RESEARCH ARTICLE

Curcumin and its Analogues (PGV-0 and PGV-1) Enhance Sensitivity of Resistant MCF-7 Cells to Doxorubicin through Inhibition of HER2 and NF-kB Activation

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Abstract

Chemoresistance of breast cancer to doxorubicin is mediated mainly through activation of NF-kB and over expression of HER2. Curcumin and its analogues (PGV-0 and PGV-1) exert cytotoxic effects on T47D breast cancer cells. Suppression of NF-kB activation is suggested to contribute to this activity. The present study aimed to explore the effects of curcumin, PGV-0, and PGV-1 singly and in combination with doxorubicin on MCF-7/Dox cells featuring over-expression of HER2. In MTT assays, curcumin, PGV-0, and PGV-1 showed cytotoxicity effects against MCF-7/Dox with IC50 values of 80 μM, 21 μM, and 82 μM respectively. These compounds increased MCF-7/Dox sensitivity to doxorubicin. Cell cycle distribution analysis exhibited that the combination of curcumin and its analogues with Dox increased sub G-1 cell populations. Curcumin and PGV-1 but not PGV-0 decreased localization of p65 into the nucleus induced by Dox, indicating that activation of NF-kB was inhibited. Molecular docking of curcumin, PGV-0, and PGV-1 demonstrated high affinity to HER2 at ATP binding site. This interaction were directly comparable with those of ATP and lapatinib. These findings suggested that curcumin, PGV-0 and PGV-1 enhance the Dox cytotoxicity to MCF-7 cells through inhibition of HER2 activity and NF-kB activation.

Keywords: Curcumin and its analogues - HER2 - MCF-7/Dox cells - NF-kB

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Introduction

Cancer is a complex disease due to the complexity of molecular mechanism. Therefore, the strategy in cancer therapy should be targeted on the proteins involved in signaling processes lead to the growth and development of cancer cells and also to the proteins involved in resistant mechanisms of cancer (Hanahan and Weinberg, 2011). Inhibition of Epidermal Growth Factor Receptor (EGFR), HER2 and Estrogen Receptor (ER) in breast cancer cells, has been shown to inhibit the growth and development of breast cancer cells (Vora et al., 2009). Overexpression of HER2 in the biliary epithelium of transgenic mice and in colorectal cancer cells led to an increase of COX-2 expression (Vadlamudi, 1999; Kiguchi et al., 2001). Doxorubicin (Dox) is a chemotherapeutic agent used to treat breast cancer. However, the use of Dox causes resistance of the cancer cells. Dox induces the expression of P-glycoprotein (Pgp) (Byun et al., 2005) that possibly correlated to the NF-κB activation (Shishodia et al., 2003). Therefore, decreasing the expression and inhibiting of HER2, EGFR, ER, GST, COX-2, NF-κB and Pgp should be an important strategy in the development of molecular-targeted agents in order to overcome the resistance of breast cancer cells.

Curcumin (Figure 1) is a compound occurring in *Curcuma longa* L. Curcumin has property of choleretic

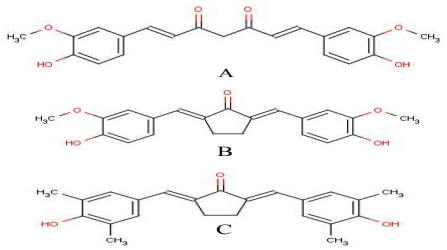

Figure 1. The Structure of Curcumin and its Analogues. A) Curcumin ((1E,6E)-1-(4-hydroxy-3-methoxyphenyl)-7-(3-methoxy-4-methylphenyl)hepta-1,6-diene-3,5-dione); **B)** PGV-0 ((2E,5E) -2- [(4-hydroxy- 3-methoxyphenyl) methylidene] -5- [(3- methoxy- 4-methylphenyl) methylidene] cyclopentan-1-one); **C)** PGV-1((2E,5E) -2- [(4-hydroxy- 3,5-dimethylphenyl) methylidene]-5-[(3-methoxy-4,5-dimethylphenyl) methylidene] cyclopentan-1-one)

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and cholagogic (Ramprasad and Sirrsi, 1957), antibacterial (Lutomsky et al., 1974; Shankar and Murthy, 1979), antihepatotoxic (Claeson et al., 1994), antiinflammation (Srimal and Dhawan, 1973; Claeson et al., 1994), antioxidant (Sharma et al., 1972), anticancer (Kuttan et al., 1985), and antivirus (Mazumder et al., 1997). Modification of curcumin molecule into some molecules to improve the pharmacological effects have been done in saveral research groups. Sardjiman, 2000 reported that two curcumin analogues, namely Pentagamavunons (PGV-1 and PGV-0) (Figure 1) performed antiinflammatory acitivity better then curcumin (Sardjiman, 2000). Moreover, Meiyanto, et al. 2006, reported that PGV-0 possesses cytotoxic activity to T47D cells with the IC_{50} of 10 μM , whereas the IC_{50} value of PGV-1 in T47D breast cancer cells is 3.16 μM, which is more toxic than curcumin (IC_{50}=19.05 μM). In addition, modification on curcumin compound possibly improves inhibitory effect of some protein kinases, such as Pgp, EGFR and HER2 thus will be more potential as chemopreventive agents (Nasiri, 2013). Therefore, further research needs to be explored in specific target especially in the application as co-chemotherapeutic agents with Dox for breast cancer.

Materials and Methods

Compounds

Curcumin, PGV-0, and PGV-1 were obtained from Curcumin Research Center (CRC) Faculty of Pharmacy, Universitas Gadjah Mada. Each sample was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma). Both 5 mg/ml Dox (Ebewe) and sample solution were then diluted in cell culture medium before being applied. DMSO was used as the co-solvent in dissolving samples in culture medium.

MCF-7 and MCF-7/Dox cells

MCF-7 cells and MCF-7/Dox cells used are the collection of Cancer Chemoprevention Research Center (CCRC), Universitas Gadjah Mada, transferred from Nara Institute of Science and Technology (NAIST), Japan.

MCF-7 cells were cultured in DMEM (Gibco) Culture Media containing 10%v/v Fetal Bovine Serum (FBS) (Gibco) and 1% 10,000 unit/ml Penicillin-10,000 mg/ml Streptomycin (Gibco), while MCF-7/Dox cells were cultured in hi-glucose DMEM (Gibco) Culture Media containing 10%v/v FBS and 1% Penicillin-Streptomycin. Trypsin-EDTA 0.25% (Gibco) was used in cell harvest. Resistant cells were originated by the method developed by Putri et al. (2012).

Cytotoxicity and combinational assay

MTT cytotoxicity assay was used to examine the effect of treatment samples alone and in combination with Dox on MCF-7 and MCF-7/Dox cells. Cells were distributed to 96-well plate with the density of 5x 103 cells/well and incubated in 37˚C with 5% CO_2 for 24 h. Certain concentrations of either single or combination of samples were then applied. After 24h incubation, 3-[4,5-dimethyl thiazole-2-yl (-2,5- diphenyltetrazoliumbromide)] (MTT) dissolved in PBS as MTT reagent was applied, followed by 4 h incubation. Stopper reagent, a solution of 10%v/v Sodium Dodecyl Sulphate (SDS) (Merck) dissolved in 0.1N HCl (Merck), was then applied. Plate was then kept with protection from light overnight, continued with absorbance determination (λ 595 nm) using ELISA reader (Bio-Rad). During the process, Phosphate Buffer Saline (PBS) pH 7.4 containing KCl (HPLC grade, Sigma), NaCl (HPLC grade, Sigma), Na_2HPO_4 (HPLC grade), and KH_2PO_4 (HPLC grade, Sigma) dissolved in aquadest was used as washing reagent.

Flow cytometric analysis

For cell cycle analysis using flowcytometry, propidium iodide solution (50 μg/ml in PBS containing 1% triton X-100) and RNAse DNAse-free (20 μg/ml) reagents were used. Cells were distributed to 6-well plate with the density of 5×10^3 cells/well. After 24 h incubation, cells were treated with Curcumin, PGV-0, and PGV-1 each solely and in combination with Dox. Following 24 h treatment, cells were trypsinized and centrifuged at 2000 rpm for 3 minutes. Cell pellets collected were then washed twice with cold PBS. Cells were resuspended in propidium iodide solution (50 μg/ml in PBS containing 1% triton X-100) and treated with RNAse DNAse-free (20 μg/ml) for 10 minutes at 37°C. Treated cells then subjected to FACS flow cytometer and data were analyzed by using ModFit LT 3.0 program.

Immunofluorescence

Cells were grown on 6 cm dish up to 80% confluent. Cell were treated with compounds, single and combination with chemotherapeutic agents and incubated for 24 hours. After 24 hours, cell were fixed by 70% ethanol and incubated for 15 min at room temperature. After rinsed with PBS, cells were incubated with blocking serum 1% BSA for 30 min at room temperature. Then, cells were incubated with primary antibody (p65) for 1 hour at room temperature. After rinsed with PBS, cells were incubated with secondary antibody conjugated by FITC for 1 hour at room temperature in the dark. Then, cell were added by DAPI solution and incubated for 10 min at room temperature in the dark. After rinsed with PBS, cell were added with mounting solution (Fluoromount), put on slide glass, and store at 4°C. The protein expressions were observed under fluorescence microscope.

Molecular docking

In silico study by molecular docking was conducted to examine the affinity of certain ligand to its docking site by evaluating drug-receptor binding energy. Evaluation of the interaction between a molecule and its docking protein involved in particular signal transduction may represent its potential biological activity and allow us to determine the possible mechanism of action. In this study, the docking of curcumin, PGV-0, and PGV-1 respectively to HER2 ATP Binding Site was observed, using lapatinib as a comparation. Ligands preparation were done by using Marvin Sketch. The structure of HER2 protein (3PPO) complex was taken from Protein Data Bank (PDB) (www.rcsb.org). Protein preparation was done by using YASARA. Molecular docking was conducted by using PLANTS (Protein-Ligand Ant System) Software, giving

ΔG as docking score result.

Analysis

Single and Combinational Cytotoxicity assay: linear regression between concentration and % cell viability giving the equation y=Bx+A were used to calculate IC_{50} value, that is the concentration inhibiting 50% cell proliferation.

Statistical analysis of combinational assay was evaluated by comparing cell viability yielded by each treatment by using one way ANOVA SPSS 16.0.

Cell Cycle: Cell cycle distribution was acquired by using ModFit LT 3.0 program.

Molecular Docking: Analysis was done on RMSD for method validity and ΔG value to evaluate sample molecules' affinity to HER2 ATP binding site. RMSD value higher than 2.0 Å is accepted, showing that the method established is valid. Lower ΔG value shows higher affinity.

Results

Cytotoxic effect of curcumin, PGV-0 and PGV-1 on MCF-7 Cells

The aims of this research are to determine the potential role of curcumin and its analogues as chemopreventive agents and underlying mechanism on breast cancer cells, especially when administered in combination with Dox. Firstly, we examined the cytotoxic effect of each compound to MCF-7 and MCF-7/dox cells using MTT assay. The efficacies of curcumin, PGV-0 and PGV-1 were presented by IC50. The result showed that, curcumin, PGV-0 and PGV-1 inhibited cell growth of MCF-7 cells in a dose dependent manner (Figure 2) with the IC_{50} value of 109 μM, 60 μM and 6 μM, respectively. Among the three compounds, PGV-1 seems to be the most potent in cytotoxic activity to MCF-7 cells. Interestingly, this result showed that curcumin performed better efficacy in MCF-7 cells compared to PGV-0. This result looks unusual if compared to the cytotoxic effect on T47D cells and HeLa cells that PGV-0 performed better efficacy than curcumin did. However the cytotoxic effect of three compounds on MCF-7/Dox, seen different pattern (Table 1). In this case, PGV-1 performed highest IC-50 value compare to the others, PGV-1 and Cur. This phenomenon may be due to the instrinsik mechanism of the cells to the compounds.

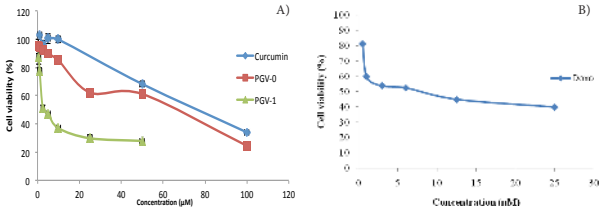

Figure 2. Cytotoxic Effect of Compounds on MCF-7 Cells. Cells were incubated with curcumin, PGV-0, PGV-1 **(A)** and dox **(B)** compounds, for 24 hours, then subjected to MTT assay as described in the method. IC_{50} values were calculated from the triplicate experiments under SE<0.05

Curcumin, PGV-0 and PGV-1 Increased Doxorubicin's Cytotoxicity on MCF-7 Cells

Curcumin, PGV-0, and PGV-1 showed cytotoxic potential to MCF-7 and MCF-7/Dox cells but not strong enough. However, inhibitory effect of those compounds to the cells, give promising to improve the cytotoxic effect of chemotherapeutic agent, e.g. Dox. Therefore, in this experiment we applied curcumin and its analogues to treat MCF-7/Dox cells in combination with Dox. The combination of curcumin, PGV-0 and PGV-1 showed synergistic effect on MCF-7 cells (Figure 3). The synergistic effect belongs to almost all of the concentration of curcumin, PGV-0 and PGV-1 and Dox combination (CI<0.9).

Table 1. IC_{50} Values of the Tested Compounds

Coumponds	IC_{50} (μM)	
	MCF-7 cells	MCF-7/Dox cells
Curcumin	109±1.915	80±2.39
PGV-0	60±2.04	21±0.008
PGV-1	6±2.02	82±3.09
Dox	0.4	7

Table 2. Cell Population in Cell Cycle Phases of Treated MCF-7 Cells

Compound	Sub G1 (%)	G1 (%)	S (%)	G2/M (%)
Untreated	4	50.22	17.43	19.37
Dox	8.21	26.08	9	52.03
Curcumin	14.15	45.14	13.44	15.63
PGV-0	12.62	18.34	5.04	53.14
PGV-1	8.72	65.25	8.96	12.01
Dox-Cur	25.01	21.87	6.59	37.52
Dox-PGV-0	3.77	60.11	11.2	16.8
Dox-PGV-1	12.62	18.34	5.04	53.14

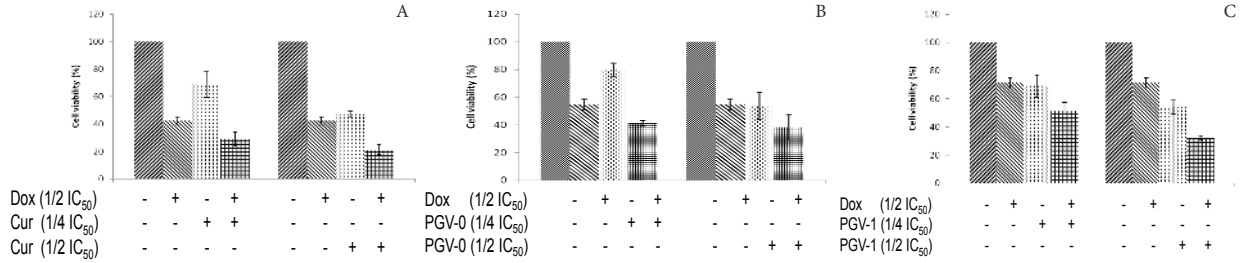

Figure 3. Effect of Combination Treatment of Curcumin and its Analogues with Dox on the Cell's Viability of MCF-7 Cells. Five thousand cells per well were incubated for 24 h and exposed with various concentration of curcumin, PGV-0, PGV-1 and Dox solely and in combination and subjected for MTT assay. (a) Combinational treatment of curcumin **(A)**, PGV-0 **(B)**, PGV-1 **(C)** and Dox yielded less cell viability compared to single treatment (p 0.05). Cytotoxicity was represented as percentage of MCF-7 cells' viability as the mean±SE of three values

Flowcytometry

To confirm the mechanism underlying the cytotoxic effect of combinatorial treatment on MCF-7 cells we performed cell cycle analysis by flowcytometry. At the sub-concentration treatment (1/10 IC_{50}) of curcumin and its analogues, the cell cycle profiles of the cells practically were not affected. On the other hand, Dox at the concentration of 1/3 IC_{50} induced cell accumulation at G2/M-phase and slightly increase sub-G-1 population. However, after combinational treatment at the same concentration the accumulation of the cells become shifted with different profile. Combination with curcumin increased G-1 and sub-G-1 population, PGV-0 increased Sub-G1 population and PGV-1 increased G2/M and Sub-G1 population (Figure 4 and Table 2). These data showed that all compounds enhanced Dox cytotoxicity to MCF-7 may be by inducing cell death. PGV-1 showed most effective as combination treatment with Dox followed by PGV-0 and curcumin.

Effect of curcumin and its analogues on NF-kB activation

Among the 3 compounds, performed different effect on NF-kB activation in MCF-7 cells. Dox significantly increase p65 expression in the nucleus of the cells. In

Table 3. Docking Score between Compound and Protein Targets

Compound	Protein							
	Pgp 1MV5	EGFR 1XKK	HER2 3PP0	ER α 3ERT	ER β 1QKM	GST 3CSH	IKK/NEMO 3BRV	COX-2 6COX
RMSD	1.247 A	1.95 Å	1.19 Å	1.42 Å	0.39 Å	1.61 Å	-	1.21 Å
Native Ligand [NL]	ATP	FMM	03Q	OHT	GEN	GSH	Withafarin	S58
Native Ligand [NL]	-97.55	-144.2	-121.1	-97.57	-95.051	-81.96	-63.94	-71.403
Curcumin	-83.8	-94.54	-102.2	-93.56	-68.72	-92.92	-66.06	-94.36
PGV-0	-74.46	-87.32	-84.31	-78.72	-19.81	-77.84	-59.23	-68.32
PGV-1	-67.93	-81.87	-82.8	-82.47	11.31	-78.2	-55.5	-76.54
ATP	-97.55	-100.8	-111.9	-93.23	-78.22	-98.78	-84.21	-82.65

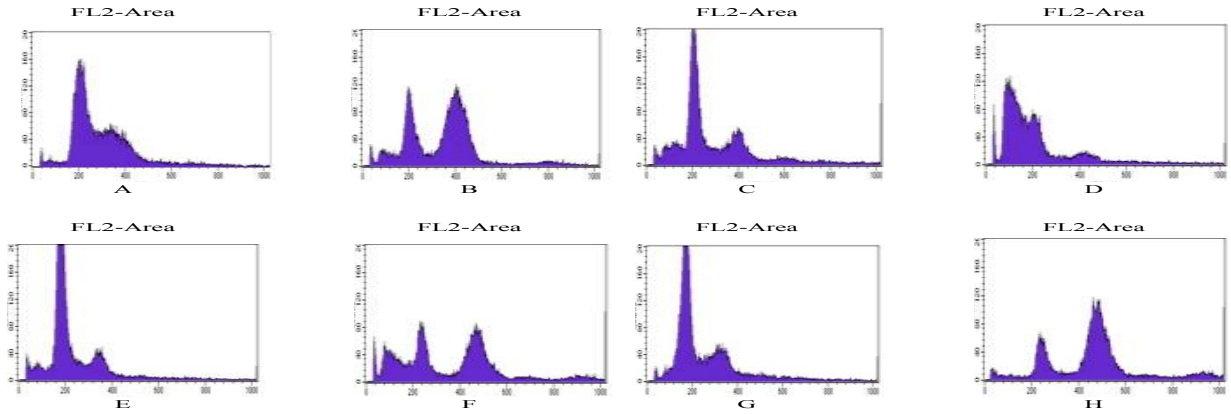

Figure 4. Cell Cycle Distribution of Treated MCF-7 Cells. A) untreated cells; **B)** Dox; **C)** Cur; **D)** Dox-Cur; **E)** PGV-0 **F)** Dox-PGV0; **G)** PGV-1; **H)** Dox-PGV-1. The cell cycle distribution assay was performed by flowcytometry

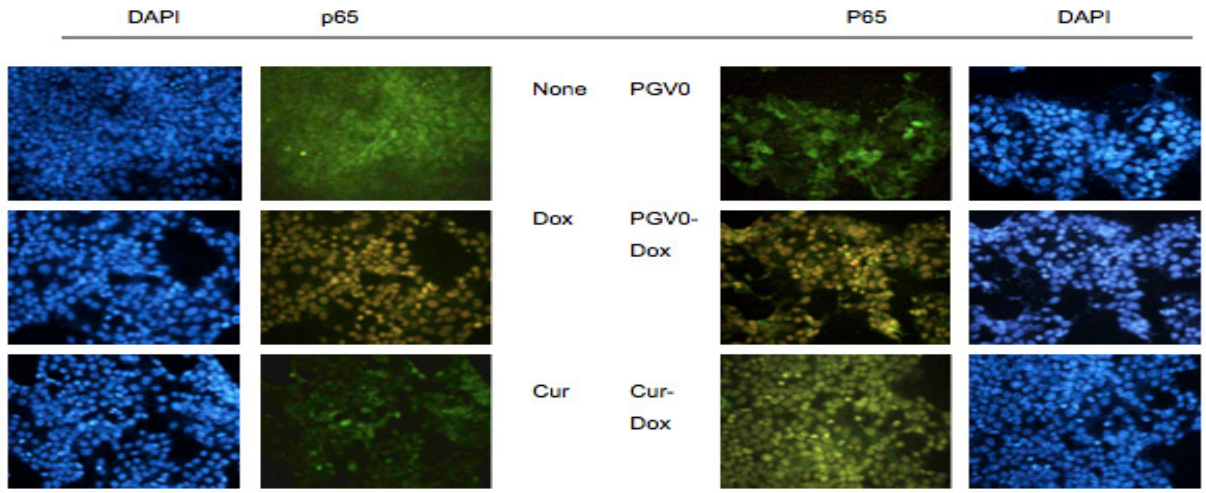

Figure 5. Effect of Curcumin and PGV-0 on NF-kB Activation on MFC-7 Cells. Cells were gorwn on coverslips and treated with compounds as indicated with the concentration of ½ IC_{50}, then subjected to immunofluorescent staining with p65 (activated NF-kB) and DAPI as described in materials and methods. Visualizations were done under microscope fluorescent with 400× magnification

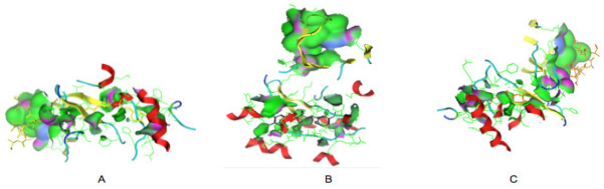

Figure 6. Interaction Model between Curcumin (A), PGV-0 (B), and PGV-1 (C) to the ATP Binding Site of EGFR

contras, curcumin and PGV-0 in single treatment did not affect the expression of p65 in the nucleus. This result showed that Dox enhance the activation of NF-kB, but curcumin and PGV-0 did not induce the activation of NF-kB. This result also showed that PGV-0 in combination with Dox did not affect the expression of p65 in the nucleus. Interestingly, curcumin attenuated the p65 expression in the nucleus of Dox-treated cells (Figure 5). In other experiment, we found that PGV-1 also decrease the expression of p65 in the nucleus (data not shown). These results indicated that curcumin and PGV-1 may inhibit the activation of NF-kB due to Dox induction.

Molecular docking

To elucidate the molecular mechanism underlying cell cycle arrest and inhibition of NF-kB activation, we then examine whether or not, curcumin and its analogues interact with some protein involved in the NF-kB signaling, e.g. HER2 and IKK-B as the upstream and Pgp as the downstream by molecular docking. Dockings were performed at the active site of the protein, such as ATP binding site and we use native ligands, including ATP for the comparation. The result showed that all of the three compounds could interact with the protein targets. Curcumin performed the best affinity to all the protein targets, especially to the HER2 protein (Table 3.). This result informed that those of three compounds may inhibit the protein by disturbing the proteins at the active sites (Figure 6).

Discussion

Curcumin has been well known to have cytotoxic activity to some cancer cells. However, due to the low efficacy, curcumin remains under exploration to obtain the suitable application, e.g. enhancing the bioavailability as well as structur modification (Aggarwal, et al., 2003; Meiyanto, et al., 2007). Exploring the potential role of curcumin as co-chemoterapeutic agent also gives good promising for cancer therapeutics application (Da'i et al., 2007). In this purpose, curcumin or its analogues do not need high efficacy to cancer cells, but those substances should posses some specific targets in the cancer cells especially to the resistance cancer cells.

In this research, we found that, based on IC50 values, PGV-1 performed highest cytotoxic activity compare to curcumin and PGV-0. Interestingly, to this resistance breast cancer cells (MCF-7/Dox), curcumin showed better efficacy compare to PGV-0. These results were not consistence with our previous results which is cytotoxicity of PGV-0 was better than curcumin (Meiyanto et al., 2007). This phenomenon could be caused by different internal mechanism in the cells due to Dox treatment that should be explored further.

The other phenomenon found in this research were that curcumin always performed the best affinity to some protein targets involved in the proliferative signal, such as HER2, EGRF, IKK, ER, but PGV-1 still performed the best citotoxic activity. This phenomenon may be caused by additional targets mechanism of PGV-1 to the cells. As mentioned in our previous report, we found that PGV-1 most effective to inhibit cell cycle at the G2/M phase leading to cell apoptosis (Meiyanto, et al., 2007). In this research we also found that PGV-1 in a low concentration as combinatorial treatment with Dox induced G2/M accumulation of the cells. In other experiment we also found that curcumin but not PGV-0 suppressed p65 expression suggesting that curcumin inhibit effectively NF-kB activation. This inhibitory effect seemed likely due to inhibitory effects to HER2 and EGFR as well as IKK as shown by docking experiment. Whereas, PGV-0, may be inhibit cell proliferation and enhance citotoxic activity of Dox through inhibiting HER2 and Pgp leading to induce apoptosis.

Taken together, this research suggests that Curcumin, PGV-0, and PGV-1 performe co-chemoterapeutic potential in combination with Dox to breast cancer cells. PGV-1 performes the best efficacy compare to curcumin and PGV-0. In a very low dose (sub toxic dose) PGV-1 still able to enhance cytotoxic effect of Dox, suggesting that PGV-1 could reduce the Dox dose effectively. All of the potential effects of three compounds as co-chemoterapeutic agents are likely mediated by inhibiting of HER2 and NF-kB activation in cancer cells. Since HER2 and NF-kB activation are usually highly marked in cancer cells, thus curcumin, PGV-0 and PGV-1 are potential to be developed further as specific targeted of co-chemotherapeutic agents for HER2 expressing breast cancer.

Acknowledgements

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