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# Anti-proliferative effects of pentagamaboronon-0-sorbitol on HER2-overexpressing breast cancer cells

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**ABSTRACT** HER2-positive breast cancer is an aggressive form of the disease that is associated with poor prognosis and chemo-resistance. As such, investigation continues into the development of a new HER2-targeted drug for breast cancer. This study investigated the anti-proliferative activities of pentagamaboronon-0-sorbitol (PGB-0-So) in HER2-overexpressing breast cancer (MCF-7/HER2) cells. The cytotoxicity of PGB-0-So was assessed via MTT assay. Flow cytometry with propidium iodide and annexin-V-FITC staining was conducted to investigate the mechanism of PGB-0-So in inhibiting the proliferation of MCF-7/HER2 cells. Finally, FACS analysis with 2′,7′–dichlorofluorescin diacetate staining was performed to examine intracellular ROS production. PGB-0-So exerted cytotoxicity towards MCF-7/HER2 breast cancer cells with an $IC_{50}$ value of 36 µM. PGB-0-So induced S-phase arrest and apoptosis in MCF-7/HER2 cells. Moreover, PGB-0-So could increase intracellular ROS production in MCF-7/HER2 cells. PGB-0-So exerted anti-proliferative activity towards MCF-7/HER2 cells. This compound may be developed as a chemotherapeutic agent against HER2-overexpressing breast cancer.

**KEYWORDS** anti-proliferative; breast cancer; HER2; PGB-0-So

## 1. Introduction

Breast cancer, a type of cancer with high prevalence, is the leading cause of death in women. Approximately 25–30% of all breast cancers show human epidermal growth factor receptor 2 (HER2) amplification (Mendes et al. 2015). HER2-positive breast cancer is an aggressive form of the disease that is significantly associated with poor prognosis (Ross et al. 2009), chemoresistance (Yokoyama et al. 2006), short survival, and high patient mortality (Mendes et al. 2015).

HER2-targeted cancer therapy is a major consideration in the development of chemotherapeutic agents for breast cancer. Several HER2-targeted drugs/antibodies, including trastuzumab (Junttila et al. 2011) and pertuzumab (De Mattos-Arruda and Cortes 2013), have been developed and accepted by the Food and Drug Administration of The United States. However, the targeted therapy of HER2-positive breast cancer with anti-HER2 agents, such as trastuzumab, has been reported to cause resistance in cancer cells (Luque-Cabal et al. 2016). Therefore, the continuous development of new chemotherapeutic agents targeting HER2 remains an urgent endeavor.

A new curcumin analog compound, namely 2,5-bis(4-boronic acids) benzylidine cyclopentanone or pentagamaboronon-0 (PGB-0), was successfully synthesized at the Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. PGB-0 is a boron-substituted compound that was initially synthesized for boron neutron capture therapy. Docking analysis showed that PGB-0 could interact with HER2 protein and reduce its expression in MCF-7/HER2 breast cancer cells (Utomo et al. 2017); the compound has also demonstrated anti-metastatic activity in 4T1 cells (Kusumastuti et al. 2019). However, similar to curcumin, PGB-0 has low solubility. In the present study, PGB-0 was formulated with the sugar alcohol sorbitol to obtain a product, hereafter referred to as PGB-0-So, with increased solubility in water. PGB-0-So is more soluble than PGB-0. The formulation of PGB-0 with sorbitol could also increase the cellular uptake of the resultant compound via the specific interaction of sorbitol with caveolar proteins, which are overexpressed in most cancer cells, including MCF-7 breast cancer cells (Waalkes et al. 2011; Nguyen et al. 2014). The increased cellular uptake/accumulation of PGB-0-So in

cells may be expected to increase the anti-cancer effect of this substance. PGB-0-So could prevent the metastasis of 4T1 triple-negative (Ramadani et al. 2018) and MCF-7/HER-2 breast cancer (Qodria et al. 2018) cells. PGB-0-So was also recently found to induce apoptosis in 4T1 cells (Ramadani et al. 2021). Thus, in the present study, the anti-cancer activity of PGB-0-So is tested on MCF-7/HER-2 cells.

## 2. Materials and Methods

### 2.1. Chemicals

PGB-0 was synthesized as previously described (Utomo et al. 2017). PGB-0-So was also prepared as previously described (Hermawan et al. 2019).

### 2.2. Cell cultures

MCF-7/HER2 cells were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology, Japan. The cells were cultured in high-glucose DMEM (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma–Aldrich), 1.5% (v/v) penicillin–streptomycin (10,000 U/mL penicillin, 10,000 µL/mL streptomycin; Gibco®), and 0.5% (v/v) Fungizone (Gibco) and incubated at 37 °C in a humidified incubator with 5% $CO_2$. The MCF-7/HER2 cells were passaged every 3–4 days.

### 2.3. Cytotoxicity assay

Cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, the cells were seeded at a density of $2\times10^3$ cells/well in 96-well plates and grown for 24 h. The spent medium was replaced with a medium containing the PGB-0-So at various concentrations, and the plates were incubated for another 24 h. On the next day, the cells were washed once with 1× phosphate-buffered saline (PBS), added with medium containing MTT solution (Sigma, USA), and incubated for 2 h. Ten percent of sodium dodecyl sulfate in 0.01 N HCl solution was added to each well, and the plates were incubated overnight in the dark. The absorbance of each well was measured using a microplate reader (BioRad, USA) at 595 nm.

### 2.4. Cell cycle analysis

Cells were seeded at a density of $2\times10^5$ cells/well in 6-well plates and grown for 24 h. The medium was replaced with medium containing the PGB-0-So at concentrations of 36 and 54 µM, and the mixture was incubated for another 24 h. Cells were harvested by trypsinization, centrifuged at 2,000 rpm for 3 min, washed once with cold PBS, fixed with 70% ethanol, and then incubated at 4 °C for 30 min. The cells were washed with cold PBS once more, stained with propidium iodide (PI) reagent containing 20 g/ml RNAse and 0.1% Triton-X 100, and counted by a FACS Calibur flow cytometer.

### 2.5. Apoptosis Assay

Cells were seeded at a density of $2\times10^5$ cells/well in 6-well plates and grown for 24 h. Then, the cells were treated with the PGB-0-So at particular concentrations based on the $IC_{50}$ value. After 24 h of incubation, cells were harvested by trypsinization and washed once with cold PBS. The cells were stained using an Annexin-V-FLUOS Staining Kit (Roche, China) containing the binding buffer, annexin-V, and PI, and then incubated for 10 min in the dark according to the manufacturer's instruction. Finally, the cell distribution was analyzed using a FACS Calibur flow cytometer.

### 2.6. Intracellular ROS Production Assay

Intracellular reactive oxygen species (ROS) production was examined according to a previous study (Larasati et al. 2018). Briefly, the cells were seeded at a density of 5×104 cells/well in a 24-well plate, incubated for 24 h, harvested in supplemented buffer (10% FBS in 1× PBS), and stained with 2',7'-dichlorofluorescein diacetate (DCFDA) at the final concentration of 25 µM. After 30 min of incubation, the cells were treated with particular concentrations of PGB-0-So based on the $IC_{50}$ value and 100 µM of $H_2O_2$ as the positive control, then incubated for another 4 h. Intracellular ROS production was analyzed using a FACS Calibur flow cytometer.

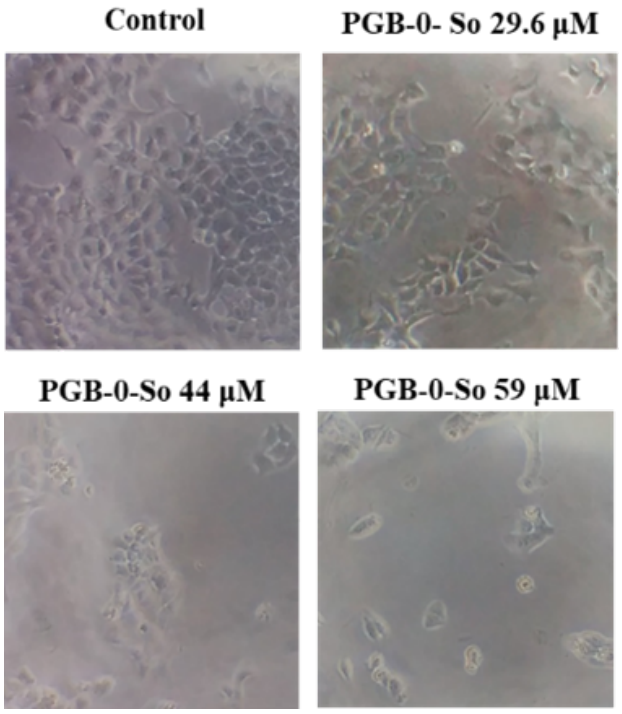
## 3. Results and Discussion

### 3.1. Cytotoxicity of PGB-0-So toward MCF-7/HER2 cells

PGB-0-So is essentially PGB-0 formulated with sorbitol to increase its solubility in water. PGB-0-So is expected to show more potent cytotoxic effects than PGB-0 on MCF-7/HER2 cells because of its increased solubility. The cytotoxicity of PGB-0-So toward MCF-7/HER2 cells was evaluated using an MTT assay. Here, the absorbance of dissolved formazan crystals was measured as an indicator of the number of live cells; the results obtained were then converted to the percentage of cell viability. The IC50 of PGB-0-So was subsequently determined from a correlation plot of the concentration of the test compound versus the percentage of cell viability.

MCF-7/HER2 cells treated with PGB-0-So at various concentrations of 2.96–59.22 µM demonstrated dose-dependent cytotoxic effects. The decrease in viability of MCF-7/HER2 cells after treatment with PGB-0-So manifested as changes in cell morphology compared with the control group (without treatment; Figure 1a). After 24 h treatment with PGB-0-So, the cells were bigger in size and became rounded in shape. Furthermore, the regression equation obtained from the graph of compound concentration versus percentage of cell viability revealed an IC50 of 36 µM (Figure 1b).

The cytotoxic effect of PGB-0-So on MCF-7/HER2 cells is related to the number of PGB-0-So molecules accumulated in the cells. Thus, a preliminary PGB-0-So ac-

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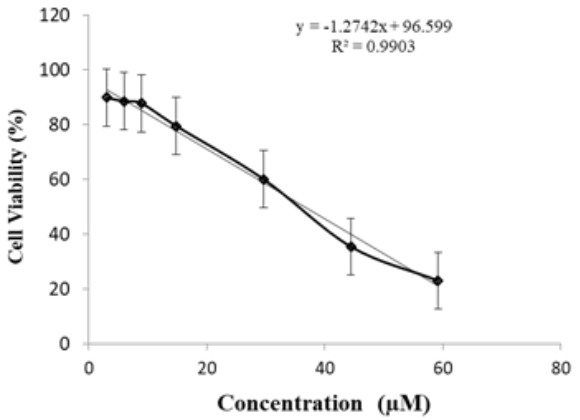



(b)

**FIGURE 1** Cytotoxic effect of PGB-0-So on MCF-7/HER2 cells. (a) Morphology of the cells after 24 h of treatment with PGB-0-So at various concentrations. (b) Cytotoxic profile of PGB-0-So in MCF-7/HER2 cells. The graph describes the correlation between the concentration of the PGB-0-So and cell viability. Results were shown as the average of three separate experiments (mean ± SD).

cumulation test was conducted by measuring the concentration of boron, one of the atoms that make up PGB-0-So molecules, in test cells. The boron concentration carried by PGB-0-So was then compared with that carried by the parent compound, PGB-0. The results showed that the concentration of boron in cells treated with PGB-0-So was much higher than that in cells treated with PGB-0 (Figure 2). This finding confirms that increased solubility could promote boron accumulation in the cells, resulting in stronger cytotoxic activity. Moreover, this result demonstrates that the cytotoxic effect of PGB-0-So on MCF-7/HER2 cells is related to the total concentration of boron in the latter.

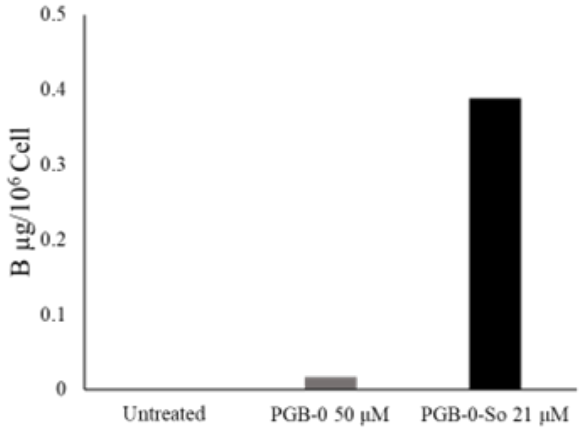



**FIGURE 2** Comparison of the level of boron incorporation due to PGB-0-So, control, and PGB-0 uptake into MCF-7/HER2 cells. Boron incorporation into cells was analyzed by inductively coupled plasma-atomic emission spectroscopy.

### *3.2. Cell cycle modulation*

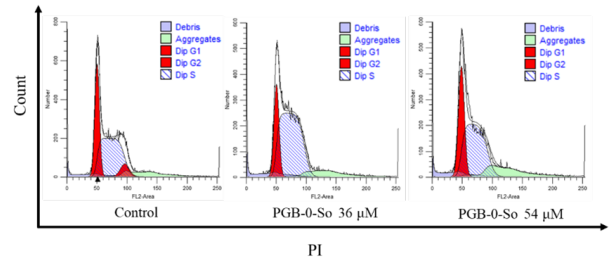
The mechanism through which PGB-0-So exerts anti-proliferative effects on MCF-7/HER2 breast cancer cells was determined by cell cycle analysis using flow cytometry. The results showed that 24 h of treatment of PGB-0-So could induce cell cycle arrest at the S-phase in MCF-7/HER2 cells (Figure 3). Treatment with PGB-So at a concentration of 36 µM caused increases in cell accumulation at the S-phase by up to 70.2% compared with that in control cells without PGB-0-So treatment.

### *3.3. Apoptosis induction*

The apoptosis induction test demonstrated that PGB-0-So could induce apoptosis in MCF-7/HER2 cells (Figure 4). Cell treatment with PGB-0-So for 24 h resulted in an increase in the number of dead cells at the early stages of apoptosis. The number of dead cells increased with increasing PGB-0-So concentration. Compared with that in control cells, the percentages of early apoptotic cells increased by 5.17% and 6.2% after treatment with the PGB-0-So at concentrations of 36 and 54 µM, respectively. Surprisingly, treatment with 36 µM PGB-0-So also increased the percentage of late apoptotic cells by 11.15% compared with that in control cells.

### *3.4. Intracellular ROS production*

Cellular ROS production was assessed to determine whether the mechanism of the anti-proliferative effects of PGB-0-So partly involves oxidative stress. Here, ROS levels in MCF-7/HER2 cells were measured after treatment with the PGB-0-So. The results showed that treatment with PGB-0-So increases ROS levels if compared with control. ROS levels are indicated by relative mean fluorescence (Figure 5). At concentrations of 15 µM and

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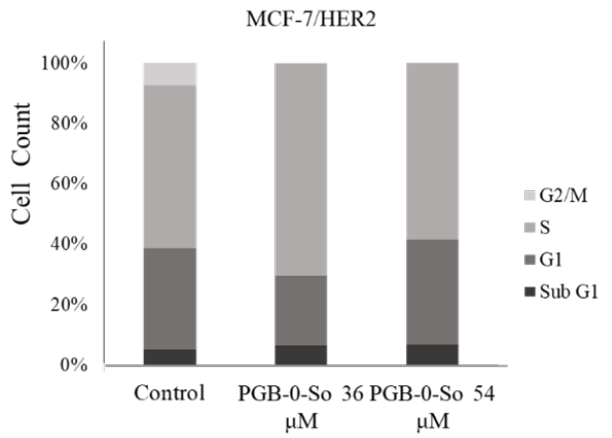



(b)

**FIGURE 3** PGB-0-So inhibits the proliferation of MCF-7/HER2 cells by inducing S-phase arrest. (a) Cell cycle phase distribution of MCF-7/HER2 cells after 24 h of treatment with PGB-0-So. (b) Summary of the cell cycle results.

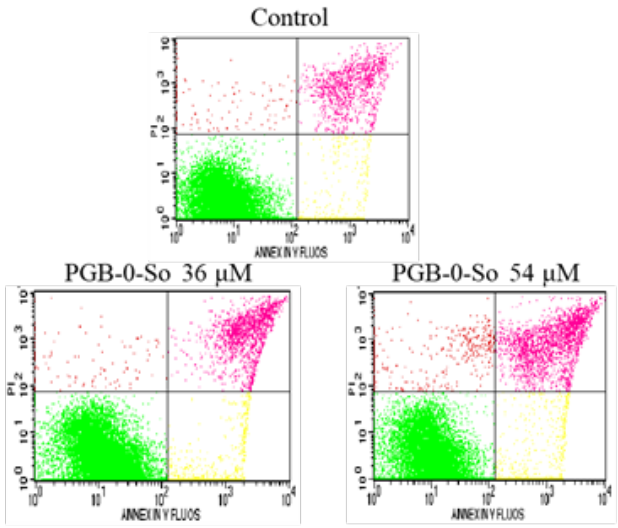
30 µM, PGB-0-So showed relative mean fluorescence values of 1.4 and 1.8, respectively. By comparison, treatment with H2O2 as a positive control yielded a relative mean fluorescence value of 1.5. The increase in ROS level after treatment with PGB-0-So confirms that the PGB-0-So may inhibit the proliferation of MCF-7/HER2 cells.

### *3.5. Discussion*

This study aimed to explore the feasibility of PGB-0-So as an anti-cancer agent against HER2-overexpressing breast cancer. HER2, a receptor protein that has a significant effect on tumor malignancy, is overexpressed in approximately 30% of all breast cancer cases (Mendes et al. 2015). Docking analysis revealed that PGB-0, the parent compound of PGB-0-So, could bind to the ATP binding site of HER2 protein; this finding was later confirmed by the observation of a decrease in HER2 protein expression in vitro in MCF-7/HER2 cells, suggested that inhibition in HER2 signaling can affect HER2 expression (Utomo et al. 2017). However, the development of PGB-0 as a chemotherapeutic agent is limited by its low solubility. Thus, a compound formulation that could increase the solubility of PGB-0 is desirable.

In this work, PGB-0 was reacted with sorbitol to increase the solubility of the parent compound. PGB-0-So demonstrated much higher solubility than PGB-0. Increases in its solubility are expected to increase the accumulation of PGB-0-So and, in turn, its anti-cancer activity, in cells. The preliminary results showed that the intracellular boron accumulation released by PGB-0-So is much higher than that induced by the parent compound, PGB-0. This finding may be attributed to the increased solubility of the latter compared with that of the former, which could encourage cellular accumulation (Shangguan et al. 2017). This result also suggests that the sorbitol molecules in PGB-0-So can promote the entry of the Boron into cells through their interaction with caveolar proteins.

Caveolae are parts of the plasma membrane enriched with lipids that have been reported to interact specifically with sorbitol and mediate endocytosis (Nguyen et al. 2014). Caveolar proteins are overexpressed in most cancer cells (Waalkes et al. 2011), including MCF-7 breast can-

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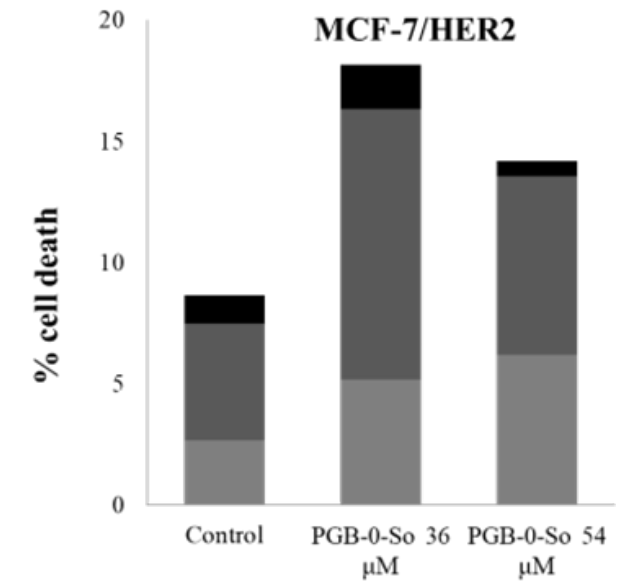
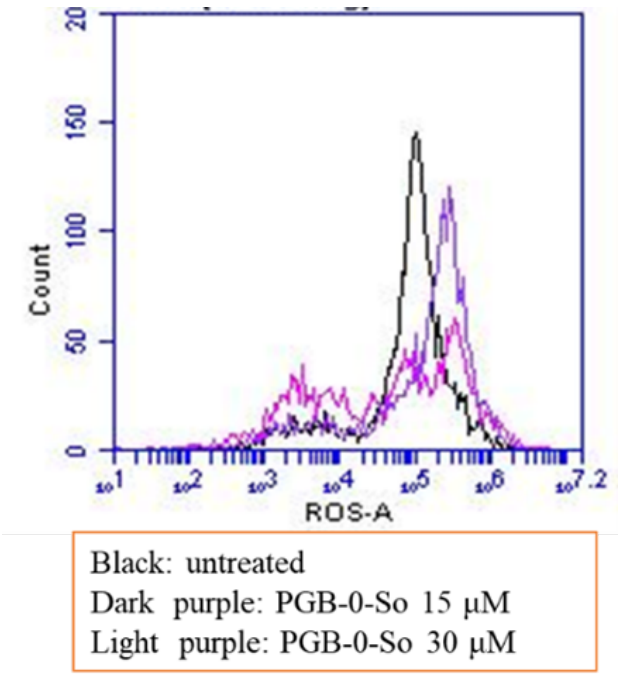



(b)

**FIGURE 4** PGB-0-So induces apoptosis in MCF-7/HER2 cells. (a) Distributions of live and dead cells after 24 h of treatment with PGB-0-So. (b) Summary of the apoptosis induction results.

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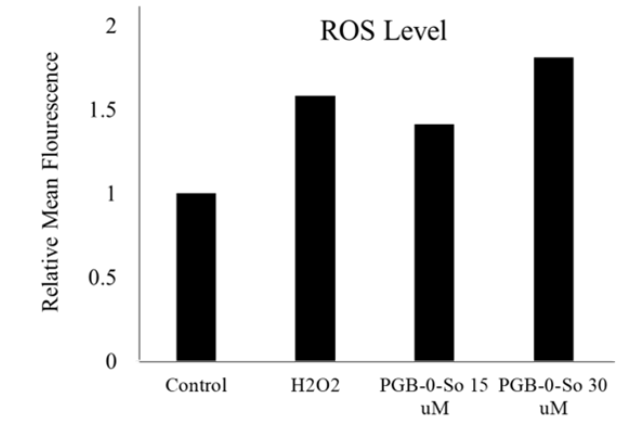


(b)

**FIGURE 5** PGB-0-So increases ROS production in MCF-7/HER2 cells. (a) Profiles of cellular ROS production. X-axis, cellular ROS levels; Y-axis, relative cell counts. (b) Quantification of intracellular ROS levels after 24 h of treatment with PGB-0-So compared with the control.

cer cells (Nguyen et al. 2014). The specific interaction of sorbitol with caveolar proteins, followed by endocytosis, could increase the uptake of PGB-0-So into MCF-7/HER2 cancer cells. However, further examination of the mechanism of entry of PGB-0-So into these cells is necessary to confirm the interaction of sorbitol in the PGB-0 with specific proteins on the surface of MCF-7/HER2 cancer cells.

The amount of PGB-0-So accumulated within the cells is undoubtedly related to its anti-cancer effect. As the accumulation of PGB-0-So increases, its bioactivity may be expected to increase. The results showed that the cytotoxic effect of PGB-0-So on MCF-7/HER2 cells is greater than that of PGB-0. PGB-0-So had an average IC50 of 35 µM (99% confidence level), while PGB-0 revealed a much higher IC50 of 270 µM (Utomo et al. 2017). The cytotoxic activity of PGB-0 may be due to the interactions of the compound with the ATP binding site of HER2 proteins in MCF-7/HER2 cells (Utomo et al. 2017). As PGB-0-So accumulates in cancer cells, the increased number of molecules available to interact with the ATP sites of HER2 could lead to stronger anti-proliferative effects against the cells. Therefore, examining the expression pattern and downstream proteins of HER2 participating in cell proliferation is necessary to confirm the relationship between the anti-proliferative activity of PGB-0-So and the HER2 signaling pathway.

Reductions in cancer cell viability induced by PGB-0-So may occur through several mechanisms of action. The mechanisms of action of PGB-0-So were determined via cell cycle modulation and apoptosis induction assays. PGB-0 is known to induce cell cycle arrest in the sub-G1 phase, leading to apoptotic events in MCF-7/HER2 cells (Utomo et al. 2017). However, unlike PGB-0, PGB-0-So (36 µM) caused cells to accumulate at the S-phase, with a cell distribution percentage of 70.2%. This finding is supported by a previous study that showed that PGB-0 could induce S-phase arrest in 4T1 breast cancer cells (Kusumastuti et al. 2019). Moreover, as a lead compound, curcumin also causes S-phase arrest in gallbladder carcinoma cells (Liu et al. 2013). Another study reported that curcumin causes G1/S arrest accompanied by decreased cyclin D1 protein expression in human osteosarcoma cells (Lee et al. 2009). The results of these previous studies indicate that PGB-0-So may be able to block cell cycle progression at the S-phase by preventing DNA synthesis, leading to the inhibition of cell proliferation.

The ability of PGB-0-So to induce apoptosis was determined, and results revealed an increase in the percentage of dead cells (11.15%) compared with that in control cells at a later stage of apoptosis following treatment with 36 µM PGB-0-So. This result is supported by several previous studies. For example, curcumin was previously reported to be able to induce apoptosis in the human pancreatic cell lines PANCI and BxPC3 (Zhu and Bu 2017) and MCF-7 cells by increasing the expression of Bax via the p53 pathway (Choudhuri et al. 2002). Demethoxycurcumin, a curcumin analog compound, has also been demonstrated to induce apoptosis in oral squamous cell carcinoma cells by triggering caspase-8/-9/-3 activity (Chien et al. 2020).The mechanism by which PGB-0-So, a curcumin analog compound, induces apoptosis is believed to resemble those of curcumin and other similar analog compounds. However, Western blot analysis may be necessary to confirm the effect of PGB-0-So on apoptotic regulatory proteins.

Oxidative stress may contribute to the mechanism of the anti-proliferative activity of PGB-0-So. Intracellular ROS production was tested using flow cytometry with DCFDA staining to confirm this supposition. The results indicated that PGB-0-So increases intracellular ROS levels if compared with control. PGB-0-So at a concentration of 30 µM showed a higher increase in ROS levels compared with the $H_2O_2$ control. Several studies have reported

that curcumin and its analogs can increase ROS production in several cancer cells, including HeLa and SiHa cells in cervical cancer (Javvadi et al. 2008). Larasati et al. (2018) reported that curcumin can bind to a series of enzymes that play a role in various ROS metabolic pathways in CML-derived leukemic cells, thereby triggering an elevation of intracellular ROS levels, which leads to cell death. In general, metabolic changes may cause cancer cells to experience an increase in ROS levels approaching a certain threshold that invokes damage to cancer cells (Holmström and Finkel 2014; Panieri and Santoro 2016). Thus, therapies that could increase ROS production to levels exceeding this threshold in cancer cells may promote cell death (Trachootham et al. 2009).

Overall, PGB-0-So showed excellent potential use as an anti-cancer agent against MCF-7/HER2 cells; the compound was demonstrated to modulate the cell cycle, induce apoptosis, and increase intracellular ROS levels. Further studies may explore the mechanism of PGB-0-So uptake into cells to confirm the interaction of sorbitol in the compound with specific proteins on the surface of cancer cells. The effect of PGB-0-So on the expression and signaling pathway of HER2 may also be assessed. Furthermore, Western blot analysis may be conducted to confirm the regulation of proteins involved in cell cycle modulation and apoptosis induction.

## 4. Conclusions

The results of this study demonstrate that PGB-0-So exerts its cytotoxic activity in MCF-7/HER2 breast cancer cells by modulating S-phase cell cycle arrest, inducing apoptosis, and increasing intracellular ROS production.

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## Authors' contributions

EM, AH designed the study. LQ, RYU carried out the laboratory work. LQ analyzed the data. LQ, AH wrote the manuscript. All authors read and approved the final version of the manuscript.

## Competing interests

All authors declare that there is no conflict of interest.

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