal Wallis depending on the normality and homogeneity of the data, and then continued with the post hoc test with the Tukey or Mann-Whitney test. The statistical analysis was performed using the SPSS version 25 program and the data was said to be statistically significant if the p value was ≤0.05.

To examine the relationship between pHER2, pRas, pSTAT3, and Ki67 variables in predicting the mechanism of action of 4TBCB, path analysis was used. From the path analysis, the β coefficients and p value will be obtained. The coefficient β shows the magnitude of the direct effect on a variable and p value shows the significance of the effect on that variable.

Results

Identification of breast cancer cells by flow cytometry and immunofluorescence is shown in Figures 1 and 2.

Figure 1 shows the number of cancer cells in the right upper quadrant, which express a combination of cell surface markers. Cells that express CD24, CD44, and CD90 are indicated as breast cancer cells. CD24, CD44, and CD90 are cell surface markers commonly found in solid tumor, one of which is breast cancer [20, 21].

Figure 2 shows that breast cancer cells fluoresce green, meaning that they express the HER2 protein. The immunofluorescence visualization of breast cancer cells expressing pHER2, pRas, pSTAT3, and Ki67 proteins and mean graph of target protein expression are shown in Figures 3–6.

Statistical analysis of pHER2 expression tested using Kruskal Wallis showed significant difference in the 95% confidence level between groups of breast cancer cells, and Mann Whitney test showed no significant difference ($p>0.05$) between the control group and the 4TBCB 0.305 mM group. This shows that 4TBCB 0.305 mM compound does not inhibit HER2 phosphorylation, so that it does not decrease pHER2 expression in breast cancer cells. The 4TBCB 0.61 mM group, 4TBCB 1.22 mM group, and the lapatinib group were significantly different from the control cell group. This suggests that 4TBCB 0.61, 1.22 mM, and lapatinib can inhibit HER2 phosphorylation thus reducing pHER2 expression. The decrease in pHER2 expression was in line with the increase in the concentration of 4TBCB (Figure 3F).

The pRas expression analyzed using one way ANOVA showed a significant difference in the 95% confidence level between cell groups, followed by the Tukey test showing significant difference ($p\leq0.05$) between all the 4TBCB compound groups and the control cell group, as well as the lapatinib group was significantly different from the control cell group. This suggests that 4TBCB compounds ranging at 0.305–1.22 mM can inhibit intracellular signals that inhibit Ras phosphorylation thus reducing pRas expression. Lapatinib can also decrease pRas expression in breast cancer cells. The decrease in pRas expression along with an increase in the concentration of 4TBCB is shown in Figure 4F.

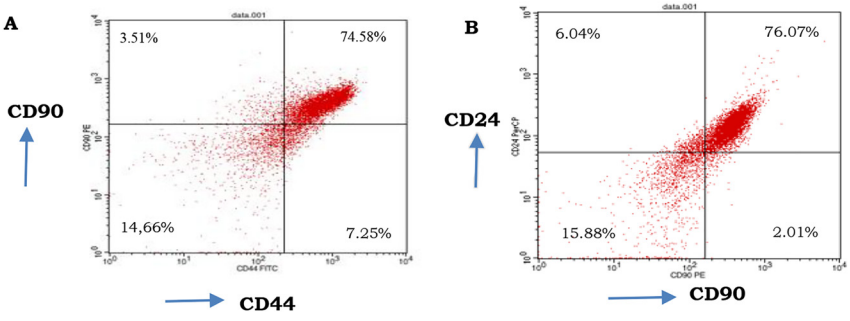

Figure 1: Identification of breast cancer cells by flow cytometry. Breast cancer cells expressing CD44/CD90 (A). Breast cancer cells expressing CD90/CD24 (B).

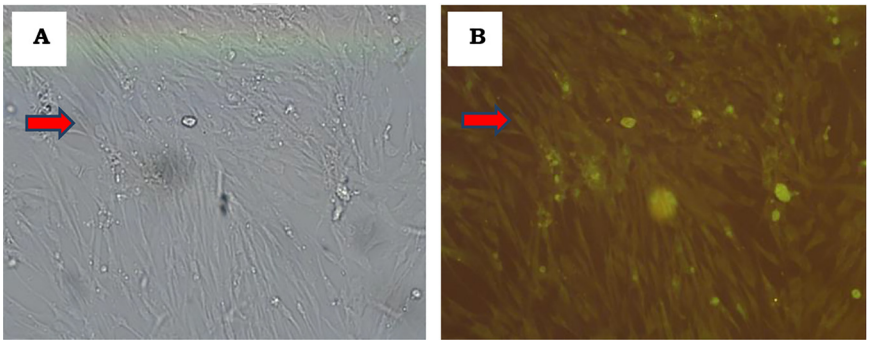

Figure 2: Identification of breast cancer cells expressing human epidermal growth factor receptor type 2 (HER2) by immunofluorescence. Phase-contrast (A) and immunofluorescence visualization (B) of HER2-expressing cells.

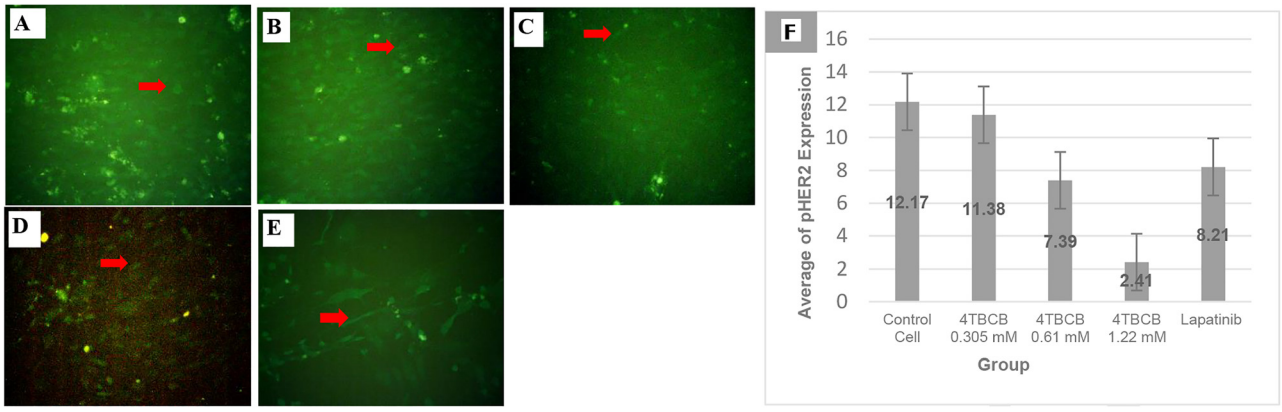

Figure 3: Visualization of immunofluorescence of breast cancer cells expressing phosphorylated human epidermal growth factor receptor type 2 (pHER2) and mean graph pHER2 expression. The red arrow shows breast cancer cells that express pHER2. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of pHER2 protein expression in breast cancer cells (F).

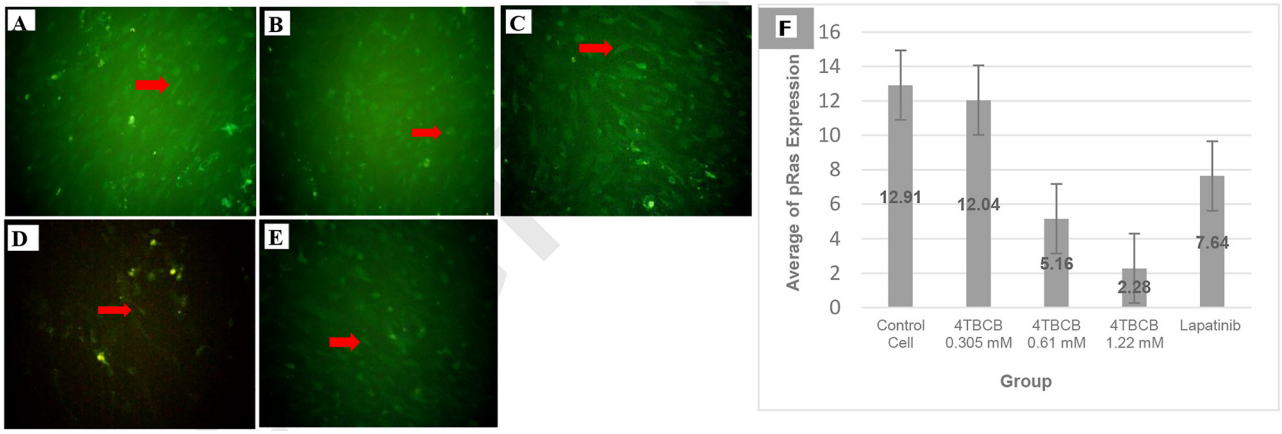

Figure 4: Visualization of immunofluorescence of breast cancer cells expressing phosphorylated Ras (pRas) and mean graph pRas expression. The red arrows indicate breast cancer cells that express pRas. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of pRas protein expression in breast cancer cells (F).

Statistical calculation of pSTAT3 expression tested using one way ANOVA showed a significant difference in the 95% confidence level between groups of breast cancer cells, followed by Tukey test showing no significant difference between the 4TBCB 0.305 mM group and the control cell group. This indicates that 4TBCB

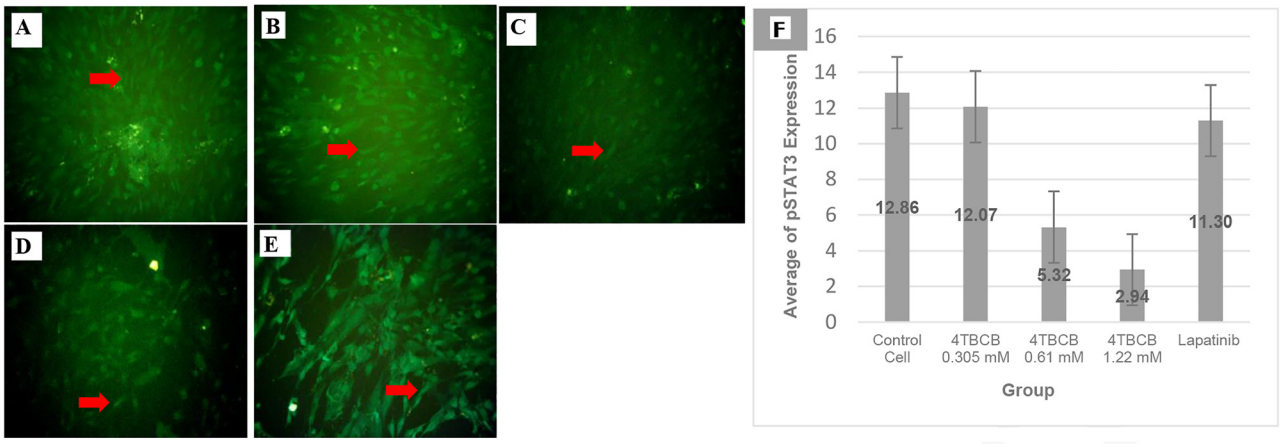

Figure 5: Visualization of immunofluorescence of breast cancer cells expressing phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and mean graph pSTAT3 expression. The red arrows indicate breast cancer cells that express pSTAT3. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of pSTAT3 protein expression in breast cancer cells (F).

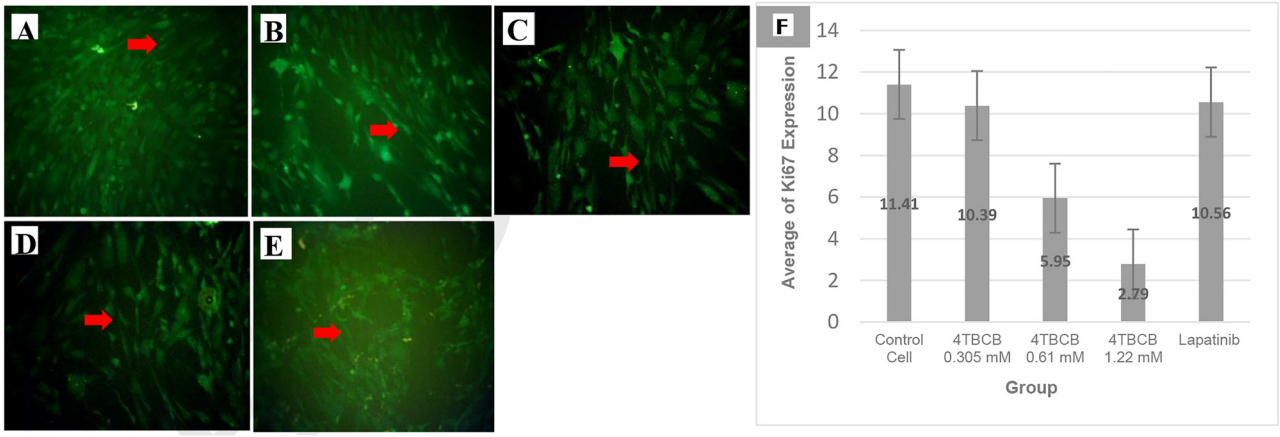

Figure 6: Visualization of immunofluorescence of breast cancer cells expressing Ki67 and mean graph Ki67 expression. The red arrows indicate breast cancer cells that express Ki67. Immunofluorescence visualization of control cells (A); 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) 0.305 mM group (B); 4TBCB 0.61 mM group (C); 4TBCB 1.22 mM group (D); lapatinib group (E); and mean graph of Ki67 protein expression in breast cancer cells (F).

0.305 mM compound does not inhibit intracellular signaling so that it does not inhibit STAT3 phosphorylation; as a result pSTAT3 expression does not decrease in breast cancer cells. The 4TBCB 0.61 mM, 4TBCB 1.22 mM, and lapatinib groups were significantly different from the control cell groups, so, it can be said that the compound 4TBCB 0.61 mM, 4TBCB 1.22 mM, and lapatinib can reduce the pSTAT3 expression in breast cancer cells (Figure 5F).

Statistical analysis using Kruskal Wallis on Ki67 expression showed a significant difference in the 95% confidence level between groups of breast cancer cells, followed by the Mann Whitney test showing no significant difference between the 4TBCB 0.305 mM and lapatinib and the control cell group. This means that 4TBCB 0.305 mM compound cannot reduce the Ki67 expression as well as lapatinib. The 4TBCB 0.61 mM and 4TBCB 1.22 mM groups were significantly different from the control cell group, meaning that the two groups of 4TBCB compounds could reduce Ki67 expression and decrease Ki67 expression along with the increase in the concentration of 4TBCB compounds (Figure 6F).

The relationship between variables was analyzed using path analysis to determine the mechanism of action of the 4TBCB compound. The path analysis chart is shown in Figure 7. Prediction of HER2 signaling inhibition on the Ras and STAT3 pathway due to 4TBCB administration is shown in Figure 8.

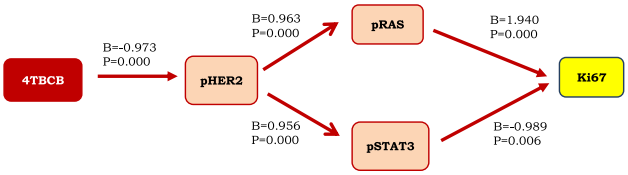

Figure 7: The path analysis of 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) compound.

Based on Figure 7, the 4TBCB compound has a negative β coefficient value (−0.973) and is significant to the pHER2 expression (p = 0.000). This means that 4TBCB has a direct effect on the pHER2 expression by reducing the pHER2 by −0.973. The decrease in pHER2 expression also has a direct effect on the decrease in praise and pSTAT3 expressions by 0.963 and 0.956, respectively. The decrease in pRas and pSTAT3 expressions directly affects the decrease in Ki67 expression.

Figure 8 is a prediction of the mechanism of action of 4TBCB in inhibiting HER2 signaling through the Ras and STAT3 pathways in breast cancer cells. By inhibiting HER2 protein phosphorylation, it is predicted that there will be inhibition of intracellular signal through the Ras and STAT3 pathways so that a decrease in cancer cell proliferation is indicated by a decrease in Ki67 expression.

Discussion

Figure 3F proves that 4TBCB 0.61 and 1.22 mM compounds work by inhibiting HER2 phosphorylation in the intracellular domain so that there is a decrease in pHER2 expression. The parameter of physical–chemical properties that plays the most important role in the distribution process of drugs in the body is the lipophilic parameter. Lipophilic parameters often used are the logarithm of the partition coefficient (log P) and the Hansh–Fujita π constant. The log P prediction of 4TBCB using the ChemDraw program was 2.26, while the *N*-carbamoylbenzamide parent compound had a predictive log P 0.56. Based on the value of the substituted constant used in aromatic substitution according to the Topliss approach model, the 4-t-butyl group has lipophilic properties with a value of π = 1.98 [22]. The presence of this group increased the lipophilic properties of the 4TBCB. Therefore, log P and π constant predicted that 4TBCB has good lipophilic properties to penetrate cell membranes well. The decreased expression of pHER2 protein causes an intracellular signal to phosphorylate Ras and STAT3 to be inhibited, thereby inhibiting DNA replication, which results in decrease in proliferation of HER2-expressing breast cancer cells.

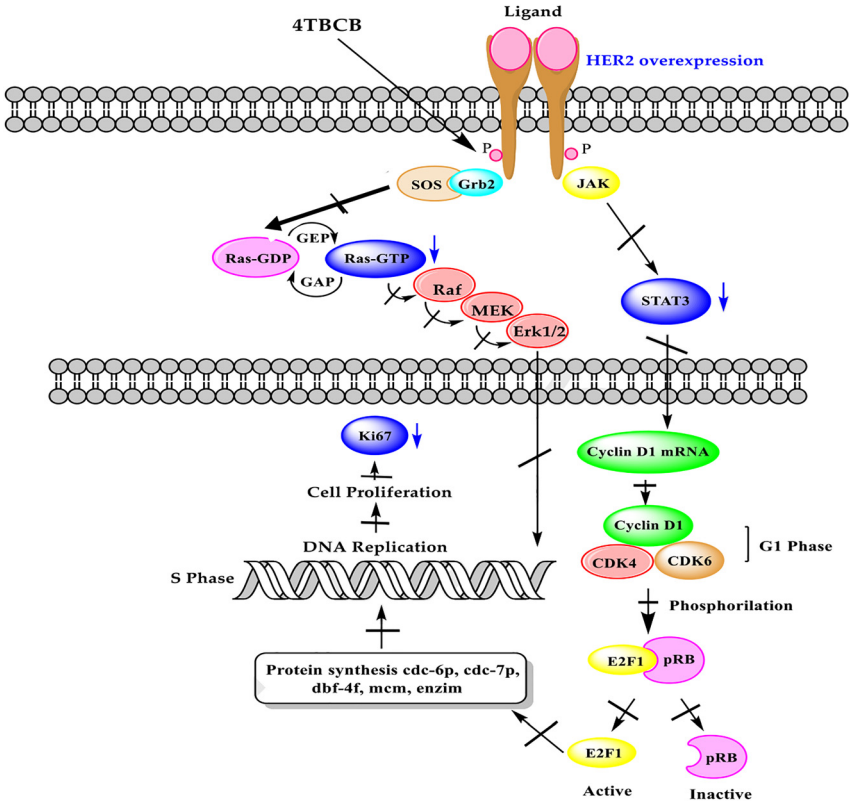

Figure 8: Prediction of HER2 signaling inhibition on the Ras and signal transducer and activator of transcription 3 (STAT3) pathway due to 4-(*tert*-butyl)-*N*-carbamoylbenzamide (4TBCB) administration.

The 4TBCB ranging from 0.305 to 1.22 mM can decrease the pRas protein expression. The decrease in pRas protein expression is in line with the increase in the concentration of 4TBCB compound (Figure 4F). Ras genes encode Ras protein that play a role in intracellular signal transduction to trigger cell multiplication. This protein displays different conformational forms, Ras-GDP (Ras-Guanine Diphosphate) when inactive, and Ras-GTP (Ras-Guanine Triphosphate) while active. The balance between Ras-GDP and Ras-GTP is regulated by guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP). Ras-GDP (in the "off" state) is transformed into Ras-GTP (in the "on" state) by the GEF enzyme. On the contrary, the "on" to "off" position is regulated by the enzyme GAP [23–25]. In breast cancer, Ras is activated by overexpressing HER2 and activating signal transduction to the nucleus by simultaneously activating the target effectors (RAF, MEK, and Extracellular Signal-Regulated Kinase [Erk]). The continuous activation of Ras-MAPK triggers an increase in the number of DNA replications and cell proliferation [5, 7]. Therefore, 4TBCB was proven to inhibit HER2 phosphorylation, which inhibits the activation of Ras pathway and causes the pRas expression to decrease.

The pSTAT3 expression analysis proved that 4TBCB at 0.61 and 1.22 mM inhibited HER2 intracellular signals which resulted in inhibition of STAT3 phosphorylation and decreased pSTAT3 expression (Figure 5F). STAT3 is a transcription factor that regulates transcription and gene expression in cellular processes. STAT3 can be activated in all breast cancer subtypes. Various tyrosine kinase receptors (EGFR, HER2, and VEGF) can phosphorylate STAT3 in breast cancer [11, 26].

The Ki67 expression analysis showed a decrease in Ki67 expression in line with the increase in concentration of the 4TBCB compound (0.61 and 1.22 mM) (Figure 6F). Ki67 is a core protein associated with cell proliferation, expressed in the cell cycle in the S, G1, G2, and M phases in the nucleus. Ki67 expression differs from phase-to-phase and peaks during mitosis. Therefore, Ki67 is used as a marker to evaluate cell proliferation to determine specific therapy for each patient [11, 27]. The classification in molecular subtypes of breast cancer states that the luminal B and HER2 overexpression had the highest Ki67 index in accordance with a study conducted by Hashmi and Ragab on patients with breast cancer in hospitals [11, 27]. This research suggested that 4TBCB lowered Ki67 expression led to lower proliferation of HER2-expressing breast cancer cells.

Lapatinib is clinically used as anticancer for HER2-positive breast cancer patients. Lapatinib is a tyrosine kinase inhibitor that selectively inhibits HER2 and EGFR [28]. The analysis results shown in Figures 3F–5F prove that lapatinib can reduce the expression of pHER2, pRas, and pSTAT3. This study has shown that lapatinib did not decrease Ki67 expression, possibly because there are other intracellular signaling pathways other than the Ras and STAT3 pathways that can activate cell proliferation so that Ki67 expression remains high with lapatinib administration. In this study, 4TBCB was compared with lapatinib which is clinically used as anticancer, so it can be said that 4TBCB has a potential to be an anticancer. However, 4TBCB required a greater concentration in reducing pHER2, pRas, pSTAT3, and Ki67, namely 0.61 mM ($1 \times IC_{50}$) compared to lapatinib at 0.05 mM ($1 \times IC_{50}$).

The mechanism of action of the 4TBCB can be explained using path analysis between variables. Based on the results of path analysis, 4TBCB is predicted to work through inhibition of HER2 signaling on the Ras and STAT3 pathways in HER2-expressing breast cancer cells.

The 4TBCB compound as a whole is predicted to inhibit HER2 signaling in the Ras and STAT3 pathways shown in the graph in Figure 8. Inhibition of HER2 phosphorylation inhibits Ras protein activation thus Raf effectors are not recruited and subsequently do not activate Mek1/2 and Erk1/2. Erk1/2 is a transcription factor that plays a role in the synthesis of proteins required for DNA replication in cancer cells. Inhibited Erk1/2 activation will decrease DNA replication, thus decreasing cell proliferation. The inhibition of HER2 phosphorylation also inhibits Janus Kinase (JAK) activation, thereby inhibiting STAT3 phosphorylation. STAT3 phosphorylation increases cyclin D1 mRNA expression, causing the formation of complex cyclin D1 bonds with CDK4 and CDK6 [29, 30]. These complex bonds cause pRB phosphorylation and then activate transcription factor E2F1, which mediate the synthesis of cdc 6p protein needed to trigger the formation of O replication bubbles that DNA replication occurs [25]. The inhibition of STAT3 activation due to HER2 phosphorylation inhibition causes DNA replication to decrease and cancer cell proliferation will also decrease. Therefore, 4TBCB compound can be said to be a candidate for antiproliferative agent for HER2-expressing breast cancer cells.

Conclusions

4TBCB compounds at concentrations of 0.61 and 1.22 mM have been shown to reduce the expression of pHER2, pRas,

pSTAT3, and Ki67 proteins and are thought to have an inhibitory mechanism of HER2 signaling through the Ras and STAT3 pathways on HER2-expressing breast cancer cells.

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Competing interests: Authors state no conflict of interest.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by Ethical Commission of General Hospital in Surabaya, Indonesia.

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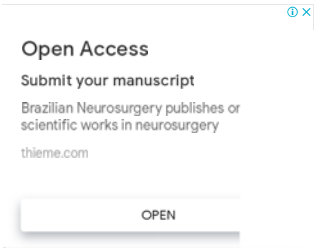

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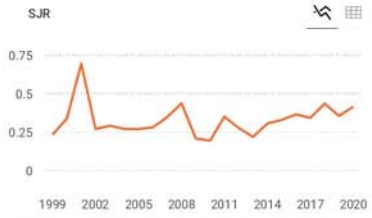

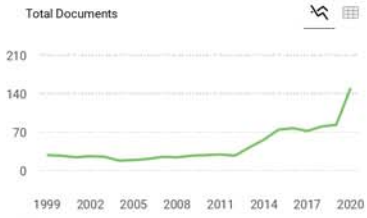

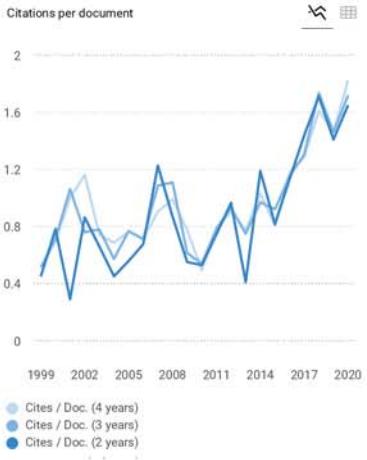

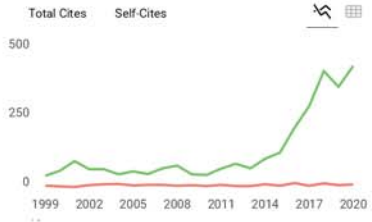

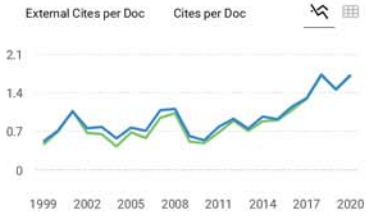

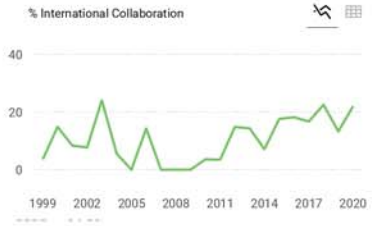

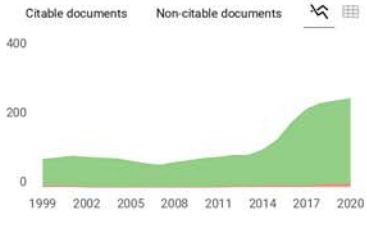

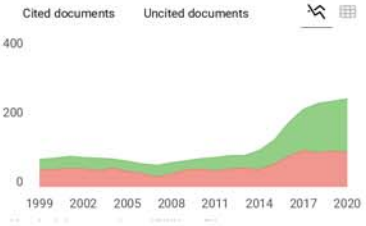

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