Inhibition of Ras and STAT3 Activity of 4-((Tert-Butyl)-N-carbamoylbenzamide
As Antiproliferative Agent in HER2-expressing Breast Cancer Cells

Aquilina Kirti Intan1, Siswandoen Siswandoen2, I Ketut Sudiana3, Desak Gede Agung Suprawati4, Ariolika Dinarayanti5

1 Department of Clinical and Community Pharmacy, Faculty of Pharmacy, University of Sari, Sari, East Java, Indonesia
2 Department of Chemistry, Faculty of Pharmacy, University of Airlangga, Surabaya, Indonesia
3 Department of Pathology, Faculty of Medicine, University of Airlangga, Surabaya, Indonesia
4 Department of Oncology Surgery, Dr. Sardjito General Hospital, Yogyakarta, Indonesia
5 Stem Cell Research and Development Center, Airlangga University, Surabaya, Indonesia

Introduction
Breast cancer is the second most malignant disease of all types of cancers in the world in 2018. It has been an alarming illness for women across the globe [IARC, 2018]. 25-30% of patients with breast cancer were expresses with steroidogenic factor receptor type 2 (HER2) excessively and this was correlated with the increase of aggression, metastasis and shorter period of survival [Hawthorne et al., 2009; Li et al., 2010; Adamczyk et al., 2017]. HER2 activation was adduced to be the active Ras phosphorlyates and activates the next Raf, and subsequently activates MEK and Erk1 and Erk2 [Iqbal and Iqbal, 2014; Eckert et al., 2004]. Raf-MAPK pathway involves protein kinase cascade which contributes to regulate cell proliferation and cell survival [Zhang and Liu, 2002; Scaltriti and Baselli, 2006]. The activation of the STAT3 pathway on HER2 overexpression has been studied by Chung in breast cancer cell culture. His research stated that STAT3 mRNA expression increased 3.62 times in MCF-7/HER2 compared to MCF-7 cell culture [Chung et al., 2014].

The (Tert-butyl)-N-carbamoylbenzamide (4TBCB) compound is this study. Harvesting cells are modification of a mother compound N-carbamoylbenzamide aiming to enhance its cytotoxic activity. The earlier studies concluded that the cytotoxic activity of 4TBCB compound toward HER2-expressing breast cancer cells using MTT (Microculture Tetrazolium) method demonstrated better results compared to those in hydroxyza [Kirti Intan et al., 2021]. This enables the compound to be used as an anticancer candidates for HER2-expressing breast cancer. This current study, on the other hand, aims at investigating the mechanism of 4TBCB compound as the inhibitor of HER2 signaling on Ras and STAT3 pathway against HER2-expressing breast cancer cells.

Materials and Methods
Harvesting HER2-expressing Breast Cancer Cells
Breast cancer cells are isolated from the biopsy tissue of breast cancer patient. Furthermore, cells were identified using immunofluorescence monoclonal antibody HER2. If breast cancer cells express HER2 it will be used in this study. Morphology of breast cancer cells were induced whenever confluence reaches 90%. Cells were rinsed 2 times in PBS and Trip and resuspended. Once separated, the cells were transferred to a sterile conical and 1 mL medium alpha MEM (Sigma Aldrich, USA) was added to the pellet medium [Dinarayanti et al., 2019].

Expressions of pHER2, pRas, pSTAT3 and Ki67 protein using immunofluorescence assay
HER2-expressing breast cancer cells that had been harvested were seeded in a 24-well plate equipped with a coverslip. The number of the seeded cells were 104 cells, each 1000 µL/well. The protein expressions to be examined are pHER2 (phosphorylated HER2), pRas (phosphorylated Ras), pSTAT3 (phosphorylated STAT3) and Ki67. Each of the protein expressions on 5 cancer groups, there were 3 cell groups added with 4TBCB treatment in 0.5x10^5 (0.305 mM), 1x10^5 (0.61 mM) and 2x10^5 (1.22 mM) concentration, 1 cell group was given lapatinib treatment (comparison compound) 1x10^5 (0.05 mM), and 1 group of control cells without being given the test and comparison compounds. Each of the groups was replicated 5 times. The result was observed under fluorescence microscope with 100x magnification (Automated Fluorescence Microscope, BX63, Olympus, USA) [Dinarayanti et al., 2019]. Green emitted fluorescent occurs whenever the cells express protein pHER2, pRas, pSTAT3 and Ki67.

Results and Discussion
Identification of breast cancer cells by immunofluorescence is show in Figure 1. Some images of pHER2, pRas, pSTAT3, Ki67 protein expressions and negative control cells (without staining monoclonal antibodies) in immunofluorescence assay are displayed on Figure 2.

Figure 1: Identification of breast cancer cells that express HER2 by immunofluorescence. Phase contrast (A) and immunofluorescence visualization (B) of HER2-expressing cells.

Based on Figure 1, it shows that the isolated cells are breast cancer cells that express HER2.

Figure 2: Photo visualization of immunofluorescence of breast cancer cells expressing target protein in the groups of 4TBCB 0.5x10^5, control cells, lapatinib and negative control cells. Cells expressing pHER2 (A). Cells expressing pRas (B). Cells expressing pSTAT3 (C). Cells expressing Ki67 (D). Red arrows show breast cancer cells that express target proteins.

Data of pHER2, pRas, pSTAT3 and Ki67 protein expressions are listed on table 1. The average expressions of pHER2, pRas, pSTAT3 and Ki67 against HER2-expressing breast cancer cells are illustrated in bar chart as shown on Figure 3. Data collected from fluorescent microscopy were transferred in ImageJ programme [Handala et al., 2019; Jensen, 2013] for the purpose of quantification.

Table 1: Fold changes of pHER2, pRas, pSTAT3 and Ki67 protein expression to control cancer cells.

![Figure 3](image_url): The average of pHER2, pRas, pSTAT3 and Ki67 protein expressions on each group of HER2-expressing breast cancer cells

The 4TBCB compound has a mechanism of action to inhibit HER2 signaling through the Ras and STAT3 pathways, thereby reducing proliferation of HER2-expressing breast cancer cells.

Figure 4: The path analysis of 4TBCB compound

The illustration chart of HER2 signaling inhibition on the Ras and STAT3 pathway due to 4TBCB administration is shown in figure 5.

Figure 5: The illustration chart of HER2 signaling inhibition on the Ras and STAT3 pathway due to 4TBCB administration

Conclusions
4TBCB Compound has been shown to work to reduce the expression of pHER2, pRas, pSTAT3, and Ki67 protein and has a mechanism of inhibiting HER2 signaling on Ras and STAT3 pathways in HER2-expressing breast cancer cells.