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Anti-metastatic Activity of Curcumin Analog Pentagamaboronon-0-Sorbitol Against HER2-overexpressed MCF-7 Breast Cancer Cells

Lailatul Qodria[1,2], Indah Hairunisa[1,2], Rohmad Yudi Utomo[2], Adam Hermawan[2,3], Edy Meiyanto[2,3,*]

[1]Master Student of Biotechnology Program, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3]Departement of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Breast cancer with Human Epidermal Growth Factor Receptor (HER)2 overexpression increases tumor progession and lead to metastasis, which is primarily cause of mortality in breast cancer. Pentagamaboronon-0 Sorbitol (PGB-0-So) is an aquoeous formulation of curcumin analog, PGB-0, with sorbitol. This compound has been developed as an anti-cancer chemotherapeutic agent and a boron carrying pharmaceutical for boron neutron capture therapy (BNCT). The aim of this study is to investigate anti-metastatic activities of PGB-0-So against HER2-overexpressed MCF-7 breast cancer (MCF-7/HER2) cells. The MTT cytotoxicity assay of PGB-0-So exhibited cytotoxic effect with an IC_{50} value of 35 μM. The testing of anti-migration activity using the scratch wound healing assay demonstrated that PGB-0-So inhibited the closure of the wound on MCF-7/HER2 cells compare to the control. Furthermore, PGB-0-So was able to suppress matrix metalloproteinase (MMP)-9 activities, based on the gelatin zymography assay. In conclusion, PGB-0-So has potency to be developed as an anti-cancer agent against metastatic breast cancer.

Keywords : *PGB-0-So, anti-metastasis, cell migration, MMP-9, MCF-7/HER2*

INTRODUCTION

Human Epidermal Growth Factor Receptor 2 (HER2) overexpression is found in approximately 20-30% of cases of breast cancer. HER2-positive breast cancer shows worse prognosis leading to aggressive disease that causes chemo-resistant (Yokoyama, *et al.*, 2006) and increased metastasis (Wolf-Yadlin, *et al*., 2006). 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

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*Corresponding author: edy_meiyanto@ugm.ac.id

use including cardiotoxicity and chemoresistance (Carvalho, *et al*., 2009; Thorn, *et al*., 2011). In addition, low dose of doxorubicin induces epithelial-mesenchymal transition (EMT) and lamellipodia formation leading to metastasis on breast cancer cells (MBC) (Bandyopadhyay, *et al*., 2010, Amalina, *et al.,* 2017). 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 metastatic HER2-positive breast cancer are treated specifically with anti-HER2 either alone or in combination with chemotherapy, especially taxanes. Unfortunately, specific anti-HER2 therapy, such as Trastuzumab, also caused resistance through various mechanisms (Luque-Cabal, *et al*., 2016). Similar to doxorubicin, taxanes in low dose induced peripheral neuropathy in breast cancer (Bhatnagar, *et al*., 2014), while platinum compound, such as cisplatin, induced EMT in ovarian cancer (Baribeau, *et al*., 2014). Thus, the effective anti-metastatic agents need to be developed further.

Curcumin analog based on benzylidine cyclopentanone backbone such as Pentagamavunon-0 (PGV-0) and Pentagamavunon-1 (PGV-1) exert potent cytotoxic and anti-metastatic activities toward several types of breast cancer cells (Meiyanto, *et al*., 2014; Putri, *et al*., 2016). Pentagamaboronon-0 (PGB-0) is a novel curcumin analog based on benzylidine cyclopentanone and boron atom substitution developed by Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada (Utomo, *et al*., 2017). This compound has been developed as an anti-cancer chemotherapeutic agent and boron carrying pharmaceutical for boron neutron capture therapy (BNCT). PGB-0 is reported to be able to interact with HER2 ATP-binding sites and decrease HER2 expression on the MCF-7/HER2 cells (Utomo, *et al*., 2017). Cytotoxicity of PGB-0 toward HER2-positive breast cancer had been determined. PGB-0 also performed anti-metastatic activity toward triple negative breast cancer cells. However, like curcumin, PGB-0 has less solubility in water. To improve the solubility of PGB-0, we synthesized the aquoeus formulation of PGB-0 with polyol sugar, sorbitol, namely PGB-0-So. As curcumin analogue, this compound is potential to be examined as an anti-cancer agent. In this study we aim to investigate the anti-metastatic activities of PGB-0-So against HER-2-overexpressed breast cancer cells.

MATERIALS AND METHODS

Cell Cultures

MCF-7/HER2 breast cancer cell lines were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. Cells were maintained with Dulbecco's Modified Eagle's Media (Gibco,New York,USA) high glucose supplemented by 10% Bovine Fetal Serum (Sigma-Aldrich, St. Leuis, USA), penicillin-streptomycin 1,5% v/v (Gibco, New York, USA), Fungizone 0,5% v/v (Gibco, New York, USA). Cells were incubated at 37°C with 5% CO_2. Samples used were sorbitol (Sigma-Aldrich, St. Leuis, USA), chemically synthesized Pentagamaboronon-0-Sorbitol (PGB-0-So) (the purity is ≥93%) was obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada and Doxorubicin (Sigma-Aldrich, St. Leuis, USA).

Cytotoxicity Assay

Cytotoxicity assay was conducted using MTT assay (Mosmann, 1983). Briefly, a 96-well microplate was seeded with 2x103 MCF-7/HER2 cells suspended in 100 µL per well and incubated overnight. On the following day, the cells were treated with PGB-0-So at various concentration and incubated for 24 hours (h) at 37°C with 5% CO_2. Then, 100 µL of 0.5 mg/mL 3-(4,5-dimethylthiazzol-2yl)-2,5-tidiphenyltetrazolium (MTT) reagent (Biovision, California, USA) were added and the cells were incubated for 2 h. The reaction was

stopped by adding SDS stopper solution containing 0.01N HCl and incubated overnight. The absorbance then being measured using a plate reader (BioRad, California, USA) at 595 nm.

Cell Migration Assay

The 8.5x10^4 MCF-7/HER2 cells were seeded into 24-well plate and incubated for 24 h. Cell starvation was performed by incubating cells on medium containing 0.5% FBS. Cells were scratched using a sterile yellow tip and treated with the different concentrations of PGB-0-So, are: ½ IC_{50} (18 µM); ¼ IC_{50} (9 µM); 1/8 IC_{50} (4.5 µM), 10 nM doxorubicin or the combination of both for 24 h. The closures of each scratched area were observed at 0, 18, 24 and 42 h after treatment under inverted microscope (Olympus, Tokyo, Japan) and captured by a digital camera (Samsung®, Seoul, South Korea). Then further analyzed using ImageJ 1.51j8 java 1.8.0_112 and IBM SPSS Statistics program.

MMP-9 Protein Activity Assay

The assay of MMP-9 activity in the MCF-7/HER2 culture media was performed using gelatin zymography. The culture media were obtained from the MCF-7/HER2 cells that were treated with estardiol (Sigma-Aldrich, St. Leuis, USA) in order to increase MMP-9 expression (Nilsson, *et al*., 2007), different concentrations of PGB-0-So, are: ½ IC_{50} (18 µM); ¼ IC_{50} (9 µM); 1/8 IC_{50} (4.5 µM), 10 nM doxorubicin or the combination of them for 24 h. Then, the culture media were subjected to 8% SDS-PAGE supplemented with 0.1% gelatin. Following the electrophoresis, gel was incubated with 2% of Triton-X 100 (Merck, New Jersey, USA) in water for 30 minutes at room temperature and the gel was then removed from solution. Furthermore, incubation buffer, consist of 40 mM Tris-HCl pH 8, 10 mM $CaCl_2$, 0.02% NaN_3, was added to gel and incubated again at 37°C for 18-20 h. Then, the gel was stained using Coomassie Brilliant Blue R-250 solution and de-stained until clear bands with blue background were observed. Those bands were documented and analyzed using ImageJ 1.51j8 java 1.8.0_112software.

RESULTS

Cytotoxic Effect of PGB-0-So

Characterization The compounds used in the cytotoxic assay in this study were sorbitol and PGB-0-So. Based on the correlation graph between the concentration of the compound and the precentage ofcell viability (Figure 1B), treatment with sorbitol did not show a decrease in the percent viability of MCF-7/HER2 cells, so it can be stated that sorbitol does not have cytotoxic effect on the cells. In contrast, PGB-0-So treatment at the concentrations of 2.96 - 59.22 µM gave cytotoxic effects toward MCF-7/HER2 cells in a dose-dependent manner with IC_{50} value of 35 µM. The decreased viability of MCF-7/HER2 cells can also be seen from the changes of cell morphology after treatment with PGB-0-So for 24 h compared to the untreated group (Figure 1A).

Anti-migratory Effect of PGB-0-So

According to the cell migration assay, a single treatment of PGB-0-So and its combination with Dox were able to inhibit the migration of MCF-7/HER2 cells (Figure 2A). At 42 h observation, a single treatment with a concentration of 1/8 IC_{50} (4.5 µM) and ½ IC_{50} (18 µM) PGB-0-So significantly inhibited cell migration compared to the control group with a percentage of closure of 58% and 71% (Figure 2B), respectively. At the same observation time (42 h), the combination treatment of 4.5 µM or 18 µM PGB-0-So with 10 nM Dox also showed significant inhibition of cell migration compared to the control group with the percentages of closure of 47% and 46%, respectively (Figure 2B).

MMP-9 Activity

MMP-9 activity analysis exhibited that a single PGB-0-So treatment and its combination with 10 nM Dox were able to inhibit MMP-9

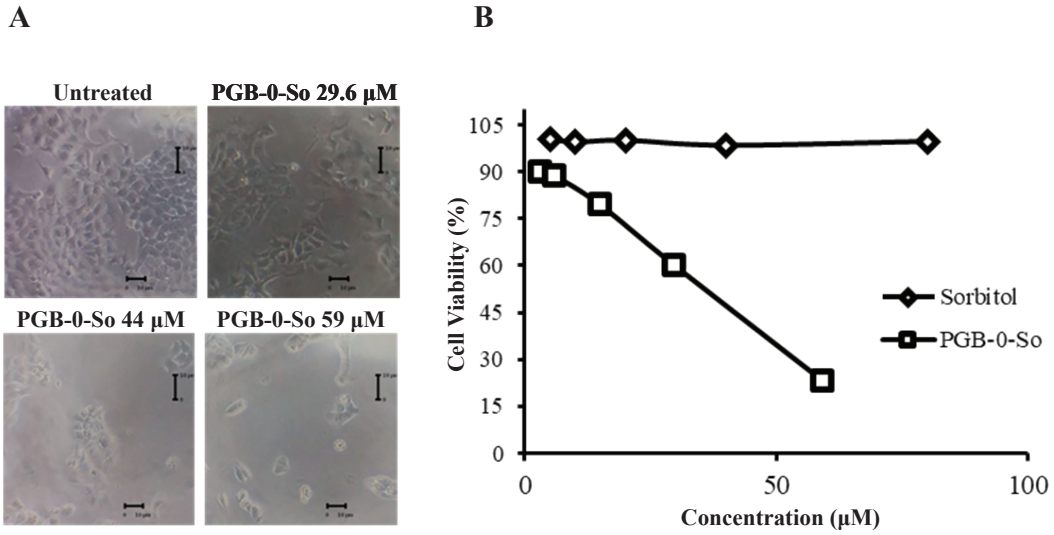

Figure 1. Cytotoxic effect of Pentagamaboronon-0-Sorbitol (PGB-0-So) toward MCF-7/HER2 cells. A: The cell morphology after treatment with PGB-0-So in various concentrations. B: Cytotoxic profile of Sorbitol and PGB-0-So on MCF-7/HER2 cells describing correlation between concentration and cell viability. PGB-0-So performed cytotoxic effect with IC_{50} value of 35 µM.

activities (Figure 3A). At 24 h of treatment, PGB-0-So both single treatment and its combination with Dox inhibited MMP-9 activity in a dose-dependent manner when compared to the control group (Figure 3B). Thus, high concentration PGB-0-So has anti-metastatic ability by inhibiting MMP-9 activity.

DISCUSSION

Cytotoxicity assay of PGB-0-So was conducted as early screening for this compound potency against MCF-7/HE2 cell lines. The results exhibited that the cytotoxic effect of PGB-0-So on MCF-7/HER2 cells was greater than that of PGB-0. PGB-0-So has an average value of IC_{50} 35 µM while IC_{50} PGB-0 was still quite large, 270 µM (Utomo, *et al.*, 2017). This may be due to the increased solubility of PGB-0-So, since the solubility of a compound has an effect on its absorption and bioactivity in the body (Shangguan, *et al.*, 2017). The cytotoxic activity of PGB-0 is probably caused by the interaction of the compound with ATP binding site of HER2 protein MCF-7/HER2 cells (Utomo, *et al.*, 2017). With the increased cytotoxic effects of PGB-0-So on MCF-7/HER2 cells compared to PGB-0 it is thought to be due to other pathways involved in its cytotoxic activity, via the caveolae-mediated pathway. Caveolae is a protein on the surface of cell membranes that is reportedly able to interact specifically with sorbitol and contribute to the occurrence of endocytosis (Nguyen, *et al.*, 2014). Caveolae is found to be overexpressed in most cancer cells (Patlolla, *et al.*, 2004; Waalkes, *et al.*, 2011) and one of them in breast cancer cells MCF-7 (Nguyen, *et al.*, 2014). The presence of a specific interaction of sorbitol with the caveolae protein followed by endocytosis allows for increased cellular uptake of PGB-0-So into MCF-7/HER2 cancer cells, thus increasing the cytotoxic effect of the compound. However, for further research it is still necessary to test the mechanism of entry of PGB-0-So compounds into

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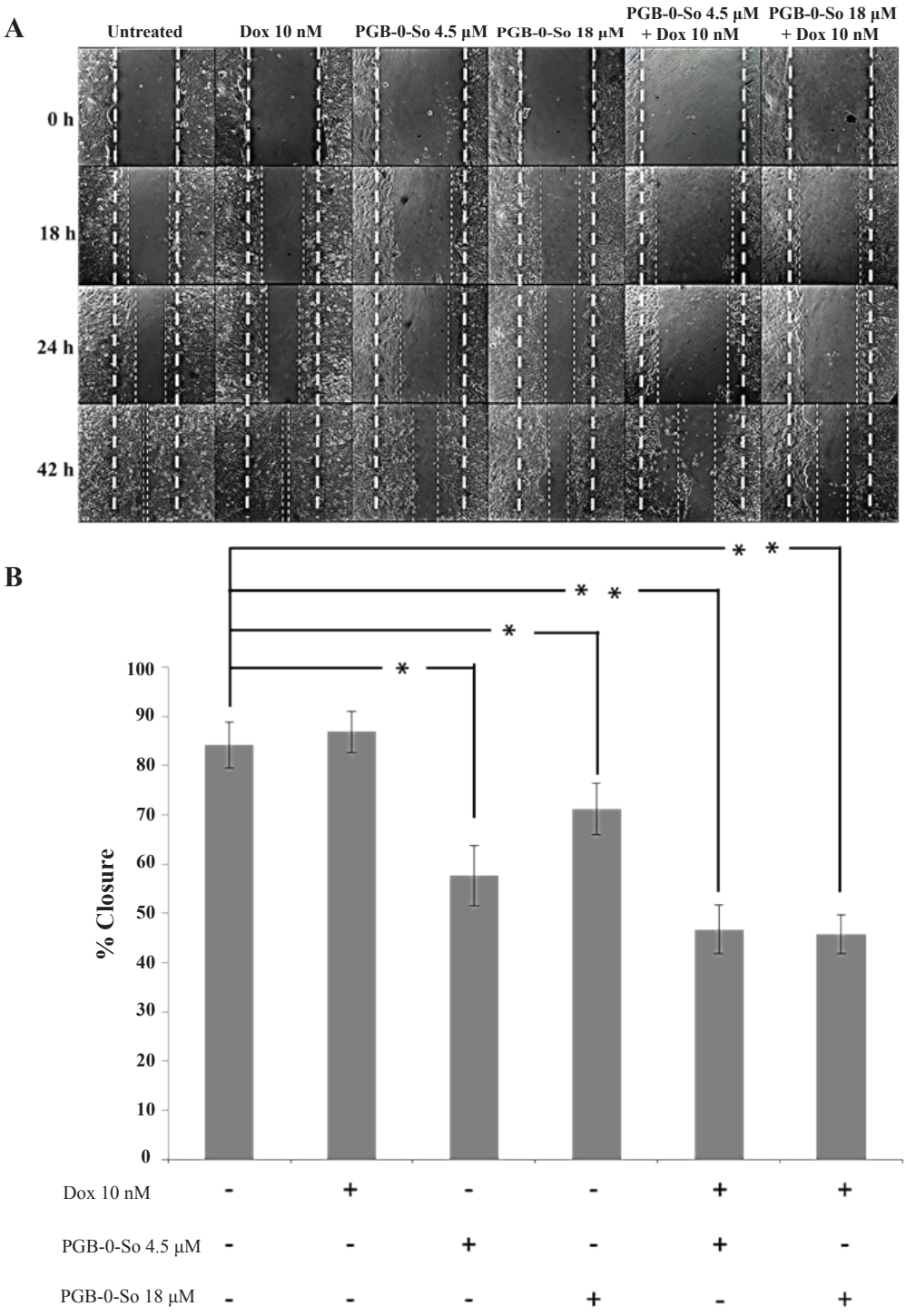

Figure 2. The effect of PGB-0-So cell migration toward MCF-7/HER2 cells. Cell migration assay was conducted using wound healing assay as described in the methods. A: Inhibitory activity of cell migration after treatment with 4.5 µM, 18 µM PGB-0-So, and its combination with 10 nM Doxorubicin. B: Quantification of closure precentage on each treatment at 42 h. Statistical analysis of % closure on each treatment at 42 h (*p<0.05) by ANOVA one way test compared to untreated cells. PGB-0-So exhibited inhibitory effect on cell migration at 4.5 µM and 18 µM concentration either in single or combination with 10 nM Doxorubicin at 42 h.

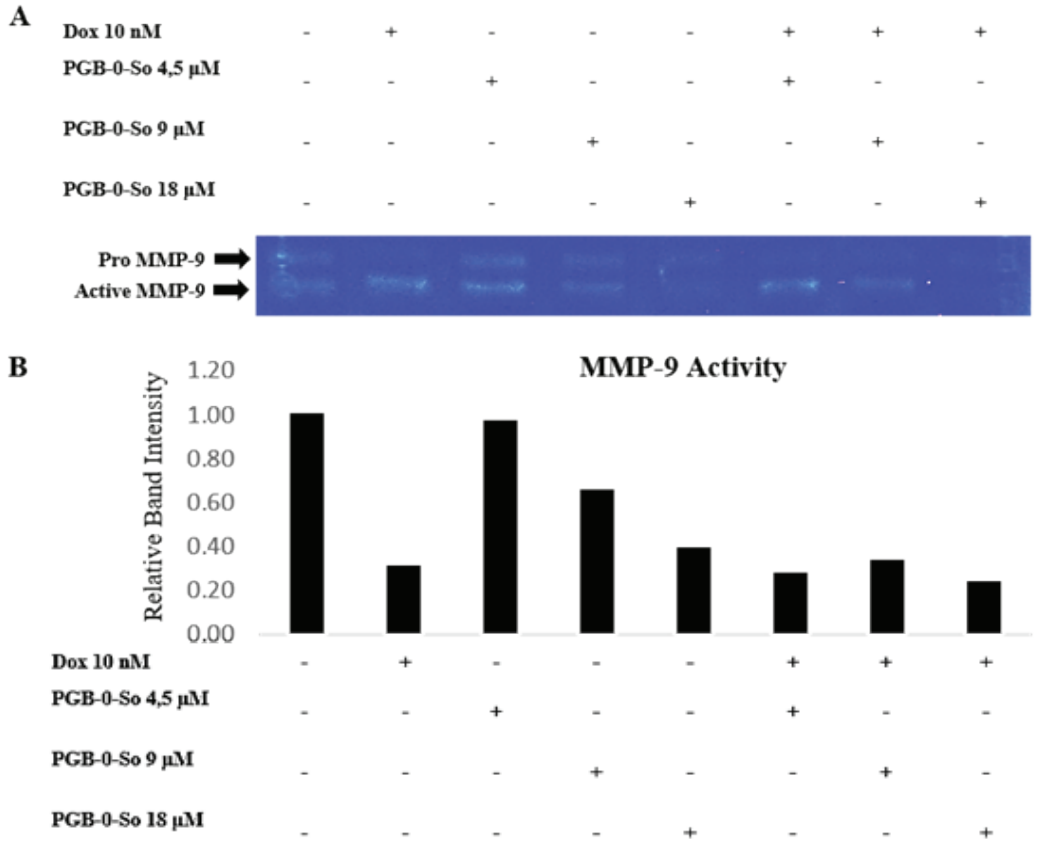

Figure 3. The effect of PGB-0-So on the MMP-9 Activity toward MCF-7/HER2 cells. Extracellular MMP-9 activity within medium was measured by analyzing MMP-9 activity using gelatin zymography assay as described in the methods. A: Band intensity of MMP-9 activity after treatment with PGB-0-So for 24 h. B: Relative band intensity of MMP-9 activity. PGB-0-So showed the inhibiting of MMP-9 activity in single and combination with Doxorubicin 10 nM after 24 h treatment.

cells to confirm the interaction of sorbitol from compounds with specific proteins on the surface of MCF-7/HER2 cells.

Furthermore, based on the cytotoxicity result, this study explored anti-metastatic activities of PGB-0-So. Anti-migratory effect of PGB-0-So was done using scratch wound healing assay. Based on this assay, PGB-0-So was combined with low concentration Dox (10 nM), because low concentration Dox can trigger cell migration by inducing epithelial-mesenchymal transition (EMT) (Bandyopadhyay, *et al.*, 2010). The results revealed that inhibition of cell migration by PGB-0-So, either in single or combination treatment with Dox. The statistical test ($p<0.05$) on a 42 hour observation showed that PGB-0-So 4.5 µM and 18 µM concentrations in single or combination treatment with Dox significantly inhibited cell migration. The results of this study indicate that PGB-0-So was able to inhibit the activity of cell migration induced by Dox.

The anti-migratory effect of PGB-0-So is in line with research by Putri, *et al.* (2016) who reported anti-migration effect of the K-PGV-1, curcumin analogue, on 4T1 breast cancer cells. Curcumin was known to be able to inhibit the

progression of MCF-7 breast cancer cell metastasis through decreasing of urocinase-type plasminogen activator (uPA) protein expression that plays a role sin extracellular molecular signalling in the cell migration process (Zong, *et al.*, 2012). The results of Lin, *et al.* (2009) also showed an inhibition of migration of lung-cell lung (A549) by curcumin through suppressing MMP-2 and -9 expression via the ER-sinyaling pathway.

Matrix metalloproteinase (MMP) is known to play an important role in the invasion, metastasis and angiogenesis of cancer cells. One type of MMP playing important role in cell invasion is MMP-9 (Bandyophadyay, 2014). Kim, *et al.* (2012) have reported that curcumin is able to suppress expression of MMP-9 induced by 12-O-tetradecanoylphorbol-13-acetate TPA on MCF-7 breast cancer cells. Therefore, to confirm migration inhibition through decreasing MMP-9 expression by PGB-0-So, the gelatin zymograph assay was performed. In this test, at each treatment was added estradiol in order to increase the resulting MMP-9 expression (Nilsson, *et al*., 2007).

The results showed that treatment for 24 and 48 hours with PGB-0-So 18 μM concentration in single or combination treatment with Dox decreased MMP-9 activity. This result is in line with previous study that PGB-0 decreased MMP-9 expression in 4T1 breast cancer cells (unplished data). The possible mechanism of inhibition of MMP-9 activity in this finding is through inhibiton of NF-κB activation, MMP-9 transcription factor. The previous research stated that curcumin inhibit MMP-9 activity through down-regulation of NF-κB and reduction of AP-1 activation (Kim et al., 2012). As an analog curcumin, the molecular mechanism of PGB-0-So in inhibiting MMP-9 activity is also expected via the NF-κB pathway.

CONCLUSION

PGB-0-So is potential to be developed as anti-cancer agent to prevent metastasis in HER2-overexpressed breast cancer cells. .

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