

Analog Curcumin, Pentagamavunon-0 (PGV-0), Induces Senescence and Increase Cytotoxic Effect of Doxorubicin on HCC 1954 Cells

Sonia Meta Angraini^{1,2}, Nadzifa Nugraheni², Edy Meiyanto^{2,3}, Adam Hermawan^{2,3*}

¹Master Student of Biotechnology Program, Graduate School, Universitas Gadjah Mada, Yogyakarta, Indonesia

²Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

³Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Senescence is an irreversible cell cycle arrest that inhibit cancer growth and suppress the progression of cancer. Some anticancer compounds are known potential to induce senescence. Senescence defence against tumour development by preventing proliferation of cells with DNA damage. The study aimed to determine the cytotoxic effects and senescence induction of Pentagamavunon-0 (PGV-0) on Human Epidermal Growth Factor Receptor 2-positive (HER2-positive) breast cancer cells, HCC 1954. Cytotoxic tests carried out with 3- (4.5-dimethylthiazol-2yl) -2.5-tidiphenyltetrazolium (MTT) assay showed that PGV-0 exhibited a potent cytotoxic effect with an inhibitory concentration (IC_{50}) value of 39 μ M. Treatment with PGV-0 at IC_{50} concentration combined with doxorubicin showed cytotoxic enhancement effects. The senescence assay using SA- β -Galactosidase assay showed that the PGV-0 alone induced senescence with a percentage of cell senescent of 15%. The combination treatment of PGV-0 at the half dose of IC_{50} with doxorubicin 100 nM was able to induce senescence with the percentage of senescent cells of 25%. Moreover, PGV-0 also increased intracellular reactive oxygen species (ROS). The results of this study indicate that PGV-0 exhibits cytotoxic effect, increases cytotoxic effect of doxorubicin and induces senescence that may correlate to the increasing of intracellular ROS in HCC 1954 cells.

Keywords: *Pentagamavunon (PGV-0), HCC 1954, Cytotoxic, Senescence*

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is highly expressed in approximately 20-30% of breast cancers and associated with more aggressive diseases, higher recurrence rates, and shorter survival (Dawood, *et al.*, 2010; Slamon, *et al.*, 1989). Several drugs targeted on this receptor are introduced and has been clinically used but become resistance due to the alteration of HER2 expression or mutations (Dawood, *et al.*, 2010).

Therefore, the development of agents in this regarding target are still a challenge to obtain the more broad spectrum for HER2 alteration with the additional of metabolic targets. In this concern, it is important to find the anti-cancer agent that kill the

Submitted: July 18, 2019

Revised: August 30, 2019

Accepted: September 2, 2019

*Corresponding author: adam_apr@ugm.ac.id

cancer cells through apoptosis in correlation with senescence evidence.

Senescence is a complex stress response when cells lose the capacity to proliferate irreversibly, followed by various changes in gene expression (Campisi, 2013). The role of senescence in cancer treatment includes senescent cells that have lost the ability to undergo cell division permanently, even though they may be fully metabolically active, therefore can suppress and reduce cancer growth (Campisi, 1997). Treatment of lapatinib in HER2-positive cell line induces senescence with accumulation of SA- β -Galactosidase (McDermott, *et al.*, 2019). Previous studies treatment using doxorubicin low-dose in colorectal cancer cells induce senescence rather than apoptosis (Sliwiska, *et al.*, 2009). Cells that cannot induce apoptosis, senescence can act as a 'backup' response and contribute to treatment outcomes (Huun, *et al.*, 2017). Collectively, senescence plays an important role in inhibiting cancer growth and contribute to cancer therapy.

Molecular national team, Faculty of Pharmacy, Universitas Gadjah Mada developed curcumin analogues, namely Pentagamavunon-0 (PGV-0) and Pentagamavunon-1 (PGV-1) that has been investigated by Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada of their potential for anticancer. Beside that, CCRC also developed many anticancer compounds from natural materials and synthetic compounds. The synthetic compounds such as Pentagamaboronon-0 (PGB-0) and Pentagamaboronon-0 complex (PGB-0) with fructose and sorbitol exhibited anticancer properties. PGB-0 reported to decreased the viability of MCF-7/HER2 cells (Utomo, *et al.*, 2017) and 4T1 cells (Kusumasuti, *et al.*, 2019). The PGB-0 in a complex form with sorbitol (PGB-0-So) increased cytotoxicity against MCF-7/HER2 cells (Qodria, *et al.*, 2018). Whereas, PGV-0 is known to have cytotoxic activities in various types of cancer cells, including breast cancer (Hermawan, *et al.*, 2011). Molecular docking studies revealed that PGV-0 can interact with several

proteins including HER2 protein (Meiyanto, *et al.*, 2014). This interaction can be directly compared to ATP and lapatinib, a chemotherapeutic agent specifically targeted on HER2. However, in some cancer cells, the cytotoxic property of PGV-0 is still inferior compared to PGV-1, the other curcumin analog (Meiyanto, *et al.*, 2019, 2018). This data suggests that the PGV-0 potential to be further developed as a specific target of co-chemotherapeutic agent for HER2-positive breast cancer.

Moreover, PGV-0 induced apoptosis and cause cell cycle arrest on MCF-7 breast cancer cells (Hermawan, *et al.*, 2011; Meiyanto, 2011). In addition to the potential for single cytotoxicity of the PGV-0, PGV-0 can increase the cytotoxic effects of doxorubicin and have the potential as a co-chemotherapy agent. PGV-0 increases cytotoxic effect of doxorubicin through suppressed HER2 and P-gp expression which leads to the induction of apoptosis (Meiyanto, *et al.*, 2014). Hence, we explore the potential of PGV-0 in inhibition of proliferation, and mechanism of action through senescence in HER2-positive HCC1954 breast cancer cells.

METHODS

Cell Culture

HCC 1954 breast cancer cell line was kindly obtained from Dr. Muhammad Hasan Bashari, Universitas Padjadjaran, Indonesia. Cell cultures were grown with the Roswell Park Memorial Institute (RPMI, New York, USA) with 10% Fetal Bovine Serum (Sigma-Aldrich, St. Louis, USA), penicillin-streptomycin 1% v/v (Gibco, New York, USA). Cells were incubated at 37°C at 5% CO₂. Curcumin and PGV-0 were obtained from the Curcumin Research Center (CRC), Faculty of Pharmacy, Universitas Gadjah Mada and doxorubicin was purchased from Wako, Japan.

Cytotoxic Test

Cytotoxicity tests were carried out using the MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-tidiphenyltetrazolium) assay referred to (Mosmann, 1983)

with modification. Briefly, a 96-well microplate was seeded with 1954 HCC cells in the amount of 8×10^3 cells in 100 μ L per well and incubated for 24 h. The next day, cells were treated with curcumin and PGV-0 and the combination with doxorubicin at various concentrations and incubated for 24 h at 37°C with 5% CO₂. Then, 100 μ L 0.5 mg/mL of MTT reagent (Biovision, California, USA) was added and cells were incubated for 2 h. The reaction was stopped by adding an SDS stopper solution containing 0.01N HCl and incubated overnight. Absorbance was then measured using a plate reader (BioRad, California, USA) at 595 nm.

Senescence-associated β -Galactosidase assay (SA- β -Galactosidase assay)

SA- β -Galactosidase assay was carried out using X-Gal staining as previously reposted (Larasati, *et al.*, 2018). In brief, HCC 1954 cells (2×10^5 cells/well) were seeded on 6 well plates, incubated for 24 h. After that, the cells were treated with curcumin and PGV-0 and the combination of PGV-0 with doxorubicin at various concentrations and incubated for 24 h at 37°C with 5% CO₂. Then cell fixation was performed with fixation buffer (4% paraformaldehyde, glutaraldehyde and aquabidest), 10 minutes incubation. Then added 2mL X-Gal solution containing X-Gal (Sigma-Aldrich, St. Louis, USA), K₃Fe [CN]₆, K₄Fe [CN]₆, MgC₁₂, PBS2X and Aquabidest. The plate was incubated in a non CO₂ incubator at 37°C. Then cells were observed after 24, 48 and 72 h staining with an inverted microscope (Olympus CKX41) (Debacq-Chainiaux, *et al.*, 2009). Further analyzed using ImageJ 1.51j8 java 1.8.0_112 and IBM SPSS Statistics program.

Intracellular Reactive Oxygen Species (ROS) Level with Flowcytometry

HCC 1954 cells was cultured with 2×10^4 in culture media (RPMI) and incubated for 24 h at 37°C. Cultured media was removed and washed 1x PBS once. Cells were treated with PGV-0 30 μ M, doxorubicin 100 nM and both. Cells were stained using the 2',7'-dichlorofluorescein diacetate

(DCFDA) Staining Kit (BD Bioscience, San Jose, California) according to the manufacturer's instructions, and analyzed with a FACSCalibur flow cytometer (BD Bioscience, San Jose, California).

RESULTS

PGV-0 Shows Cytotoxicity on HCC 1954 Cell Line

The purpose of this research was to find out the cytotoxic potential of PGV-0 as chemopreventive agent and the combined effects with doxorubicin. First, we evaluated the cytotoxic activity of the PGV-0 and compared it with Curcumin on HCC 1954 cells. The result showed that the treatment of PGV-0 and curcumin at the concentration of 5-100 μ M exhibited a cytotoxic effect on HCC 1954 cells in a dose-dependent manner with IC₅₀ value of 39 μ M and 36 μ M respectively (Figure 1A). Whereas the Cytotoxic effect on Vero cells showed the IC₅₀ value of 235 μ M and 74 μ M respectively. Curcumin more toxic than PGV-0 on Vero cells. The selectivity of PGV-0 against normal vero cells (Figure 1B) compared to HCC 1954 cells, with selectivity index value of 6.02. This value reached the minimum selective number which is 3, so that the PGV-0 is selective for normal cells. Decreasing viability of HCC 1954 cells after PGV-0 treatment can be seen from changes in cells morphology after 24 h of treatment compared to the untreated group (cell control) (Figure 1C).

PGV-0 Increases Cytotoxicity of Doxorubicin

PGV-0 and curcumin exhibited a weak cytotoxic effect on HCC 1954 cells, yet this phenomena still potential to be studied further as doxorubicin co-chemotherapeutic agent. The results of cytotoxic combinations of PGV-0 with doxorubicin on HCC 1954 cells (Figure 2A) showed that the combination of sub-doses of PGV-0 (1/2, 1/4 and 1/8 IC₅₀) with doxorubicin 100, 300, 600 nM had CI values of <1 (Figure 2B). These CI values show synergistic effect between PGV-0 and doxorubicin. The most synergistic combination of the sub-dose

concentration treatment was $1/2$ IC_{50} PGV-0 with doxorubicin 600 nM. The combination of the lowest concentration of sub-dose $1/8$ IC_{50} with Doxorubicin 100 nM showed a synergistic effect and increased the cytotoxic effect of doxorubicin.

PGV-0 Induced Senescence

To determine the mechanism of proliferation inhibition, we evaluated the induction of senescence on HCC 1954 cells following single dose treatment of IC_{50} $1/2$ and $1/4$ IC_{50} treatment. Also, we tested the effect of the combination of PGV-0 with doxorubicin on induction of senescence. Senescent cell were characterized by the green coloration of cells by X-gal dyes. This indicates the accumulation of β -galactosidase in senescent cells. Green colour formed in the cells identified as senescent positive cells (Figure 3B). The results showed that the sub-dose concentration $1/2$ IC_{50} of PGV-0 increased senescent positive cells higher than that of the untreated group (Figure 3A).

The senescent cell treatment with $1/2$ IC_{50} and $1/4$ IC_{50} PGV-0 sub-dose increased by 16% and 15% compared to the untreated group. Combination treatment PGV-0 $1/2$ and $1/4$ IC_{50} with doxorubicin 100 nM increased senescent positive cells by 25% and 18% compared to doxorubicin 100 nM.

PGV-0 Increases Intracellular Reactive Oxygen Species (ROS) Levels

To determine the mechanism of further induction senescence and inhibit proliferation cells, we evaluated intracellular reactive oxygen species levels in HCC 1954 cells. This result showed that treatment with PGV-0 30 μ M significantly increased 0.2 fold mean fluorescence intensity compared with untreated. Combination treatment PGV-0 30 μ M with Doxorubicin 100 nM significantly increases ROS levels compared treatment with doxorubicin 100 nM. PGV-0 and combination with doxorubicin increases intracellular ROS in HCC 1954 cells.

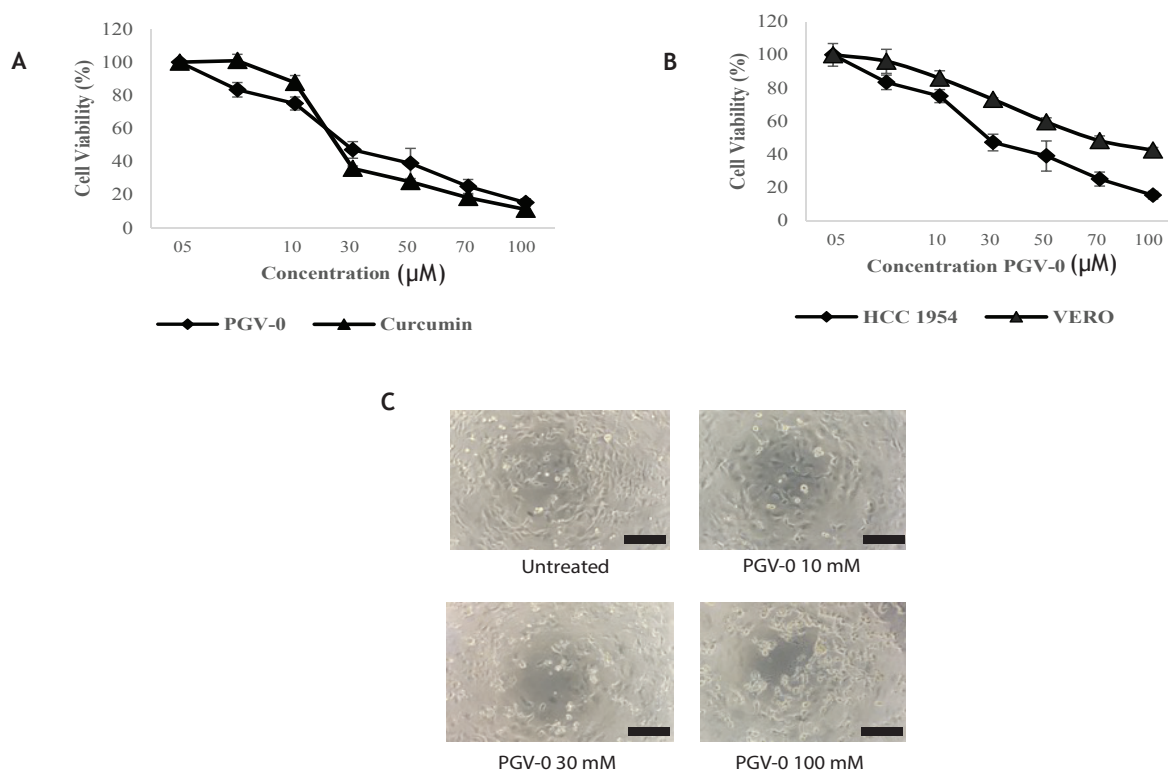


Figure 1. Cytotoxic effect of Pentagamavunon-0 (PGV-0) on HCC 1954 cells. Cytotoxic profile of Curcumin and PGV-0 on HCC1954 cells describing correlation between concentration and cell viability (A) Curcumin and PGV-0 performed cytotoxic effect with IC_{50} value of 36 μ M and 39 μ M. Cytotoxic profile of PGV-0 on HCC1954 and Vero cells (B). The cell morphology after treatment with PGV-0 in various concentrations and scale bar 100 μ M (C),

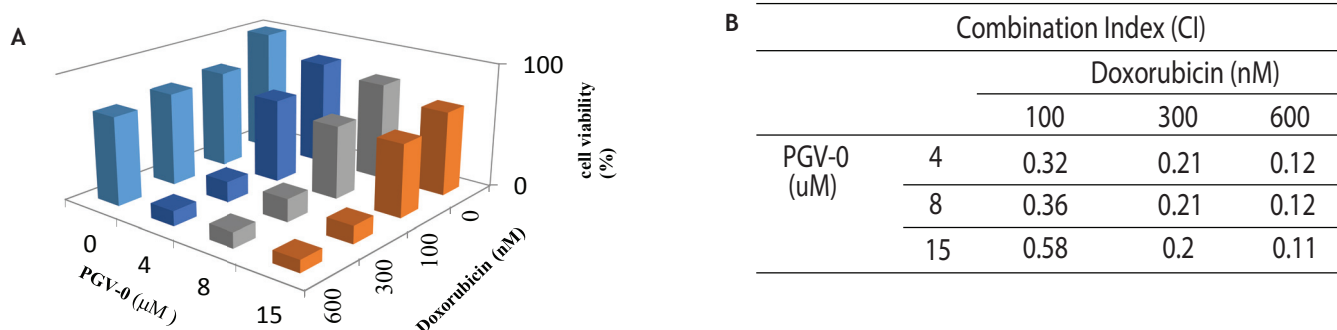


Figure 2. Cytotoxic effect of Combination Pentagamavunon-0 (PGV-0) and Doxorubicin on HCC 1954 cells (A) and Combination Index (CI) value (B). A Cytotoxic profile of Combination of PGV-0 and doxorubicin with various Concentration on HCC1954 cells describing correlation between concentration and cell viability. PGV-0 performed cytotoxic effect with combination index (CI)<1.

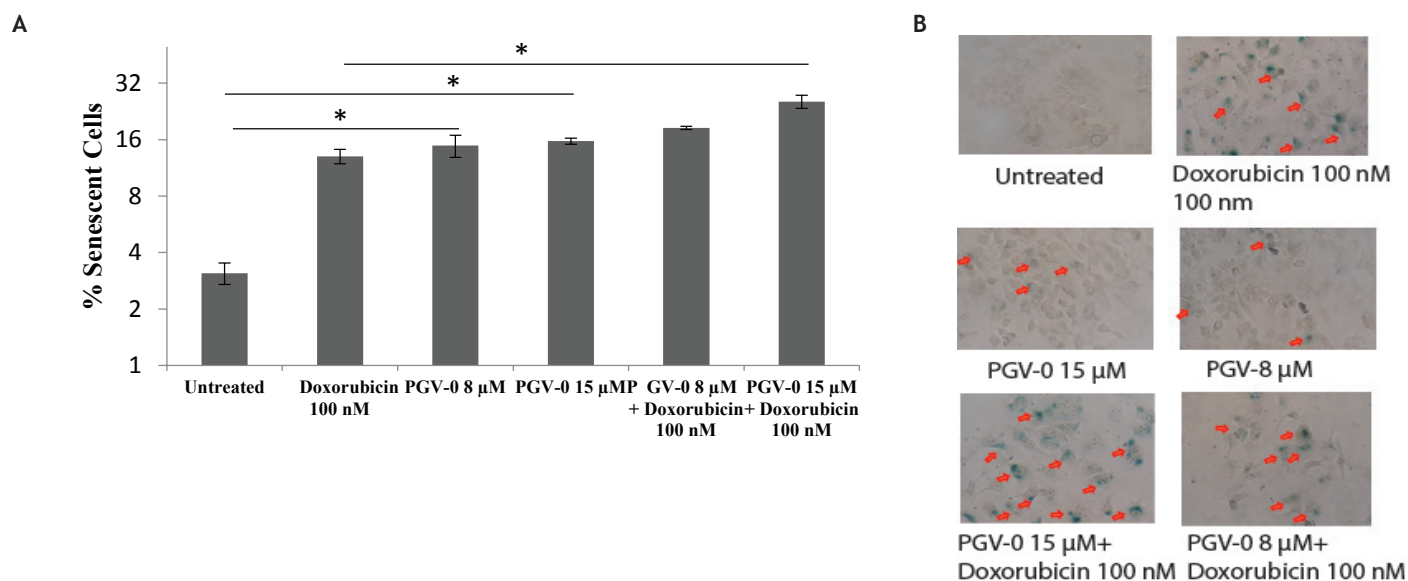


Figure 3. Senescence induction of Single and Combination Treatment Pentagamavunon-0 (PGV-0) on HCC 1954 cells (A) Quantification of cell senescent percentage on each treatment at 24 h. Statistical analysis of % senescent cell on single treatment at 24 h (* $p < 0.05$) by ANOVA one way test compared to untreated cells and combination treatment compared to doxorubicin. PGV-0 induced senescence on HCC 1954 at 8 μ M and 15 μ M concentration either in single or combination with 100 nM Doxorubicin at 24 h. (B) Red arrows show accumulation of SA- β -Galactosidase on the cells.

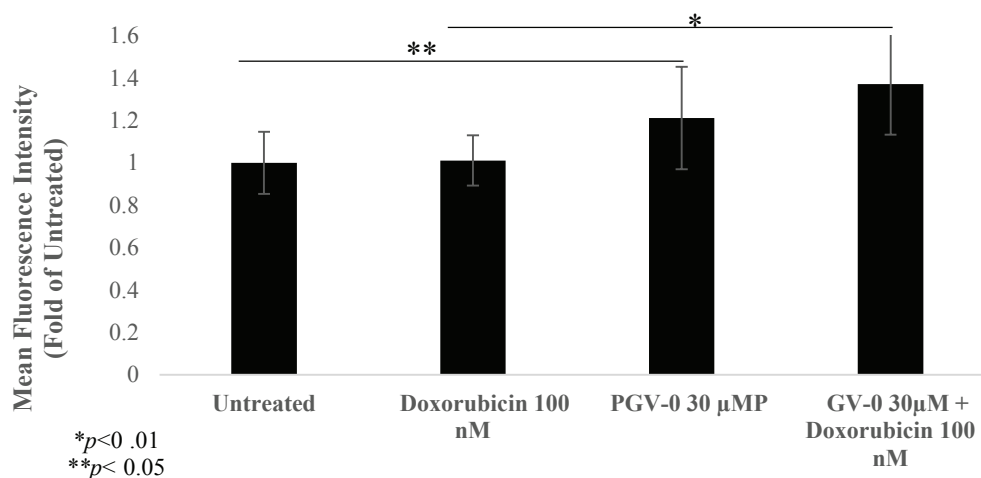


Figure 4. PGV-0 increases intracellular ROS levels to inhibit HCC 1954 cell proliferation. Cells (2×10^5 cells/mL) were treated with PGV-0 (30 μ M), Doxorubicin 100 nM and both for 24h, and subjected to the ROS detection analysis using FACS. Results are the average of three independent experiments (means \pm SD).

DISCUSSION

Curcumin and Curcumin Analog, PGV-0, have been known to possess cytotoxic or antiproliferative effects on several cancer cell lines such as MCF-7, T47D, HeLa, Raji, Myeloma and WiDr (Hermawan, *et al.*, 2011; Ikawati and Septisetyani, 2018; Meiyanto, 2011). Exploration of curcumin and its analogues as co-chemotherapeutic agents provides new insight for reducing chemoresistance and side effects toxicity toward normal cells. Results of this study showed that the PGV-0 performed cytotoxic effect in HCC 1954 cells, a cell model of HER2 positive cancer cells. Cytotoxic effect of curcumin and PGV-0 are almost similar, with the IC_{50} values of 36 μ M and 39 μ M, respectively. These IC_{50} values indicated that both compounds are categorized to be weak cytotoxic effects. However based on the selectivity index, those compounds could be developed as adjuvant therapy for metastatic cancer or co-chemotherapeutic agents.

Curcumin deserves a special mention among the list of adjuvants due to its better success rate in cancer chemotherapy (Sa and Das, 2008). Curcumin is a nontoxic agent toward normal cells

and has been proposed to increase the therapeutic efficiency of chemotherapeutics. Curcumin and its analogues PGV-0 in combination with doxorubicin increases the sensitivity of MCF-7 cells that are resistant to doxorubicin (Meiyanto, *et al.*, 2014). This study demonstrated that the combination of PGV-0 with doxorubicin increased cytotoxic effect synergistically. Results of this study supported the previous studies which showed that PGV-0 increased cytotoxic doxorubicin in MCF-7 cells, and also declare the potential of PGV-0 as a co-chemotherapeutic agents of doxorubicin.

Moreover we found that both PGV-0 and curcumin alone and combination induced senescence in correlation with their cytotoxic activities. PGV-0 is potential to be further developed as a specific target of co-chemotherapy agents for positive HER2 breast cancer. It was discovered that curcumin arrested cells in the G2/M phase and induces apoptosis and senescence (Larasati, *et al.*, 2018). In this regard, curcumin binds to several enzymes that function in the metabolic pathways of ROS and regulate levels of intracellular ROS, which trigger the checkpoints that are dependent on p53, resulting in apoptosis or aging (Larasati, *et*

al., 2018). The increasing ROS under PGV-0 treatment suggested the marked point of its cytotoxic property. PGV-0 might induced senescence through targets that with curcumin which are ROS metabolizing enzymes. However the cytotoxic effect in this HER2 positive cells may be also correlated to its affinity to bind and inhibit HER2. All of these possibilities are subjected to be explored further.

CONCLUSION

PGV-0 exhibits cytotoxic effect, increases cytotoxic effect of doxorubicin and induces senescence in HCC 1954 cells. PGV-0 is potential to be developed as a co-chemotherapeutic agent.

REFERENCES

- Campisi, J., 2013, Aging, cellular senescence, and cancer, *Annu. Rev. Physiol.* **75**, 685-705.
- Campisi, J., 1997, Aging and cancer: the double-edged sword of replicative senescence. *J Am Geriatr Soc.*, **45**, 482-488.
- Dawood, S., Broglio, K., Buzdar, A.U., Hortobagyi, G.N. and Giordano, S.H., 2010, Prognosis of women with metastatic breast cancer by HER2 status and trastuzumab treatment: an institutional-based review, *J. Clin. Oncol.*, **28**, 92-98.
- Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J. and Toussaint, O., 2009, Protocols to detect senescence-associated beta-galactosidase (SA-beta-gal) activity, a biomarker of senescent cells in culture and in vivo, *Nat Protoc.*, **4**, 1798-1806.
- Hermawan, A., Fitriyanti, A., Junedi, S., kawati, M., Haryanti, S., Widaryanti, B., Da'i, M., and Meiyanto, E., 2011, PGV-0 and PGV-1 Increased Apoptosis Induction of Doxorubicin on MCF-7 Breast Cancer Cells, *Pharmakon*, **12**(2), 55-59.
- Huun, J., Lønning, P.E. and Knappskog, S., 2017. Effects of concomitant inactivation of p53 and pRb on response to doxorubicin treatment in breast cancer cell lines, *Cell Death Discov.*, **3**, 17026.
- Ikawati, M. and Septisetyani, E.P., 2018, Pentagamavunone-0 (PGV-0), a Curcumin Analog, Enhances Cytotoxicity of 5-Fluorouracil and Modulates Cell Cycle in WiDr Colon Cancer Cells, *Indonesian Journal of Cancer Chemoprevention*, **9**(1), 23-31.
- Kusumastuti, R., Utomo, R.Y. Khumaira, A., Putri, H., Jenie, R.I. and Meiyanto, E., 2019, Pentagamaboronon-0 increased cytotoxicity of and inhibited metastasis induction by doxorubicin in breast cancer cells, *Journal of Applied Pharmaceutical Science*, **9**(6), 043-051.
- Larasati, Y.A., Yoneda-Kato, N., Nakamae, I., Yokoyama, T., Meiyanto, E. and Kato, J., 2018, Curcumin targets multiple enzymes involved in the ROS metabolic pathway to suppress tumor cell growth, *Scientific Reports*, **8**, 2039.
- McDermott, M.S.J., Conlon, N., Browne, B.C., Szabo, A., Synnott, N.C., O'Brien, N.A., Duffy, M.J., Crown, J. and O'Donovan, N., 2019, HER2-Targeted Tyrosine Kinase Inhibitors Cause Therapy-Induced-Senescence in Breast Cancer Cells, *Cancers (Basel)* **11**(2), 197.
- Meiyanto, E., 2011, PGV-0 and PGV-1 Increased Apoptosis Induction of Doxorubicin on MCF-7 Breast Cancer Cells, *Pharmakon: Jurnal Farmasi Indonesia*, **12**(2), 55-59.
- Meiyanto, E., Nakamae, I., Kawaichi, M., Kato, J.-Y., Lestari, B., Ikawati, M., Yoneda-Kato, N., Utomo, R., Jenie, R., Putri, H. and Larasati, Y., 2019, Anti-proliferative and Anti-metastatic Potential of Curcumin Analogue, Pentagamavunon-1 (PGV-1), Toward Highly Metastatic Breast Cancer Cells in Correlation with ROS Generation, *Advanced Pharmaceutical Bulletin*, **9**(3), 445-452.
- Meiyanto, E., Putri, D.D.P., Susidarti, R.A., Murwanti, R., Sardjiman, null, Fitriyanti, A., Husnaa, U., Purnomo, H. and Kawaichi, M., 2014, Curcumin and its analogues (PGV-0 and PGV-1) enhance sensitivity of resistant MCF-7 cells to doxorubicin through inhibition of HER2 and NF-κB activation, *Asian Pac. J. Cancer Prev.*, **15**, 179-184.
- Meiyanto, E., Septisetyani, E.P., Larasati, Y.A. and Kawaichi, M., 2018, Curcumin Analog Pentagamavunon-1 (PGV-1) Sensitizes WiDr Cells to 5-Fluorouracil through Inhibition of NF-κB Activation, *Asian Pac J Cancer Prev.*, **19**(1), 49-56.
- Mosmann, T., 1983, Rapid colorimetric assay for cellular growth and survival: applica-

- tion to proliferation and cytotoxicity assays, *J. Immunol. Methods*, **65**, 55-63.
- Qodria, L., Hairunisa, I., Utomo, R.Y., Hermawan, A. and Meiyanto, E., 2018, Anti-metastatic Activity of Curcumin Analog Pentagamaboronon-0-Sorbitol Against HER2-overexpressed MCF-7 Breast Cancer Cells, *Indonesian Journal of Cancer Chemoprevention*, **9**(3), 118-125.
- Sa, G. and Das, T., 2008, Anti cancer effects of curcumin: cycle of life and death, *Cell Div* **3**, 14.
- Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J. and Ullrich, A., 1989, Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer, *Science*, **244**(4905), 707-712.
- Sliwinska, M.A., Mosieniak, G., Wolanin, K., Babik, A., Piwocka, K., Magalska, A., Szczepanowska, J., Fronk, J. and Sikora, E., 2009, Induction of senescence with doxorubicin leads to increased genomic instability of HCT116 cells, *Mech. Ageing Dev.*, **130**(1-2), 24-32.
- Utomo, R.Y., Putri, H., Pudjono, P., Susidarti, R.A., Jenie, R.I. and Meiyanto, E., 2017, Synthesis and Cytotoxic Activity of 2,5-Bis(4-Boronic Acid)Benzylidene Cyclopentanone on HER2 Overexpressed-Cancer Cells, *Indonesian Journal of Pharmacy*, **28**(2), 74-81.