

RESEARCH ARTICLE

Combination of Potassium Pentagamavunon-0 and Doxorubicin Induces Apoptosis and Cell Cycle Arrest and Inhibits Metastasis in Breast Cancer Cells

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Abstract

A salt compound of a curcumin analogue, potassium pentagamavunon-0 (K PGV-0) has been synthesized to improve solubility of pentagamavunon-0 which has been proven to have anti-proliferative effects on several cancer cells. The purpose of this study was to investigate cytotoxic activity and metastasis inhibition by K PGV-0 alone and in combination with a chemotherapeutic agent, doxorubicin (dox), in breast cancer cells. Based on MTT assay analysis, K PGV-0 showed cytotoxic activity in T47D and 4T1 cell lines with IC_{50} values of $94.9 \mu\text{M}$ and $49.0 \pm 0.2 \mu\text{M}$, respectively. In general, K PGV-0+dox demonstrated synergistic effects and decreased cell viability up to 84.7% in T47D cells and 62.6% in 4T1 cells. Cell cycle modulation and apoptosis induction were examined by flow cytometry. K PGV-0 and K PGV-0+dox caused cell accumulation in G2/M phase and apoptosis induction. Regarding cancer metastasis, while K PGV-0 alone did not show any inhibition of 4T1 cell migration, K PGV-0+dox exerted inhibition. K PGV-0 and its combination with dox inhibited the activity of MMP-9 which has a pivotal role in extracellular matrix degradation. These results show that a combination of K PGV-0 and doxorubicin inhibits cancer cell growth through cell cycling, apoptosis induction, and inhibition of cell migration and MMP-9 activity. Therefore, K PGV-0 may have potential for development as a co-chemotherapeutic agent.

Keywords: K PGV-0 - doxorubicin - breast cancer - cell lines - apoptosis - cell cycling - metastasis

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Introduction

Long-term use of chemotherapeutic agent, doxorubicin for the treatment of cancer cause several side effects including toxicity and drug resistance (Tacar et al., 2013). Therefore, there is development of chemotherapy to reduce its side effects, called co-chemotherapy. Combination of chemotherapeutic agent with chemopreventive agent (co-chemotherapy) is an alternative to overcome the drug resistance, improve efficacy, and reduce its toxicity (Parhi et al., 2012).

Curcumin, a natural compound has been extensively explored and has the potential to be developed as co-chemotherapeutic agent. Single treatment of curcumin inhibited cancer cell growth through various mechanisms (Yoysungnoen et al., 2005; Lin et al., 2009; Prasad et al., 2009). However, based on previous study, curcumin was found to be poorly soluble in water, thus curcumin has low bioavailability (Tønnesen et al., 2002). To improve the bioavailability of curcumin in the body, curcumin has been developed to a salt compound, namely sodium

curcumin (Mukhopadhyay et al., 1982). Sodium curcumin has been shown to have anti-inflammatory activity better than curcumin (Ghatak and Basu, 1972).

Faculty of Pharmacy Universitas Gadjah Mada developed a curcumin analogue as chemopreventive agent, namely [2,5-bis (4'-hydroxy-3'-methoxybenzylidene) cyclopentanone] or pentagamavunon-0 (PGV-0) (Meiyanto et al., 2006). PGV-0 has been proven to have several pharmacological activities, especially to inhibit cancer cell growth (Meiyanto et al., 2006; Hermawan et al., 2011; Meiyanto et al., 2014). The salt compound of PGV-0, potassium pentagamavunon-0 (K PGV-0) had been developed to increase its bioavailability. This compound has a good solubility in water and is expected to have same or better activity than PGV-0 (Margono and Verawati, 2005)

In this study we found that combination of K PGV-0 and doxorubicin (dox) showed synergistic effect and decreased the viability of the breast cancer cells. The combination also caused cell accumulation in G2/M phase, induction of apoptosis either early or late apoptosis, and

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inhibition of the activity of MMP-9. Our results suggest that K PGV-0 is potential to be developed as an effective co-chemotherapy to inhibit the breast cancer cells metastasis.

Materials and Methods

Cell culture

T47D and 4T1 breast cancer cells were obtained from Prof. Masashi Kawaichi (NAIST, Japan). Cells were grown in Dulbecco's Modified Eagles Medium (DMEM) high glucose (Sigma) supplemented with 10% FBS (Sigma), HEPES, sodium bicarbonate, 1.5% Penicillin-Streptomycin and Fungizone (Gibco). Cells were incubated in 37°C with 5% CO₂.

MTT Cytotoxicity assay

MTT assay was performed according to (Mosmann, 1983), with slight modification. Cells were treated with various concentration of K PGV-0 (obtained from Curcumin Research Center, Faculty of Pharmacy, Universitas Gadjah Mada), doxorubicin (dox) (Sigma, Germany) or the combination of K PGV-0 and dox. After 24 h of treatment, cells were treated with 0.5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biovision) and incubated further for 4 h. Cells were lysed using SDS stopper containing 0.01 N HCl and incubated in the dark condition for overnight. After incubation, cells absorbance was measured by ELISA reader plate at λ 595 nm. Cells absorbance was converted to % cell viability. Linear regression between concentration and % cell viability giving the equation $y = Bx + A$ were used to calculate IC₅₀ value, that is the concentration inhibiting 50% cell proliferation.

Apoptosis assay

Cells were treated with K PGV-0, doxorubicin and the combination for 24 h. After the treatment, cells were harvested by trypsinisation and stained with Annexin-V-FLUOS staining kit (Roche) consisting of 100 mL of binding buffer, 2 mL of 2 mL Annexin V and propidium iodide (PI) and incubated for 10 minutes in the dark room, according to manufacturer's instruction, and analyzed by flow cytometer (BD FACS Calibur, BD Bioscience)

Cell cycle analysis

Cells were seeded, treated, harvested and stained with the staining solution contains propidium iodide (PI) 1 mg/mL protease inhibitor, 10 mg/mL RNase and 0,1% (v/v) Triton X-100 (Merck). Cells were incubated for 5 minutes in the dark room, transferred into a flow cytometric tube and analyzed by BD FACS Calibur (BD Bioscience, USA). Cell cycle distribution was acquired by using Flowing software.

Wound healing assay

The 4T1 cells (7.5×10^4 cells) were seeded in 24-well plate and incubated for 24 hours. Cells starvation was done by incubating cells in culture medium supplemented with 0,5% FBS for 24 hours. After starvation, cells were scratched with sterile tip, and treated with various

concentration of samples. The cells were documented at 0, 18, 24, and 42 h after treatment. The results were analyzed by ImageJ software and presented as % of wound closure.

Gelatin zymography

The 8% of SDS-PAGE supplemented with 0.1% of gelatin was used to determined the activity of MMP-9 in the culture medium. After electrophoresis, gels were washed and incubated with distilled water containing 2% of Triton-X 100 (Merck) for 30 min at room temperature. The solution was removed from gels. 100 mL of reaction buffer (40 mM Tris-HCl pH 8, 10 mM CaCl₂, 0.02% Na₃N) was added and incubated for 24 h at 37°C. After removal of reaction buffer, gels were stained by Coomassie Brilliant Blue R-250 solution and destained by destaining solution (20% methanol, 10% acetic acid and

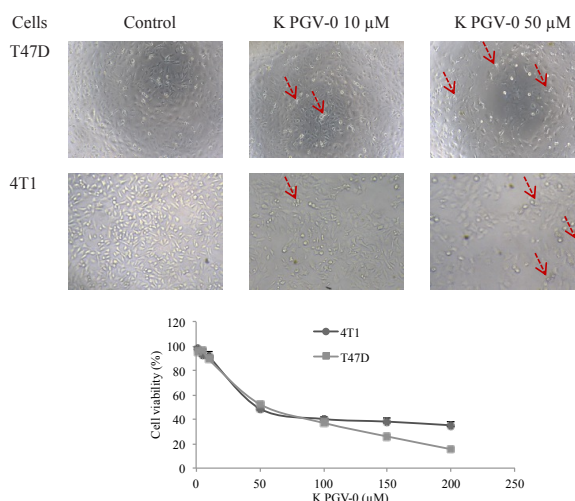


Figure 1. Cytotoxic Effect of K PGV-0 on T47D and 4T1 cells. T47D cells (8×10^3 cells/well) and 4T1 cells (5×10^3 cells/well) were seeded in 96 wellplate and incubated for 24h, then treated with K PGV-0. Cells viability was determined by using MTT assay as described in methods. (A) T47D and 4T1 cells morphology after treatment for 24 h. Arrows indicate cells morphological changes. (B) Cell viability profile after single treatment of K PGV-0 for 24 h on T47D and 4T1 cells. Profiles of cell viability were means \pm SE from 3 independent experiments

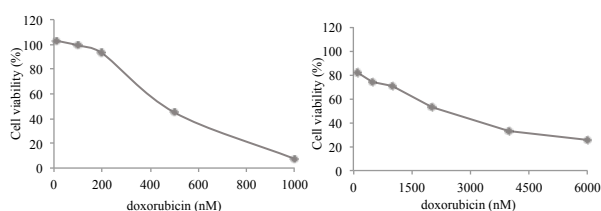


Figure 2. Cytotoxic effect of Doxorubicin on T47D and 4T1 cells. T47D cells (8×10^3 cells/well) and 4T1 cells (5×10^3 cells/well) were seeded in 96 well-plate and incubated for 24h, then treated with doxorubicin. Cells viability was determined by using MTT assay as described in the methods. (A) Cell viability profile after single treatment of Doxorubicin for 24 h on T47D cells. (B) Cell viability profile after single treatment of Doxorubicin for 24 h on 4T1 cells. Profiles of cell viability were means \pm SE from 3 independent experiments

70% water) until clear bands with dark blue background appear. The results were documented and analyzed by ImageJ software.

Statistical analysis

Data were expressed as mean \pm SE. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test were used for statistical analyses. P-values less than 0.05 were considered significant.

Results

Single Treatment of K PGV-0 and doxorubicin Revealed Cytotoxic Effect on T47D and 4T1 cells

Cytotoxic assay was conducted to analyse cytotoxicity of K PGV-0 and Dox on T47D and 4T1 breast cancer cells with IC_{50} parameter. Cytotoxic effect of K PGV-0 was observed after 24 h of treatment. Single treatment of K PGV-0 for 24 h induced morphological changes. K PGV-0 10 μ M induced cell shrinkage. At

higher concentrations, K PGV-0 50 μ M there were more number of cell shrinkage and fragmentation. In addition, single treatment of K PGV-0 for 24 h revealed cytotoxic effect on T47D and 4T1 cells with IC_{50} value of 94.9 μ M dan 48.97 \pm 0.2 μ M, respectively (Figure 1).

We also examined the cytotoxic activity of doxorubicin on T47D and 4T1 cells. Doxorubicin performed cytotoxic effect with IC_{50} value of 383.67 \pm 83.70 nM and 2.65 \pm 0.27 μ M, respectively. Subsequently, doxorubicin was combined with K PGV-0 to enhance the effectiveness and decrease side effects of chemotherapeutic agents for cancer therapy (Figure 2).

Combination K PGV-0 and Doxorubicin Decreased Cells Viability on T47D cells

Combination of doxorubicin and K PGV-0 reduced cell viability of T47D cells. The results showed that the combination of cytotoxic K PGV-0 in combination with doxorubicin for 24 hours decreased cell viability up to 84.69% (Figure 3), in combination of K PGV-0 48 μ M (1/2 IC_{50} K PGV-0) with 200 nM doxorubicin (1/2

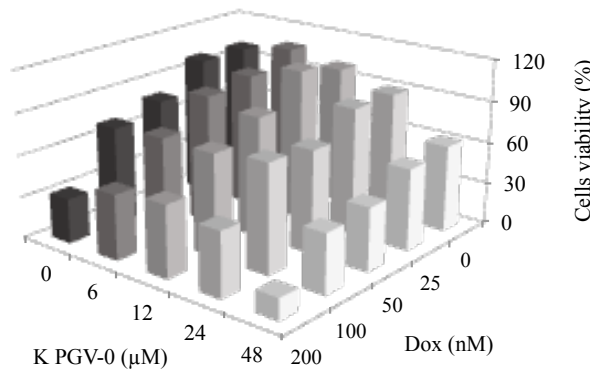


Figure 3. Cytotoxic Effect of Combination of K PGV-0 and Doxorubicin on T47D Cells. T47D cells (8×10^3 cells/well) were seeded in 96 wellplate and incubated for 24h, then treated with doxorubicin and K PGV-0 as indicated in the figure. Cells viability was determined by using MTT assay as described in the methods

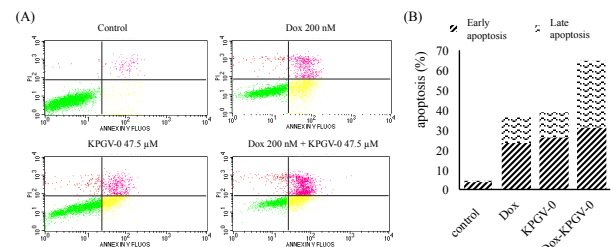


Figure 5. Effect of Combination of K PGV-0 and doxorubicin on Apoptosis Induction on T47 D Cells.

Cells were harvested after treatment of the sample (200 nM doxorubicin, 48 μ M K PGV-0, or a combination of both) for 24 hours, stained with reagents annexin V and propidium iodide (PI) and analyzed using flowcytometer. (A) The distribution profiles of living cells (bottom-left), early apoptotic (bottom-right), late apoptosis (upper-right), necrosis (upper-left) in various indicated treatments. (B) Quantification of cells undergoing early and late apoptosis

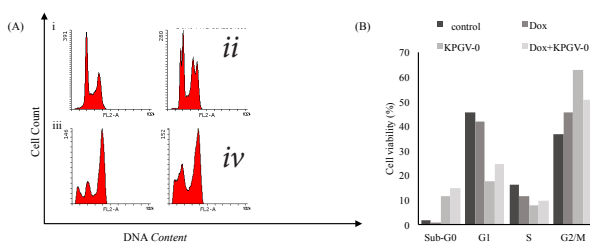


Figure 4. Effects of Combination of Doxorubicin and KPGV-0 on Cell Cycle Modulation of T47D Cells. Cells were harvested after 24 h of treatment, stained with propidium iodide and DNA content were analyzed by using flowcytometry. (A) The profiles of cells in the phases of sub-G1, G1, S and G2-M (i) control cells, (ii) doxorubicin 200 nM, (iii) K PGV-0 48 μ M, (iv) a combination of doxorubicin 200 nM - K PGV-0 48 μ M. X-axis showed the relative content of DNA and the Y axis showed the relative cell numbers. (B) Quantification of cells distribution in each phase of the various treatments. The percentage of sub-G1 phase cells showed apoptotic cell population

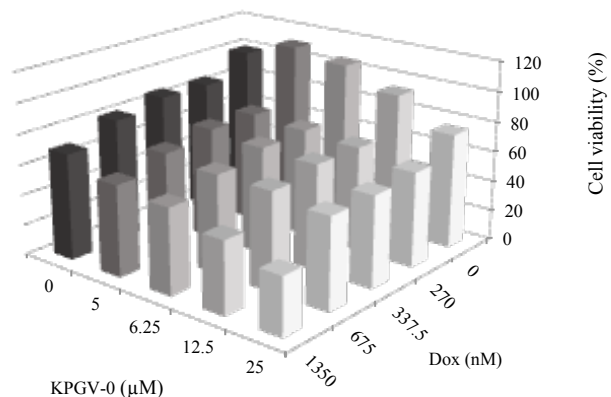


Figure 6. Cytotoxic Effect of Combination of K PGV-0 and Doxorubicin on 4T1 Cells. 4T1 cells (8×10^3 cells/well) were seeded in 96 wellplate and incubated for 24h, then treated with doxorubicin and K PGV-0 as indicated in the figure. Cells viability was determined by using MTT assay as described in the methods

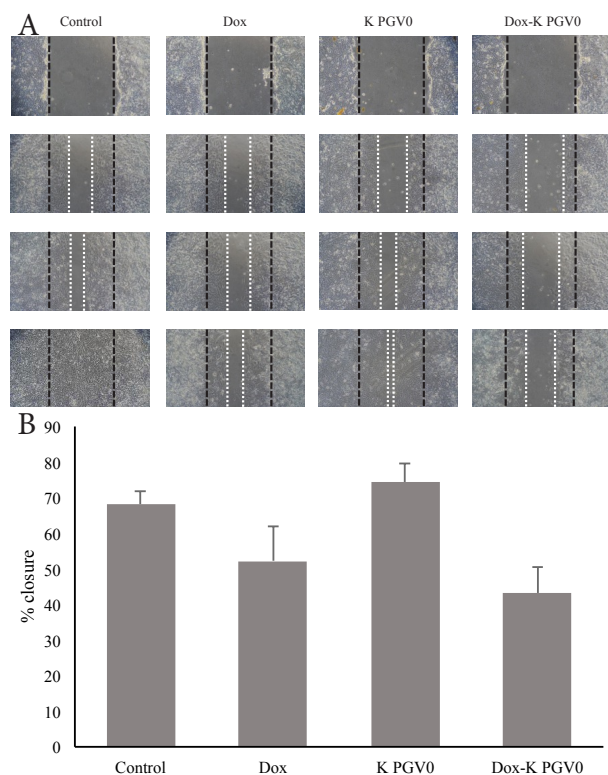


Figure 7. Effect of Single Treatment of K PGV-0 and its Combination with Doxorubicin on 4T1 Cells Migration. (A) The morphology of the cells after scratch and treated with 675 nM doxorubicin, K PGV-0 12.5 μ M and their combination. Observations were made after 0, 18, 24 and 42 hour of treatment under an inverted microscope with magnification of 100x. (B) The percentage of 4T1 cells closure after 24 h of treatment. Values were means of % closure \pm SD from 3 independent experiments. The area of the scratch were analyzed using ImageJ software then % closure was calculated in accordance with the procedures of the analysis. The asterisk (*) indicates $p < 0.05$; $n = 3$. Black dashed line showed the boundary area of scratches (scratch) h-0 and white dotted line indicated scratching area at the time of observation

Table 1. CI Values of Combination of K PGV-0 and Doxorubicin on T47D Cells

		CI			
		Doxorubicin (nM)			
		25	50	100	200
K PGV-0 (μ M)	6	3.23	17.45	0.69	0.52
	12	-0.73	1.45	0.8	0.65
	24	3.28	0.8	1.32	0.7
	48	0.71	0.59	0.66	0.58

Table 2. CI Values of Combination of K PGV-0 and Doxorubicin on 4T1 Cells

		CI			
		Dox (nM)			
		270	337.5	675	1350
PGV-0 (μ M)	5	0.45	0.53	0.65	0.79
	6.25	0.45	0.49	0.58	0.74
	12.5	0.62	0.67	0.76	0.7
	25	0.83	0.81	0.97	0.78

IC₅₀ doxorubicin). Several combination K PGV-0 with doxorubicin showed synergistic effect with value of CI

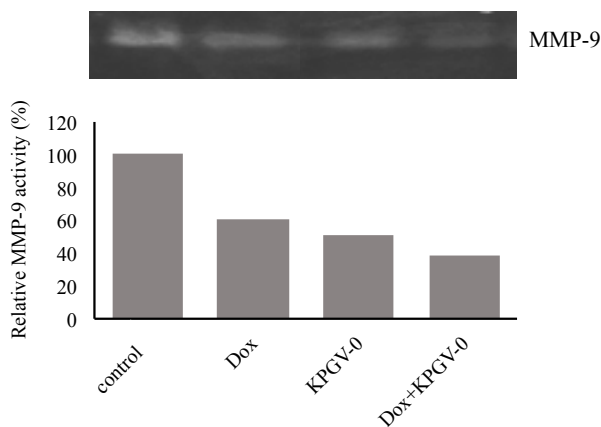


Figure 8. Effect of Single Treatment of K PGV-0 and its Combination with Doxorubicin in MMP-9 Activity on 4T1 Cells. Cells were treated with 675 nM doxorubicin, K PGV-0 12.5 μ M and their combination. MMP-9 activity assay was conducted using gelatin zymography according to the method. Analysis of the results were done by using ImageJ software to measure the intensity of gelatin degradation by MMP-9 in the gel

<1 (Table 1).

Modulation of Cell Cycle of Single Treatment of K PGV-0 and Its Combination with Doxorubicin

Based on the analysis of the cell cycle, single treatment of K PGV-0 increased T47D cells accumulation in G2/M phase. Combination of K PGV-0 and doxorubicin also increased cells accumulation in G2/M, however, with less percentage compared to single treatment of K PGV-0 (Figure 4). This phenomenon probably due to the differences of the target of K PGV-0 and doxorubicin in modulating cell cycle. Furthermore, combination of K PGV-0 and doxorubicin increased the accumulation of cells in sub-G1 phase. Cells in sub-G1 phase were hipodiploid cell with low DNA content due to the DNA fragmentation. Hipodiploid cells were the sign of cell death. Thus, the combination of K PGV-0 with doxorubicin increased the cell death of T47D.

K PGV-0 and its combination with Doxorubicin Induced Apoptosis on T47D cells

Results showed that treatment of K PGV-0 combination with doxorubicin increased the number of apoptotic cells up to 66.92% (Figure 5). We suggested that the combination of K PGV-0 with doxorubicin induced cell death through apoptosis mechanism.

Combination K PGV-0 and Doxorubicin Revealed Synergistic Effect on 4T1 cells

Combination of Doxorubicin with K PGV-0 reduced cell viability greater than single treatment of K PGV-0 and doxorubicin on 4T1 cells. Treatment of K PGV-0 in combination with doxorubicin for 24 hours reduced the viability of 4T1 cells up to 62.46% (Figure 6). In addition, combination of doxorubicin and K PGV-0 showed synergistic effect. Synergistic effect that caused cell viability less than 50% were performed by combination of K PGV-0 12.5 μ M (1/4 IC₅₀ K PGV-0) with doxorubicin 1350 nM (1/2 IC₅₀ doxorubicin) and combination of K

PGV-0 25 μM (1/2 IC_{50} K PGV-0) with doxorubicin 1350 nM (1/2 IC_{50} doxorubicin) with CI values of 0.70 and 0.78, respectively (Table 2).

Combination of K PGV-0 with Doxorubicin Inhibited 4T1 Cell Migration

Results of scratch wound healing assay up to 42 h showed that single treatment of K PGV-0 did not inhibit 4T1 cell migration. In addition, combination of K PGV-0 with doxorubicin up to 24 h inhibited the migration of 4T1 breast cancer cells with % closure up to 43.21% (Figure 7).

Combination of K PGV-0 with Doxorubicin Decreased MMP-9 Activity on 4T1 Cells

Results showed that single treatment of 1.5 μM K PGV-0 and 675 nM doxorubicin decreased gelatinase activity of MMP-9 (Figure 8). Combination treatment of doxorubicin and K PGV-0 decreased activity of MMP-9 greater than single treatment of K PGV-0 and doxorubicin. We suggested that combination of K PGV-0 and doxorubicin plays a role in breast cancer invasion process.

Discussion

Single treatment of K PGV-0 showed cytotoxic effect on T47D and 4T1 breast cancer cells with IC_{50} value 94.94 μM and 48.97 μM , respectively. Single treatment of doxorubicin also performed cytotoxic effect on T47D and 4T1 cells with IC_{50} value 400 ± 71.76 nM and 2.7 ± 0.16 μM , respectively. The IC_{50} value of K PGV-0 in T47D was about 10-fold greater than IC_{50} value of curcumin analogue. PGV-0. The IC_{50} values of PGV-0 on T47D cells was 11 μM (Meiyanto et al., 2006). This phenomenon occurs due to the polarity difference between PGV-0 and K PGV-0. The polarity difference will cause a difference in the process of drug diffusion into the cell. The process drug diffusion into the cell is important since the protein targets of both curcumin and its analogues are in the cell.

Combination of K PGV-0 with doxorubicin reduced cell viability greater than single treatment of K PGV-0 and doxorubicin on T47D cells and 4T1 cells. In general, the combination of various concentrations of K PGV-0 and doxorubicin showed synergistic effect and reduced cell viability up to 84.69% on T47D cells and 62.6% on 4T1 cells. Synergistic effect of combination PGV-0 and doxorubicin in breast cancer cells has been proven in the previous studies, PGV-0 increased the effect of doxorubicin on MCF-7 cells (Hermawan et al., 2011). Based on the result of synergistic effect of combination K PGV-0 and doxorubicin, this present study was continued to investigate possibility pathway in growth cells inhibition through cell cycle modulation and apoptosis induction.

This study showed that K PGV-0 and doxorubicin inhibited T47 breast cancer cell growth through cell cycle modulation. Single treatment of K PGV-0 and doxorubicin caused cell accumulation in G2/M phase, while combination treatment of K PGV-0 and doxorubicin caused cell accumulation at G2/M and subG1 phase. This findings indicated that the cell cycle modulation mechanism of K PGV-0 on T47D cells is similar with

curcumin analogue. PGV-0 which also cause cell accumulation in G2/M phase. Probably, molecular mechanism occurred after treatment of K PGV-0 and PGV-0 is similar with curcumin. Curcumin cause cell cycle modulation through down-regulation of cyclin A and up-regulation of cyclin-dependent kinase (CDK) inhibitor p21 (Park et al., 2006). Cell accumulation in subG1 phase is one of apoptosis property. Therefore, to prove it apoptosis induction, flow cytometric apoptosis assay was conducted.

Single treatment of K PGV-0 and doxorubicin inhibited T47D cell growth through apoptosis mechanism. Combination of K PGV-0 with doxorubicin increased apoptotic cells up to 64.58%. The molecular mechanisms causing apoptosis on T47D cells is through p53-independent pathway. Doxorubicin cleavage PARP and activate caspase-8 (Lee et al., 2002). In addition, doxorubicin induced caspase-independent cell death (Moreira et al., 2014), while curcumin analogue PGV-0 has been proven to cause apoptosis through PARP-cleavage mechanism (Da'i, 2008). Therefore, K PGV-0 alone or its combination with doxorubicin probably induced apoptosis in the same pathway with PGV-0.

In this present study, inhibition of cancer metastasis after treatment of combination K PGV-0 and doxorubicin was also observed. Cell migration is part of the process of metastasis. Single treatment of K PGV-0 did not inhibit 4T1 cells migration, while combination K PGV-0 and doxorubicin inhibited cell migration. Single treatment of K PGV-0 did not inhibit cell migration because K PGV-0 did not inhibit FAK pathway. Therefore, lamellipodia formation and actomyosin that have a pivotal role in cell migration are still occur. Combination K PGV-0 and doxorubicin inhibited cell migration because the migration inhibitory activity of doxorubicin is greater than K PGV-0. This findings is consistent with previous research which perform that doxorubicin inhibit M5076 cell migration (Sugiyama and Sadzuka, 1999) and hepatocellular cancer HepG2 cells (Yang et al., 2014)

Although single treatment of K PGV-0 did not inhibit cell migration, this compound inhibited cancer cell invasion process by reducing the activity of matrix metalloproteinase-9 (MMP-9). The possible molecular mechanism which cause this finding is inhibition of NF- κ B activation. MMP-9 transcription factor. Previous research states that curcumin and its analogue inhibit the activation of transcription factor NF- κ B (Sandur et al., 2007).

Taken together, combination of K PGV-0 and doxorubicin inhibit cancer cell growth through cell cycle inhibition, apoptosis induction, and inhibition of cell migration and MMP-9 activity. Therefore, K PGV-0 performs potency to be developed as co-chemotherapeutic agent.

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