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Curcumin Analog Pentagamaboronon-0-Sorbitol Inhibits Cell Migration Activity of Triple Negative Breast Cancer Cell Line

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Abstract

Mortality in cancer is primarily due to failure of metastasis prevention. One strategy to target the cancerous cell is Boron Neutron Captured Therapy which showed high affinity toward cancer cells and reported to have anti-proliferative as well as anti-metastatic activities. Cancer Chemoprevention Research Center Faculty of Pharmacy Universitas Gadjah Mada, has developed boron-containing substance namely pentagamaboronon-0 (PGB-0) which is known to exhibit anticancer activity towards breast cancer cell. The purposes of this research are focused to explore the anti-migratory activities of PGB-0-So against triple negative breast cancer cell. The MTT cytotoxicity assay of PGB-0-So against 4T1 breast cancer cell line were found to exert potential effect in dose-dependent manner with IC50 values of 39 μM. The study of cell migration inhibition using in vitro wound healing assays and gelatin zymography on highly metastasis breast cancer cell line 4T1, following the treatment of sub IC50 doses of PGB-0-So complex slightly inhibited cell migration through the inhibition of matrix metalloproteinase-9 expression. These findings suggest that PGB-0-So is potential as an anticancer agent.

Keywords : *curcumin analogue, PGB-0-So, 4T1 Cells, migration, MMP-9*

INTRODUCTION

Breast cancer is the most common type of cancer causing mortality for women. In 2012, there were registered 1.67 million new cases of breast cancer mortality in women by 198,000 (Ferlay, *et al*., 2012). It was estimated that approximately 10-15% of breast cancer was known to be triple negative breast cancer (TNBC) (Dawood, 2010). This breast cancer subtype is positive-metastatic breast cancer (MBC) which have worse prognosis leading to aggressive disease. Moreover, failure of metastasis prevention primarily caused mortality in breast cancer.

The treatment of metastasis in breast cancer was conducted by chemotherapy, such as doxorubicin which performed strong cytotoxicity against cancer cells. Despite its potent anticancer activity, doxorubicin had several limitations for long-term use including cardiotoxicity and chemoresistance (Carvalho, *et al.*, 2009; Thorn, *et al.*, 2011). In addition, low dose of doxorubicin induces

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epithelial-mesenchymal transition (EMT) leading to metastasis on breast cancer cells (MBC) (Bandyopadhyay, *et al.*, 2010). Hence, several anti-metastatic agents had been developed to treat MBC.

Development of anti-metastatic agents as potential candidate of chemotherapeutic agents has been established over the years. Patients characterized as triple negative breast cancer are treated with taxanes or platinum compounds (Gavilá, *et al.*, 2015). Similar to doxorubicin, taxanes in low dose induced peripheral neuropathy in breast cancer (Bhatnagar, *et al*., 2014), while platinum compound (cisplatin) induced EMT in ovarian cancer (Baribeau, *et al.*, 2014). Thus, the effective anti-metastatic agents need to be developed further.

Curcumin analogues based on benzylidine cyclopentanone backbone such as Pentagamavunon-0 (PGV-0) and Pentagamavunon-1 (PGV-1) exert potent cytotoxic (Meiyanto, *et al*. (2006); Nurulita, *et al*. (2006); Dai, *et al*. (2007); Dai, *et al*. (2011); Hermawan, *et al*. (2011); Meiyanto, *et al*. (2014) and anti-metastatic activities toward several types of breast cancer cells (Putri, *et al*., 2016). Pentagamaboronon-0 (PGB-0) is a novel curcumin analogue based on benzylidine cyclopentanone developed by Faculty of Pharmacy Universitas Gadjah Mada. Cytotoxicity of PGB-0 toward HER2 positive breast cancer had been determined and showed to decrease HER2 expression (Utomo, *et al*., 2017). PGB-0 also performed anti-metastatic activity toward triple negative breast cancer cells (unpublished data). However, similar to curcumin, PGB-0 is less soluble in water. To improve solubility of PGB-0, we synthesized the complex form of PGB-0 with polyol sugar, sorbitol, namely PGB-0-So.

This study aims to develop PGB-0-So as novel anti-cancer agent especially through the inhibition of cell migration. 4T1 cells were used as a model of highly metastatic breast cancer cell line. Possible anti-metastasis activities of PGB-0-So were analyzed by using scratch wound healing assay and gelatin zymography. The result of this study will be used for further experiment in order to develop novel anti-migratory agents from PGB-0-So.

MATERIALS AND METHODS

Chemicals

PGB-0-So was synthesized by Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. Doxorubicin was purchased from Sigma.

Cell Culture

4T1 breast cancer cells were obtained from Prof. Masashi Kawaichi, M.D., Ph.D (Nara Institute of Science and Technology, NAIST, Japan). The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) high glucose (Sigma, St. Louis, CA, USA) with 10% FBS (Sigma), HEPES, sodium bicarbonate, 1000 U/mL of Penicilin-1000 U/ml of Streptomycin and 0.5 µg/mL Fungizone (Gibco, New York, USA).

Scratch Wound Healing Assay

The 4T1 breast cancer cells were seeded $7.5x10^4$ cells per well in 24 well-plate. Cells were incubated for 24 hours until 80% confluent. Media was removed and well was washed with 100 µL PBS (Sigma). Then cells were added with media contained 0.5% FBS for starvation and incubated for 22 hours. Each well was scratched vertically by using yellow tip and treated with Doxorubicin 10 nM as positive control, PGB-0-F, and combination of both compounds. The closures of cell migration were observed in 0, 18, 24 and 42 hours under inverted microscope (Olympus, Tokyo, Japan) and captured by Handphone (Samsung, Seoul, South Korea).

Gelatin-Zymography Methods

A total of 2×10^5 4T1 cells were planted in a 6-well plate with 2 mL of culture medium and incubated for 24 hours. The samples solution was carried out using culture medium containing

0.5% FBS. After incubation, the media is removed and washed with PBS 1 mL 1 times. Cell then treated with various concentration of PGB-0-So and doxorubicin. Estradiol was used to induce the expression of MMP-9. The cells then were incubated again for 48 hours. Culture medium then collected in 1.5 mL microtube and centrifuged at 4°C and then take the supernatant. The sample has been obtained by loading the buffer and running in appropriate concentration electrophoresis. Gel that has gelatin mold. The checking process with electrophoresis (V = 120V, I = 60A) was carried out for 130 minutes. After that, the gel is done and renaturation using a renaturing solution containing Triton-X 100 to remove SDS for 30 minutes. Continued with incubation for 20 h using an incubation solution at 37°C. Then, it should be stained with coomasie brilliant blue for 30 minutes, and through destaining using a destaining solution until the transparent blue band appears.

Data Analysis

Scratch wound healing analysis was figured out by measuring the distance between scratch edges using ImageJ software then comparing the untreated and treated cells. Data from multiple scratch within the same test group were analyzed using Analysis of Variance test to analyze the difference between experimental group. Data were presented as mean±S.D and analyzed by one-way ANOVA. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Anti-migratory Effect of PGB-0-So and Its Combination with Doxorubicin against 4T1 cell

The purpose of this study is to develop a novel anti-cancer agent which especially plays role in inhibiting cancer cell migration. In this study, we used highly metastatic breast cancer cell model, 4T1, to explore the potency of PGB-0-So in inhibiting cell migration. Low concentration of Doxorubicin (Dox) in this study was used to induce migration (Bandyopadhyay, *et al.*, 2010). Scratch wound healing assay was conducted to observe cells migration following the treatment of several concentrations under IC50 value of 1/8, 1/4, and 1/2 of IC_{50}, which are nontoxic and appropriate concentration to observe cell migration activity. The IC_{50} value of PGB-0-So against 4T1 cell was 40 μM. After 42 hours observation, Dox increased the % closure up to 100% indicating the cell migration activity. On the other hand, single treatment of 1/2 IC_{50} PGB-0-So (20 μM) showed significant inhibitory activity against 4T1 cell line whereas the concentrations of 1/4 and 1/8 IC_{50} value (10 and 5 μM) of PGB-0-So showed unsignificant inhibitory anti-migratory activity compared to the untreated group. Interestingly, combination of Dox with all concentrations of PGB-0-So showed significant anti-migratory activity against doxorubicin-induced 4T1 cell (Figure 1). The result needs to be confirmed with the expression of certain protein played role on cells migration or invasion.

Effect of PGB-0-So and Its Combination with Doxorubicin against MMP-9 Expression

Secondary Cancer cells migration and invasion was tightly regulated by certain proteins especially Matrix Metalloproteinase 9 (MMP-9). Role of MMP-9 on cells migration and invasion was to degrade extracellular matrix (ECM) around cancer cells (Yabluchanskiy, *et al*., 2013; Reunanen, *et al*., 2013). To observe possible effect of PGB-0-So on the decreasing expression of MMP-9, gelatine zymography was performed. Low concentration of Dox (10 nM) showed decreased expression of MMP-9 expression indicating the different possible effector of anti-migratory activity might be induced by Dox. Amalina, *et al*. (2017) reported that low dose of Dox induced EMT through Rac1 independent-lamellipodia formation by which the initial progression of cancer cell migration. In contrast, both single treatment of PGB-0-So and

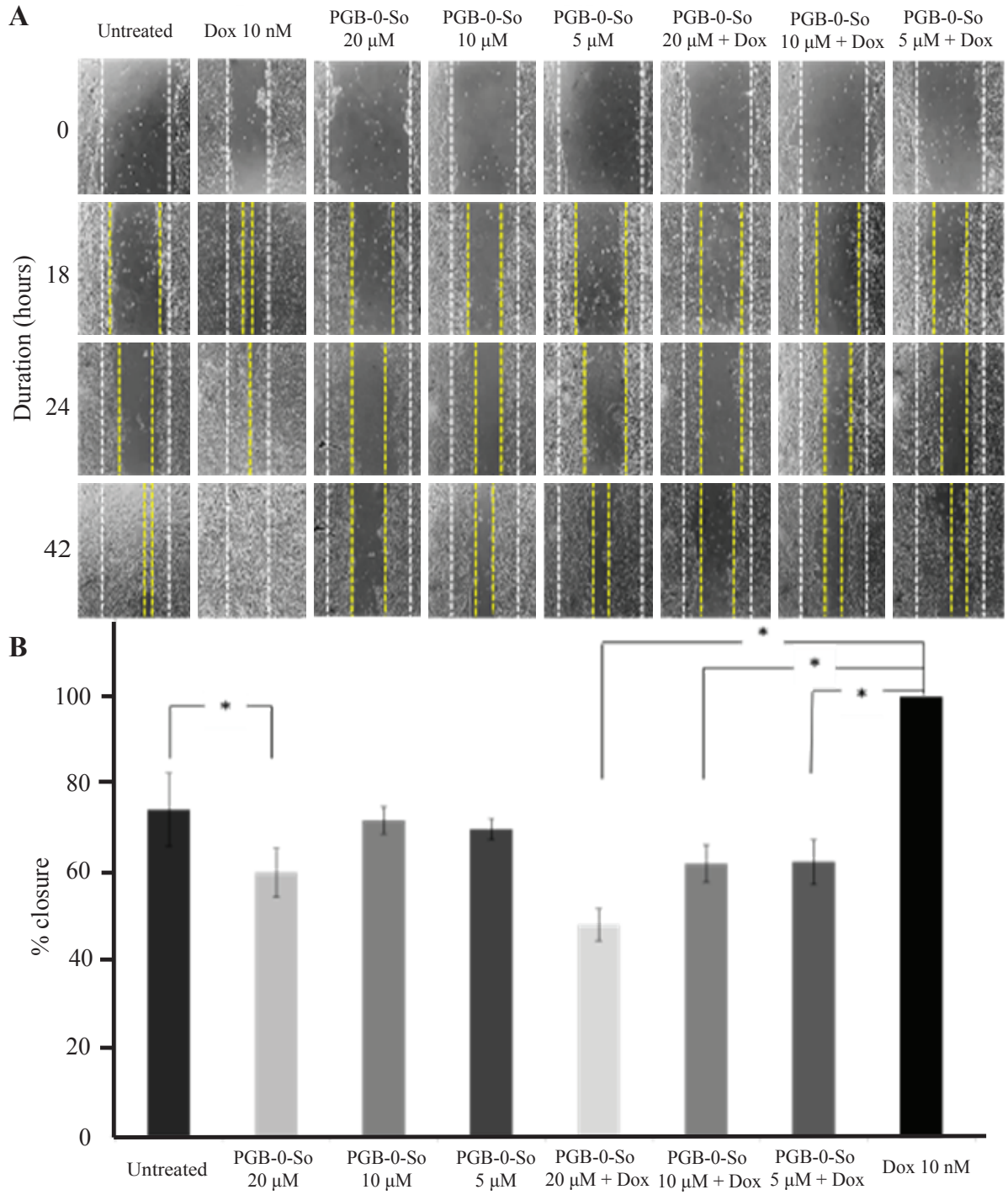

Figure 1. Anti-migratory Effect of PGB-0-So against Highly Metastatic, 4T1, Cells Migration. 4T1 cells (7.5x10^4 cells/well) were treated with PGB-0-F in the concentration as indicated in the figure, then subjected for scratch wound healing assay. A: The morphology of the cells after scratch and treated with PGB-0-So. Observations were made after 18, 24 and 42 hour of treatment under an inverted microscope with magnification of 100x. B: The percentage of 4T1 cells closure after treatment. The area of the scratch were analyzed using ImageJ software then % closure was calculated in accordance with the procedures of the analysis (p<0.05).

its combination with Dox showed the decreasing expression of MMP-9 protein in dose dependent manner (Figure 2). Hence, anti-migratory effect of PGB-0-So against 4T1 cell might be through the suppression of MMP-9 expression.

DISCUSSION

Inhibition of tumor cell migration is crucial in the therapy and inhibition of cancer spread, especially in metastasis. Thus, it is necessary to develop anti-migratory agent to overcome this situation. Previous research showed that PGB-0 exhibited anti-migratory effect as well as inhibited MMP-9 expression (unpublished data). MMP-9 is a family member of zinc- and calcium-dependent endopeptidases, 88 kDa protein which has numerous cell activities, involving in various physiological functions, such as cell-cell contact, tissue remodeling cell migration and cellular differentiation (Yabluchanskiy, *et al*., 2013; Vandooren, *et al*., 2013).

Other study using boron containing compound, phenylboronic acid showed that this compound has potency as selective inhibitor of cancer cell migration and viability without effecting non-tumorigenic cell lines (Bradke, *et al*., 2008; McAuley, *et al*., 2011). The main purpose of this study is to explore the potential metastasis-inhibitory of PGB-0-So on triple negative breast cancer cells, 4T1. In this study, 4T1 cells were used as the model of human metastatic breast cancer cells because it is highly metastatic breast cancer cells. This cells can metastasize to liver, lung, bone and brain, making it a good model of human metastatic breast cancer (Pulaski, *et al*., 2000; DuPré, *et al*., 2007).

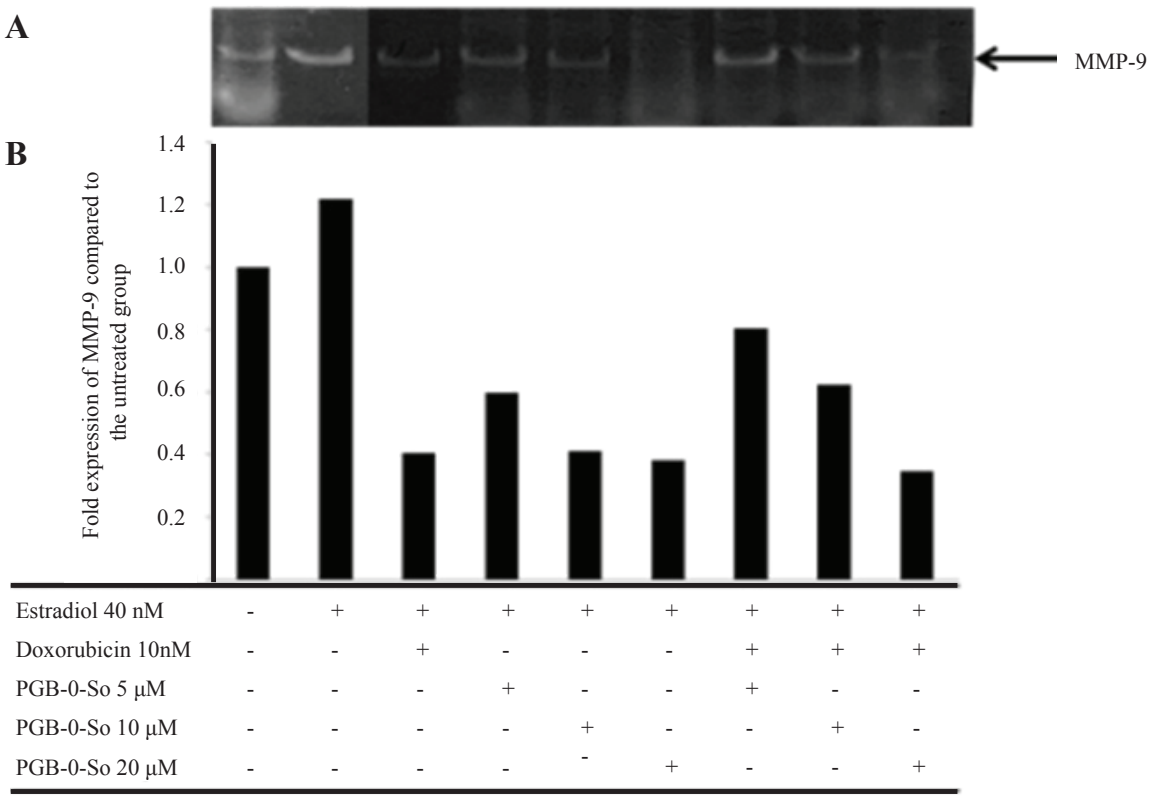

Figure 2. Result of MMP-9 Expression Following The Treatment of PGB-0-So, Doxorubicin, and PGB-0-So Combination with Doxorubicin (48 hours). A: Clear protein bands formed indicating the presence of MMP-9 protein. B: Intensity Quantification of MMP-9 band compared to the untreated groups.

Doxorubicin is usually used as chemotherapeutic first-line treatment of several type of cancer especially triple negative breast cancer. Prolonged use of doxorubicin showed toxicity effect such as cardiotoxicity and hepatotoxicity (Pedrycz and Kramkowska, 2016). On the other hand, another research reported that doxorubicin at low dose can enhanced cancer cell migration by inducing lamellipodia formation (Amalina, *et al*., 2017). Previous studies showed the migration inbitory activity of PGB-0-So (Pentagamaboronon-0-sorbitol) in 4T1 cells (unpublished data). PGB-0-So showed cytotoxic effect on 4T1 cells with IC_{50} values of 39 μM (unpublished data). While PGB-0 has IC_{50} value 300 μM in 4T1 cells (unpublished data), and 270 μM in MCF-7/Her2 cells (Utomo, *et al*., 2017).

In this present study, we also observed the inhibition of cancer cell migration as the one of parts of metastasis process by treatment of PGB-0-So through scratch wound healing assay. Cell migration is part of the metastasis process. Based on the percent graph of 4T1 cell closure (Figure 1A) the treatment of PGB-0-So with concentration 5 and 10 μM had demonstrated unsignificant migration inhibitory activity in all time course compared to the negative control group (untreated). On the other hand, 20 μM concentration of PGB-0-So began to affect significantly the inhibition of cell migration especially after 42nd hours.

The treatment of doxorubicin 10 nM showed higher % closure than untreated cells, it means that doxorubicin induced cell migration. Previous study reported that doxorubicin induced lamelipodia formation and cell migration in 4T1 and MCF-7/Her2 cells (Amalina, *et al*., 2017). Furthermore, combination treatment of PGB-0-So and doxorubicin showed inhibition of 4T1 cell migration 42 hours after incubation time. At this incubation time both single PGB-0-So 1/2 IC_{50} treatment and its combination with 10 nM doxorubicin showed significant differences with the difference in closing percentages compared to the control of cells without treatment. Whereas when PGB-0-So treatment with doxorubicin 10 nM compared with a single treatment doxorubicin 10 nM resulted in lower closing percentage indicating the inhibitory activity of PGB-0-So.

It has been reported in the breast cancer patients where there is a significant association between high MMP9 expression and poor survival (Song, *et al*., 2013) so that MMP-9 expression would be potential therapeutic agents to inhibit development of cancer metastasis. Curcumin analogue PGB-0-So showed tendency of inhibitory effect of MMP-9 activity in gelatin zymograph assay 48 hours after treatment. Previous study also showed that PGB-0 inhibited MMP-9 expression (soon will be published). Curcumin itself showed the inhibition of MMP-9 expression by inhibiting Mitogen- activated Protein Kinase (MAPK) phosphorylation (Cao, *et al*., 2014). Other research showed that simultaneous silencing of MMP-9 in breast cancer cells decreased the wound healing, migratory, invasive and adhesive capacity of the cells by increasing cell-cell adhesion and modulating EMT genes (Moirangthem, *et al*., 2016).

CONCLUSION

PGB-0-So exhibits anti-migration effect against doxorubicin treatment cells. PGB-0-So also inhibits MMP-9 activity which has role in tumor invasion.

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