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 \( \mu \)M, 21 \( \mu \)M, and 82 \( \mu \)M 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-\( \kappa \)B activation (Shishodia et al., 2003). Therefore, decreasing the expression and inhibiting of HER2, EGFR, ER, GST, COX-2, NF-\( \kappa \)B 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 \textit{Curcuma longa} L. Curcumin has property of choleretic

![Figure 1. The Structure of Curcumin and its Analogues.](image)

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-dimethylphnyle) methylidene]cyclopentan-1-one)
and chologagic (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 several research groups. Sardjiman, 2000 reported that two curcumin analogues, namely Pentagamavunons (PGV-1 and PGV-0) (Figure 1) performed antinflammatory activity better then curcumin (Sardjiman, 2000). Moreover, Meiyanoto, 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 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 (Gibico), while MCF-7/Dox cells were cultured in hi-glucose DMEM (Gibico) 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 10^3 cells/well and incubated in 37°C with 5% CO2 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), NaHPO4 (HPLC grade), and KH2PO4 (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 5x10^5 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, cells 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, cells 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 laptatinib 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, which 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 from the triplicate experiments under SE<0.05 assay as described in the method. IC and dox

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 compared to the others, PGV-1 and Cur. This phenomenon may be due to the intrinsic mechanism of the cells to the compounds.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
<th>MCF-7 cells</th>
<th>MCF-7/Dox cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>109±1.915</td>
<td>80±2.39</td>
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<tr>
<td>PGV-0</td>
<td>60±2.04</td>
<td>21±0.008</td>
<td></td>
</tr>
<tr>
<td>PGV-1</td>
<td>6±2.02</td>
<td>82±3.09</td>
<td></td>
</tr>
<tr>
<td>Dox</td>
<td>0.4</td>
<td>7</td>
<td></td>
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</tbody>
</table>

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

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

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

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.
Table 3. Docking Score between Compound and Protein Targets

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pgp</th>
<th>EGFR</th>
<th>HER2</th>
<th>ER α</th>
<th>ER β</th>
<th>GST</th>
<th>IKK/NEMO</th>
<th>COX-2</th>
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<tbody>
<tr>
<td>1MV5</td>
<td>RMSD</td>
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<td>A</td>
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<td>1.19 Å</td>
<td>1.42 Å</td>
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<td>1.61 Å</td>
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<td>3ERT</td>
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<td>3CSH</td>
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<tr>
<td>3BRV</td>
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<td></td>
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<tr>
<td>6COX</td>
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Effect of curcumin and its analogues on NF-κB activation

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

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, EGFR, 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 in 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 performs 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-chemoterapeutic agents for HER2 expressing breast cancer.

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