

PGV-0 AND PGV-1 INCREASED APOPTOSIS INDUCTION OF DOXORUBICIN ON MCF-7 BREAST CANCER CELLS

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ABSTRACT

As chemotherapeutic backbone for breast cancer therapy, doxorubicin showed various side effects and induced resistancy of breast cancer cells. Development of targeted therapy on breast cancer focused on combinatorial therapy of doxorubicin and molecular targeted agents. PGV-0 and PGV-1, a curcumin analogue showed potency as co-chemotherapeutic agent with doxorubicin. Our previous study of PGV-0 and PGV-1 showed cytotoxic activity in T47D cells. Therefore, the aim of this research is to examine the synergistic effect of PGV-0, PGV-1 on the cytotoxic activity of doxorubicin through cell cycle modulation and apoptotic induction on MCF-7 breast cancer cell lines. The cytotoxic assay of PGV-0, PGV-1, doxorubicin, and their combination were carried out by using MTT assay. Cell cycle distribution and apoptosis were determined by flowcytometer FACS-Calibur and the flowcytometry data was analyzed using Cell Quest program. Single treatment of PGV-0, PGV-1 and doxorubicin showed cytotoxic effect on MCF-7 with cell viability IC50 value 50 μ M, 6 μ M and 350 nM respectively. Single treatment of Doxorubicin 175 nM induced G2/M arrest. Single treatment of PGV-0 5 μ M induced G2/M arrest while in higher dose 12.5 μ M, PGV-0 induced apoptosis. Combination of doxorubicin 175 nM and PGV-0 5 μ M induced apoptosis. Combination of doxorubicin 175 nM and PGV-0 12.5 μ M also increased apoptosis induction. Single treatment of PGV-1 0.6 μ M induced G1 arrest while in higher dose 1.5 μ M, PGV-1 induced apoptosis. Combination of doxorubicin 175 nM and PGV-1 0.6 μ M induced apoptosis. Combination of doxorubicin 175 nM and PGV-0 1.5 μ M also increased apoptosis induction. PGV-0 and PGV-1 are potential to be developed as co-chemotherapeutic agent for breast cancer by inducing apoptosis and cell cycle modulation, but the molecular mechanism need to be explored detail.

Key words: PGV-0, PGV-1, doxorubicin, co-chemotherapy, breast cancer, cell cycle arrest, apoptosis

INTRODUCTION

Breast cancer is one of most the death-cause cancer in women in the world (Jemal *et al.*, 2007). Doxorubicin is one of chemotherapeutic agent mostly used in breast cancer therapy. Using of doxorubicin at high dose causes side effect on normal tissue, primarily on heart, and suppression of immune system. Breast cancer cells also showed resistance to chemotherapeutic agent such as doxorubicin. To improve the antitumor efficacy of chemotherapy, a combination with chemopreventive agent (co-chemotherapy) would be interesting to evaluate.

PGV-0 [Pentagamavunon-0 (2,5-bis-(4'-hydroxy-3'-methoxybenzylidene)-cyclopentanone)] and PGV-1 [Pentagamavunon-1 (2,5-bis-(4'-hydroxi,3',5'-dimethyl)-benzylidin-cylopentanone)], a curcumin analogue, shows stronger cytotoxic effect than curcumin in several cancer cell lines. Our previous study of PGV-0 cytotoxic activity on T47D cells with IC50 of 9.4 μ M seemingly potential to be developed as combinatorial agent with doxorubicin. PGV-1 also showed cytotoxic activity on T47D cells with IC50 1.8 μ M and represent a new microtubule inhibitor.

Those researches showed the potency of PGV-0 and PGV-1 as chemopreventive agent and became a basic for the development of PGV-0 and PGV-1 as co-chemotherapeutic

agent to increase the cytotoxic activity and reduce the side effects of doxorubicin. Therefore, the purpose of this research is to examine the effect of PGV-0 and PGV-1 and their combination with doxorubicin on cell cycle and apoptosis of MCF-7 breast cancer cells.

METHODE

Cytotoxic Assay (MTT Method)

The method was modified from Mosmann (1983). MCF-7 cells were cultured in *Dulbecco's Modified Eagle's Medium* (DMEM) containing *Fetal Bovine Serum* (FBS) 10% (v/v) and penicillin-streptomycin 1 % (v/v). Cells (5×10^3 cells/well) were transferred to 96-well plate and incubated for 24 hours (70-80% confluent). Cells were treated by PGV-0 (Curcumin Research Center UGM), PGV-1 (Curcumin Research Center UGM) doxorubicin (Ebewe), and their combination, and incubated for 24 hour. At the end of the incubation, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] 0,5 mg/ml were added to each wells and the cells were incubated for 4 hours in 37°C. Viable cells reacts with MTT to form purple formazan crystal. After 4 hours, stopper sodium dodesil sulphate 10% in 0,1 N HCl solution were added to dissolve formazan crystal. Cells were incubated over night and protected from light. Cells were shaken for 10 minutes before read by ELISA reader at λ 595

nm. The absorbance of each well converted to percentage of viable cells :

% Viable cells =

$$\frac{\text{Treated cells abs} - \text{Medium control abs}}{\text{Cells control abs} - \text{Medium control abs}} \times 100\%$$

Flowcytometry Assay

Cells (5×10^5 cells/well) were transferred into 6-well plate and incubated until the cells return to normal condition. Cells were treated by PGV-0, PGV-1, doxorubicin, and their combination, and incubated for 24 hours. At the end of the incubation, the media containing free cells suspension were taken and transferred into 1.5 ml eppendorfs, then it were centrifugated (2000 rpm, 3 minutes) and the supernatant were removed. The cell in 6-well plate were added by PBS, and the PBS were transferred into previous eppendorfs. The eppendorfs were centrifugated and the supernatant were removed again. This steps were repeated before the cells harvested by trypsin-EDTA 0,025%. Harvested cells were transferred into the eppendorfs and centrifugated (2000 rpm, 30 seconds). The remaining cells in the 6-well plate were rinsed with PBS and transferred into the eppendorfs. The eppendorfs were centrifugated and the

supernatant were removed. Pellet cells in eppendorfs washed by cold PBS and added by propidium iodide (PI) reagents. The eppendorfs were wrapped in aluminum foil and incubated in 37°C for 10 minutes. After 10 minutes, cell suspension were homogenated and transferred into the flowcyto-tube to be analyzed by flowcytometer.

Result and Discussion

PGV-0 and PGV-1 have been proved to have cytotoxic and antiproliferative effect against several cancer cell lines such as T47D, HeLa, Raji and Myeloma. In this research, single treatment of PGV-0 and PGV-1 showed cytotoxic effect on MCF-7 cells with IC_{50} 50 μM and 6 μM (Figure 1A and Figure 1B). This result showed that the PGV-0 and PGV-1 potential as anticancer agent.

While doxorubicin showed strong cytotoxic effect in MCF-7 breast cancer cell lines (Figure 1C) with IC_{50} 350 nM. This is due to the characteristic of MCF-7 which is resistant to doxorubicin by overexpressing anti-apoptotic protein Bcl-2 and P-glycoprotein efflux pump (Davis *et al.*, 2003). To increase the sensitivity of MCF-7 cells towards doxorubicin, we combined doxorubicin with PGV-0 and PGV-1

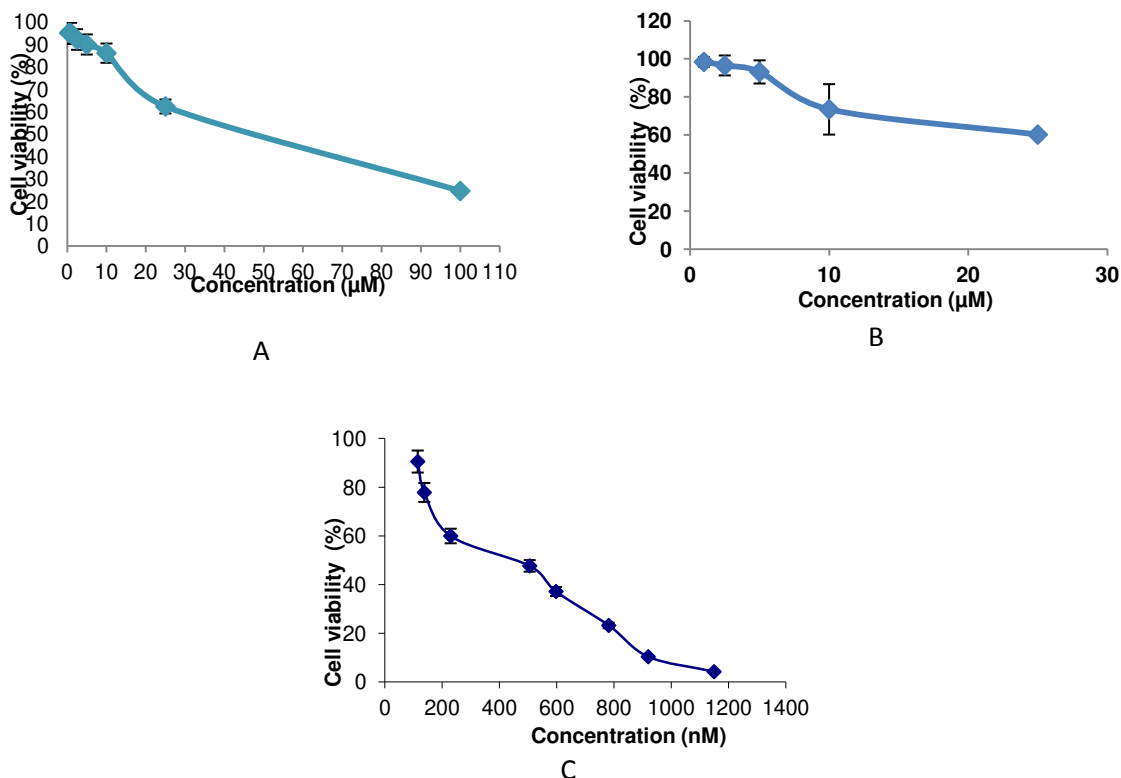


Figure 1—The effect of PGV-0 (A), PGV-1 (B) and doxorubicin (C) to the viability of MCF-7 breast cancer cells. The assay performed by incubating 5×10^5 cells/well with PGV-0 (0.5-100 μM), PGV-1 (1-25 μM) and doxorubicin (100-1100 nM) on MCF-7 for 24 hours. After 24 hours, cells were added by MTT reagent to calculate the absorbance which represent viable cells. IC_{50} value were calculated from linier regression of log concentration vs % viable cell ($p < 0,05$).

Synergistic effect of combination between PGV-0, PGV-1 and doxorubicin could be occurred via cell cycle modulation (Table 1 and Figure 2). Cell cycle analysis of both breast cancer cell lines showed that single treatment of Doxorubicin 175 nM induced G2/M arrest. Single treatment of PGV-0 5 μ M induced G1 arrest, while in higher dose (12.5 μ M) PGV-0 induced apoptosis. Combination of doxorubicin 175 nM-PGV-0 5 μ M induced apoptosis.

Combination of doxorubicin 175 nM-PGV-0 12.5 μ M also increased apoptosis induction compared with control and doxorubicin single treatment. These results showed that the combination increased apoptosis induction of doxorubicin single treatment with dose dependent mechanism. The higher dose of PGV-0 in combination, the higher of apoptosis induction occurred.

Table 1–MCF-7 Cell distribution after treatment of PGV-0, PGV-1, doxorubicin, and their combination for 24 hours

Treatment	Concentration	Sub G1 (%)	G1 (%)	S (%)	G2/M (%)
Control	-	4.00	50.22	17.43	19.73
Dox	175 nM	8.21	26.08	9.00	52.03
PGV-0	5 μ M	8.72	65.25	8.96	12.01
PGV-0	12.5 μ M	19.74	27.39	19.50	27.28
PGV-0+Dox	5 μ M - 175 nM	25.01	21.87	6.59	37.52
PGV-0+Dox	12.5 μ M - 175 nM	44.33	34.15	6.67	11.57
PGV-1	0.6 μ M	3.77	60.11	11.20	16.80
PGV-1	1.5 μ M	24.58	18.68	12.09	25.92
PGV-1+Dox	0.6 μ M - 175 nM	12.62	18.34	5.04	53.14
PGV-1+Dox	1.5 μ M - 175 nM	34.13	27.92	6.89	29.07

Single treatment of PGV-1 0.6 μ M induced G1 arrest while in higher dose (1.5 μ M) PGV-1 induced apoptosis. Combination of doxorubicin 175 nM-PGV-1 0.6 μ M induced apoptosis. Combination of doxorubicin 175 nM and PGV-0 1.5 μ M also increased apoptosis induction than combination in lower dose of PGV-1. Single treatment both of PGV-0 and PGV-1 in lower dose induced G1 arrest, but in the higher dose both of them induced apoptosis. These results showed that the combination increased apoptosis induction of doxorubicin single treatment with dose dependent mechanism. The higher dose of PGV-1 in combination, the higher of apoptosis induction occurred.

concentration of chemotherapeutic agent inside of cell will decrease and also decrease the efficacy of chemotherapeutics. Resistance of chemotherapeutics in cancer cells also caused by cell cycle arrest, so combination of chemotherapeutics with an agent which induce apoptosis directly without cell cycle arrest mechanism is better than an agent which only acts in cell cycle arrest.

MCF-7 cells showed overexpression of Bcl-2 and P-glyco Protein (PgP) so that it is resistance to apoptosis. Single treatment of doxorubicin induced overexpression of PgP in MCF-7 cells (Mealey *et al.*, 2002). Transport of drug using PgP need ATP to bring the drug outside the cells (Chung *et al.*, 2005), so that

Single treatment of PGV-0 and PGV-1 in lower dose showed G1 arrest. Curcumin induced apoptosis via ROS generation (Moussavi *et al.*, 2005) so that PGV-0 and PGV-1 probably induced apoptosis in the same pathway. In the lower dose, curcumin and its analogue probably does not enough to induce apoptosis and induce G1 arrest, but in the higher dose curcumin and its analogue induce apoptosis. In the high dose, curcumin upregulate p53 expression (Choudhuri *et al.*, 2005) so that the apoptosis induction will be occurred

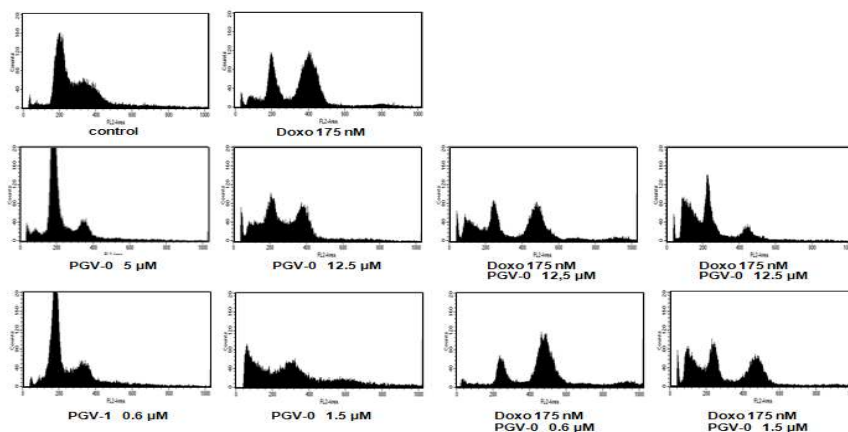


Figure 2–MCF-7 cell cycle analysis after treatment of PGV-0, PGV-1, doxorubicin, and their combination. MCF-7 cell were treated by PGV-0, doxorubicin, and their combination for 24 hours and stained by PI reagent before analyzed by flowcytometer.

Apoptosis could be occurred through mitochondrial independent pathway via Fas receptor by activating caspase 8, or mitochondrial dependent pathway via activation p53 to transcript pro apoptotic regulator protein (Bad, Bax and Bid). MCF-7 cells expressed p53 so that apoptosis pathway occurred via p53 pathway. Doxorubicin induces apoptosis through intrinsic mechanism via p53 pathway which is induce transcription of pro-apoptosis protein (Bax) (Minotti *et al.*, 2004). Single treatment of doxorubicin 200 nM in MCF-7 cells induced expression of Bax (Li *et al.*, 2007). Curcumin upregulate Bax in MCF-7/TH cells (Ramachandran and You, 1999). PGV-0 and PGV-1 probably upregulate Bax and increased apoptosis induction, but it is need to be explore details.

Resistance of cancer cells is regulated by NF- κ B (Olivier *et al.*, 2006). Doxorubicin induced overexpression of PgP via activation of NF- κ B (Wang *et al.*, 2002). Doxorubicin also increase expression of phosphorilated Akt kinase in MCF-7 cells (Li *et al.*, 2005) which is also take a place in activation of NF- κ B (Simstein *et al.*, 2003). Activation of NF- κ B will increase expression of Bcl-2 so that cancer cells also resistant to apoptosis (Ruddon, 2007). Curcumin inhibited activation of NF- κ B, downregulated Bcl-2 and downregulated Akt (Agarwal and Shishoida, 2006). Inhibition of

NF- κ B activation will down regulated PgP and Bcl-2, so that apoptosis will occurred. Combination of PGV-0 and PGV-1 with doxorubicin probably inhibit activation of NF- κ B, down regulated PgP and Bcl-2, but the mechanism of combination need to be explored further.

This result showed the potency of PGV-0 and PGV-1 to be developed as co-chemotherapeutic agent for doxorubicin by inducing apoptosis and cell cycle arrest. The use of doxorubicin together with PGV-0 and PGV-1 is expected to increase the activity and reduce the side effects of doxorubicin. However, the molecular mechanism of apoptotic induction and cell cycle arrest by this combination need to be explored further.

CONCLUSION

This research shows that combination of PGV-0, PGV-1 and doxorubicin synergically increase the effect of doxorubicin through apoptotic induction and cell cycle arrest. Based on this result, PGV-0 and PGV-1 are potential to be developed as co-chemotherapeutic agent for doxorubicin.

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