The potency of Pentagamavunone-0 (PGV-0) as chemopreventive agent for the formation and growth of breast cancer as revealed in 3D model

Wulandari¹,², Muthi’ Ikawati²,³, and Edy Meiyanto²,³,*

¹Master Student of Biotechnology Program, Graduate School, Universitas Gadjah Mada, Jl. Teknika Utara, Yogyakarta 55281, Indonesia
²Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia
³Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia
*Corresponding author: edy_meiyanto@ugm.ac.id

SUBMITTED 22 November 2019  REVISED 16 February 2020  ACCEPTED 24 April 2020

ABSTRACT Pentagamavunone-0 (PGV-0) or 2,5-bis(4'-hydroxy-3-methoxybenzylidine)-cyclopentanone is a curcumin analogue that exhibits anticancer activity in breast cancer cells. However, most of previous reports are limited to the use of two-dimensional (2D) cell culture. The use of three-dimensional (3D) cell culture model in cancer research can represent the real condition of cancer growth in patients better than the 2D culture. The purpose of this study was to determine the anticancer activity of PGV-0 on a 3D model of HCC 1954 breast cancer cells. HCC 1954 cells were grown in the 3D culture in the presence of PGV-0, and the spheroid formation and growth of formed spheroids were observed using microscope at 24 and 96 h, respectively. The cytotoxic effects were measured by MTT assay. PGV-0 inhibited the formation and growth of spheroids at the concentration as low as 60 µM. The cytotoxic effect of PGV-0 appeared in a dose-dependent manner with the IC₅₀ value of 70.9 µM. The results of this study indicate that PGV-0 has an anticancer activity on a 3D model of HCC 1954 breast cancer cell line. Therefore, the result supported the potency of PGV-0 as cancer chemopreventive agent.

KEYWORDS 3D model; chemoprevention; cytotoxic; HCC 1954 breast cancer cells; Pentagamavunone-0 (PGV-0)

1. Introduction

Chemoprevention is the process of preventing or inhibiting the development of cancer by using chemopreventive agents, either natural or synthetic chemical substances (Meiyanto et al. 2012). Natural compounds are widely used as chemopreventive agents because of their safety, low toxicity, antioxidants, and used as food supplements. Curcumin, epigallocatechin 3-gallate (EGCG), resveratrol, sulforaphane, and withaferin-A are natural compounds that have potential as chemopreventive agents (Pistollato et al. 2017).

Pentagamavunone-0 (PGV-0) or 2,5-bis(4'-hydroxy-3-methoxybenzylidine)-cyclopentanone is an analogue of curcumin. Synthesis of PGV-0 was carried out to increase the solubility level of curcumin in water and maintain its stability at pH>6.5 (Mohammadian et al. 2019; Utomo et al. 2017). It was reported to have similar or better anti-inflammatory activities than curcumin. PGV-0 can inhibit the growth of T47D breast cancer cells through apoptosis and has anti-proliferative effects on myeloma cells (Da’i et al. 2004, 2007). PGV-0 also shows cytotoxic activity in WiDr colon cancer cells (Septisetyani et al. 2008) and MCF-7 breast cancer cells (Hermawan et al. 2011). The previously reported anti-cancer research of PGV-0 is limited to the use of two-dimensional (2D) cell culture and had not been carried out on three-dimensional (3D) cell culture.

The 3D cell culture performs better abilities to represent the real condition of cancer cells in the patients compared to the 2D culture. These better abilities include cell morphology, cell-cell communication, gene expression, and biological and stress response (Boutin et al. 2018). In 2D cell cultures, cancer cells attach and grow on a flat surface resulting one layer of proliferating cells; while cancer cells in 3D cultures form aggregates or spheroids, 3D-shaped cancer cells, consisting of proliferation, quiescent, and necrotic zones (Edmondson et al. 2014). Concerning the stress response, the 3D model is also suitable to explore the resistance characteristic or the stem cell-like phenomenon.

The physiological mechanism of PGV-0 as a candidate for chemopreventive agent needs to be explored further, mainly focusing on the stress response. Curcumin has been widely explored for its cytotoxic activities to many types of cancer cells. The high selectivity of curcumin to cancer cells rather than normal cells possibly a correlates with the inhibitory activities against some reactive oxygen species (ROS) metabolizing enzymes that contribute...
to the induction of cell apoptosis as well as cell migration (Larasati et al. 2018). Therefore, PGV-0 may have the same mechanism as curcumin that triggers cell death in a 3D system. This research was carried out to determine the biological activity of PGV-0 as a candidate for chemopreventive agents for breast cancer by using HCC 1954 breast cancer cells in a 3D model.

2. Materials and Methods

2.1. Materials
Curcumin and PGV-0 in powder forms were obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. Curcumin (271.5 mM) and PGV-0 (283.8 mM) stock solutions were prepared by dissolving them in dimethyl sulfoxide (DMSO) (Sigma Aldrich). The stock solutions were then dissolved in culture medium at a serial concentration 10, 20, 30, 60, 80, and 160 µM to treat the cancer cells.

2.2. Cell culture
HCC 1954 breast cancer cell line, given by Dr. med. Muhammad Hasan Bashari, M.D., M.Kes., Universitas Padjadjaran, Indonesia, was grown in the RPMI (Gibco) medium supplemented with 10% v/v FBS (Sigma) and 1% v/v penicillin-streptomycin (Gibco) (Bashari et al. 2019). The cells were incubated in an incubator with 5% CO₂ at 37°C. At 80% confluence, cells were harvested by using 0.025% trypsin-EDTA (Gibco).

2.3. Spheroid formation
The formation of spheroids was carried out by growing HCC 1954 cells (9 × 10³ cells/well) in the RPMI medium on 96-well plates that had been coated with 1.5% agarose matrix (Sigma). The cells were incubated for 96 h until the spheroids were formed and ready to be used for experiments (Bashari et al. 2019). The morphological appearances of the spheroids were observed by using an inverted microscope (Boeco) at 24, 48, 72, and 96 h. The diameter of the spheroids was measured using Image Raster 3.0 software while the density of the spheroids was measured using ImageJ software.

2.4. Inhibitory effect of spheroid formation
To find out the inhibitory effect of PGV-0 and curcumin on spheroid formations, HCC 1954 cells (9 × 10³ cells/well) were grown in RPMI medium on an agarose-coated 96-well plate. The cells then were treated with 100 µL/well of PGV-0 or curcumin at a serial concentration (0, 10, 20, 30, 60, 80, 100, and 160 µM). Spheroids were formed after 24 h incubation.

2.5. Inhibitory effect of spheroid growth
The effect of PGV-0 and curcumin on the growth of spheroids can be observed after the spheroids had been formed. The spheroids were treated with PGV-0 or curcumin at a serial concentration and incubated for 96 h. During the incubation period, the spheroids were observed using an inverted microscope (Boeco) at 0, 24, 48, 72, and 96 h.

2.6. Cytotoxicity assay
The cytotoxic effect of PGV-0 and curcumin were measured using MTT assay according to the previous report (Ho et al. 2012) with a slight modification to the protocol. Briefly, the spheroids which had been treated with PGV-0 or curcumin for 96 h were then transferred to a new 96-well plate using a micropipette. Ten microliters of MTT reagents were added to each well-containing spheroid in 90 µL of the transferred medium. The reaction was stopped by adding an SDS stopper solution containing 0.01 N HCl to the spheroids that had been incubated for 4 h. Furthermore, the spheroids were incubated overnight at room temperature in a dark place. Absorbance was measured using an ELISA reader with a wavelength of 595 nm. Percentage of the cell viability was calculated to determine IC₅₀ values by a linear regression analysis (Ikawati et al. 2018).

3. Results and Discussion

3.1. Results

3.1.1 Spheroid formation
The spheroids of HCC 1954 breast cancer cells began to form at 24 h, but the compactness was relatively low. The compactness increased as the incubation time increased. It caused a decrease in the diameter of spheroids during the incubation period (Figure 1B). The highest reduction of spheroids diameter occurred from 24 h to 48 h, which was 7%, while the total decrease in spheroid diameter during the incubation period was 12% (Figure 1C). The cohesiveness of spheroids was obtained by measuring the spheroid density based on the color of the observed images. The color of spheroids was getting darker during the incubation period (Figure 1A), which means that the thickness of the spheroids was increasing (Figure 1D).

3.1.2 PGV-0 inhibits spheroid formation
Spheroids were formed after incubation for 24 h (Figure 2A). PGV-0 began to inhibit the formation of spheroids at 60 µM. It could be seen that at this concentration, HCC 1954 cells could not form compact aggregates. Some HCC 1954 cells partially scattered at the edge of non-compact aggregates as a single cell (Figure 2B). Under 60 µM, PGV-0 resulted in spheroids with the cohesiveness decrease as the increase of the concentrations. Curcumin could inhibit the formation of spheroids at a lower concentration than PGV-0, which is 30 µM. At this concentration, HCC 1954 cells formed smaller compact aggregates than untreated spheroids and were surrounded by non-compact aggregates.
FIGURE 1 Spheroid formation of HCC 1954 cells. (A) HCC 1954 cells (9 × 10^3 cells/well) were grown in a 96-well plate coated with 1.5% agarose matrix. The formation of the spheroids was observed using an inverted microscope up to 96 h. (B) The diameter of the spheroids was measured using Image Raster 3.0 software and (C) the decrease in diameter was measured at the indicated time. (D) The density of the spheroids was measured using ImageJ software. Each point on B and D was measured from three wells and is presented as mean ± standard deviation; while point on C was calculated based on the data from B and is represented in average. The asterisk (*) indicates a significant difference (P<0.05).

FIGURE 2 Inhibition of spheroid formation by PGV-0 or curcumin at 24 h. (A) HCC 1954 cells (9 × 10^3 cells/well) were grown and treated with PGV-0 or curcumin at a serial concentration for 24 h until spheroids formed. Figures depicted at 0 h after spheroid formation. (B) After 24 h of spheroid formation, the inhibitory effect of PGV-0 started to be observed at a concentration of 60 µM. Yellow arrow shows the single cells at the edge of non-compact aggregates.
FIGURE 3 Effect of PGV-0 or curcumin on the spheroids of HCC 1954 cells. (A) HCC 1954 spheroids that have been formed, subsequently were treated by PGV-0 or curcumin at a serial concentration for 96 h. (B and C) The spheroid diameter was measured using Image Raster 3.0 software. (D) The density of the spheroids was measured using ImageJ software. Each point on B, C, and D was measured from three wells and is presented as mean ± standard deviation, except for curcumin 0 h on B that was measured from one well.

FIGURE 4 The cytotoxic effect of PGV-0 or curcumin on the HCC 1954 spheroids. The formed spheroids were treated by PGV-0 or curcumin for 96 h. Cell viability was measured using MTT assay in triplicate. The cytotoxic effects of PGV-0 or curcumin were shown by the percentage of cell viability (mean ± standard deviation).
3.1.3 PGV-0 inhibits spheroid growth

The data showed that PGV-0 and curcumin caused decreases and increases, respectively, in the formed spheroids (Figure 3A). PGV-0 increased the spheroid diameter at a concentration of 60 µM while curcumin at a concentration of 30 µM, but not at the lower concentration (Figure 3B). After 96 h, at the lower concentration, the highest total reduction in spheroid diameter was 4% by 30 µM PGV-0; while 30 µM curcumin caused the highest increase as many as 8% (Figure 3C).

3.1.4 PGV-0 has cytotoxic activity on spheroids

The cytotoxic activity of PGV-0 and curcumin in spheroids was measured by MTT assay. PGV-0 and curcumin performed a cytotoxic effect on a dose-dependent manner in HCC 1954 breast cancer cells with the IC50 of 70.9 µM and 60.6 µM, respectively (Figure 4).

3.2. Discussion

This study aimed to determine the anticancer activity of PGV-0 on a 3D model of HCC 1954 breast cancer cells by inhibiting the formation, growth, and viability of spheroids. Therefore, it was started by growing the spheroids without being treated to find out the period of spheroids to be formed, the incubation period of spheroids, and the spheroid appearance during the incubation period. The result showed that spheroids began to be formed at 24 h with the compactness increased as the incubation period increased. It was indicated by the decreasing diameter and increasing density of spheroids during 96 h of incubation. Spheroids were ready to use after being incubated for 96 h in accordance to the previous study (Bashari et al. 2019). The spheroids are ready to use when there is a decrease in diameter, but the size of the spheroids is relatively the same.

The use of 2D culture in cancer research has limitations as it consists of one layer of cells so that it cannot represent the real condition of cells in patients which consists of several layers of cells. The 3D cell culture has a better ability to represent cell morphology, cell-cell communication, gene expression, and biological response compared to 2D culture (Boutin et al. 2018). In this study, PGV-0 has tested on 3D models of HCC 1954 cells. Besides PGV-0, curcumin was also used in this study as a comparison.

The results indicated that PGV-0 could inhibit the formation of spheroids, starting at a concentration of 60 µM. The inhibitory effect on spheroids increased as the concentration increased. Curcumin treatment at a serial concentration (0-160 µM) started to inhibit at a concentration of 30 µM. Although curcumin was able to inhibit at a lower concentration than PGV-0, the aggregates coming from PGV-0 treatment were more fragile by pipetting. It may indicate that PGV-0 had a better ability to reduce adhesion between cancer cells than curcumin. Epithelial-cadherin (E-cadherin) is a transmembrane glycoprotein that has a pivotal role in maintaining cell to cell adhesion (Liu and Chu 2014). The inhibitory effect on spheroids formation might be caused of PGV-0 and curcumin effects on the E-cadherin expression. However, the molecular mechanism of this phenomenon needs to be explored further.

PGV-0 showed the ability to inhibit the growth of spheroids by decreasing and increasing the diameter. The spheroid diameter started to increase at a concentration of 60 µM. Furthermore, the inhibitory effect of spheroid growths had not been seen under a concentration of 60 µM. This can be occurred because the spheroids were experiencing the same condition as untreated spheroids that were decreasing in diameter. Besides, the cohesiveness of spheroid increased, and only cells in the outer part of the spheroids began to detach. In contrast, an increase of the spheroid diameter might be caused by a decrease in adhesion between cells due to PGV-0 so that the cells detach in all parts of the spheroids. The reduced sticking ability between cells resulted in the decreased spheroid cohesiveness. This result also happened to the treatment with curcumin, but at a lower concentration of 30 µM.

The previous study reported that a single treatment of PGV-0 exhibited anticancer activities in several types of 2D breast cancer cells, namely T47D (Da’i et al. 2007), MCF-7 (Hermawan et al. 2011), and MCF-7/HER2 (Meiyanto et al. 2014). In combination with other chemotherapy agents, PGV-0 enhances their cytotoxic effects in a 2D model of MCF-7 and WiDr cells (Hermawan et al. 2011; Ikawati and Septisetyani 2018) so that it can be served the candidate of a co-chemotherapy agent. This study showed that the treatment of PGV-0 exhibits cytotoxic effects on spheroids of HCC 1954 breast cancer cells with the IC50 value of 70.9 µM. This value was about twice the IC50 of PGV-0 in 2D models of HCC 1954 cells (CCRC unpublished data, 2019). PGV-0 affects spheroids with a higher concentration than monolayer cells because the spheroids consist of several layers so that the toxic effects are not evenly distributed on all cells. The outermost part of spheroids obtained greater influence than the inner part (Sant and Johnston 2017). The toxic effects increase as the concentration increases meaning that the higher the concentration, the fewer the number of living cells. Meanwhile, the treatment of curcumin at the same serial concentration (0-100 mM) gave an IC50 value of 60.6 µM. The results showed that curcumin has a higher cytotoxic effect on a 3D model of HCC 1954 cells than PGV-0. Taken together, PGV-0 and curcumin have a cytotoxic activity at an intermediate level in the range of 21-200 µM.

In this study, curcumin performs a better effect than PGV-0 as it affects the spheroids at a lower concentration. Anticancer activity of PGV-0 is not always better than curcumin in all types of cancer cells. In WiDr colon cancer cells, curcumin had a higher cytotoxic effect than PGV-0 with an IC50 value of 27 µM; while PGV-0 is 45 µM (Septisetyani et al. 2008). Curcumin has been well known to have anticancer activity (Meiyanto 1999). It is proven to inhibit the growth of cancer cells by inducing apoptosis (Da’i et al. 2017) and increasing ROS levels beyond the threshold (Larasati et al. 2018). Therefore, PGV-0 may
inhibit the growth of cancer cells in the same pathway as curcumin. PGV-0 still has an opportunity to be used as a candidate of chemopreventive agent for breast cancer treatment because the active concentration is similar to curcumin but has more stable and better bioavailability than curcumin. Nevertheless, further investigation is needed to reveal the molecular mechanism of cells death caused by PGV-0.

4. Conclusions
PGV-0 performed the anticancer activity in the 3D model of HCC 1954 breast cancer cell line. It was seen from the ability of PGV-0 to inhibit the formation and growth of spheroids and its toxic effect. Based on the results, PGV-0 can be promoted as a candidate of chemopreventive agent for breast cancer treatment.

Acknowledgments
The authors express the gratitude to Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada Indonesia, who funded and supported this research.

Authors’ contributions
W, MI, and EM designed the study. W carried out the laboratory work. W and MI analyzed the data. W wrote the original version of the manuscript. MI reviewed and edited the manuscript. EM oversaw all aspects of the study and provided final approval for submission.

Competing interests
The authors declare there is no conflict of interest.

References


