PGV-O INDUCES APOPTOSIS ON T47D BREAST CANCER CELL LINE THROUGH CASPASE-3 ACTIVATION *)[1]

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Abastrak

Pentagamavunon-0 (2,5-bis-(4'-hidroksi-3'-metoksi)-benzilidin-siklopentanon), dikenal sebagai PGV-0, merupakan analog kurkumin (1,7-bis-(4'-hidroksi-3' metoksifenil)-1,6-heptadiena-3,5-dion) yang telah banyak diteliti aktifitas antikankernya. Kurkumikn telah diteliti memiliki aktifitas menginduksi terjadinya apoptosis pada beberapa sel kanker payudara. Penelitian ini dilakukan untuk membuktikan kemampuan menginduksi apoptosis senyawa PGV-0 terhadap sel kanker payudara T47D dengan analisis morfologis menggunakan *double staining*, analisis DNA fragmentation assay, immunositokimika dan *western blot*. Hasil uji sitotoksisitas PGV-0 terhadap sel kanker payudara diperoleh nilai IC50 10,91 μM. PGV-0 terbukti pula mampu memacu terjadinya apoptosis pada pengamatan DNA fragmentation assay dan analisis double staining. Pengamatan Immunositokimia. PGV-0 terbukti mampu menurunkan level ekspresi Bcl-2 dan meningkatkan level ekspresi BAX protein. Penelitian ini menyimpulkan bahwa PGV-0 mampu memacu terjadinya apoptosis sel T47D melalui aktivasi caspase 3.

Kata kunci : PGV-0, apoptosis, caspase-3

Abstract

Pentagamavunon-0 or PGV-O (2,5-bis-(4'-hidroxy-3'-methoxy)-benzilidine-cyclopentanone) is recognized as curcumin (1,7-bis-(4'-hidroxy-3'-metoksyphenyl)-1,6-heptadiena-3,5-dion) analogue that has been studied as anti-cancer. On breast cancer cell lines, curcumin shows apoptotic activity. The aim of this research is to study the potency of PGV-O to induce apoptosis on breast cancer cell line, T47D. Double staining methods with etidium bromide and acridine orange, DNA fragmentation assay, immunocytochemistry and western blot analyses were carried out to know the molecular mechanism of PGV-0 activity in apoptotic effect on T47D. The IC50 value of PGV-0 was 10,91 μM. PGV-0 induced the DNA laddering in DNA fragmentation assay and apoptotic activity was demonstrated by morphological analysis by using double staining methods. In accordance of the apoptotic effect , PGV-O increases the expression of BAX, decreases the expression BCl-2 and activate the caspase-3.

Keywords : PGV-0, apoptosis, caspase-3

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Introduction

In previous study, Pentagamavunon-0 atau PGV-0 (2,5-bis-(4'-hydroxy-3'-metoxy)-benzilidin-cyclopentanone), a curcumin analogue, shows antiproliferative activity through modulation of S-phase progression (Meiyanto, 2006). The alternative mechanism of antiproliferative effect of PGV-0 on T47D that may occur is inducing apoptosis. Curcumin has been reported to induce apoptosis in some breast cancer cell lines. Apoptotic induction by curcumin on MDA-MB 468 breast cancer cell line is associated with Akt/PKB (protein kinase B) activity (Squires *et al.,* 2003). Inhibition of PKB could prevent inactivation of Bad, a pro-apoptotic protein. Bad induces apoptotic process through mitochondrial pathway by releasing cytochrome C leading to caspase 9 activation, the main executor protein of apoptosis (Hanahan and Wienberg, 2000). Apoptotic induction by curcumin in breast cancer cell line also involves the p53 pathway with the downstream effector of BAX protein (Choudhuri et al., 2002). Curcumin also induces apoptosis by Bcl-2 inhibition in Leukemia cell line (Kuo *et al*., 1996; Anto, 2002). Curcumin induces apoptosis by the products of two other p53-sensitive genes. Inhibition of Bcl-2 and stimulation of Bax expression can induce the apoptotic process (Chen and Huang, 1998; Joe *et al*., 1998). Those results conclude that curcumin can inhibit cell growth by inducing apoptosis process.

The cytotoxic activity of PGV-O in myeloma cell line is better than curcumin as lead compound (Nurrochmad, 2001). Antiproliferative effect of PGV-0 on Raji, HeLa and Myeloma cells also conclude that PGV-0 can inhibit the cell proliferation by inducing cell cycle arrest and apoptosis (Da'i, 1998). Based on the studies above, PGV-0 as curcumin analogue should have the some anticancer properties as well. This research was done to elucidate the molecular mechanism of PGV-0 to induce apoptosis on breast cancer cell line T47D.

Materials and Methods

Materials

PGV-0 and curcumin were synthesized and elucidated by National Molecule Team of the Faculty of Pharmacy Gadjah Mada University(2001). T47D cell line was obtained from Prof. Tatsuo Takeya collection (Nara Institute of Science and Technology, Japan). RPMI 1640 medium (GIBCO BRL) that contains 10% FBS (*Fetal Bovine Serum*)(Sigma Chem. CO. St. Louis. USA), etidium bromide, RNA-se, DMSO, natrium carbonate (E.Merck), Fungizon, penicilin dan streptromicyn antibiotics (Sigma Chem. CO. St. Louis. USA), hepes dan tripsyn (Sigma Chem. CO. St. Louis. USA) and monoclonal antibodies of BAX, Bcl-2, P53 (Santa cruz) were used in this research.

Methods

Morphological changes on apoptosis

Cells were cultured on cover slips at 1,5 X 10^4 cells/well onto 24 wells plate until 50 % confluent. The medium was replaced with fresh medium containing sample with concentration as indicated above. Cells then were incubated for 48 h in humidified atmosphere at 37oC in CO2 5%. The medium was removed and added by *Working Solution* ethidium bromide/acridin oranye (1X) for 5 min. *Cover slip* containing cells was removed and covered on the object glass, then assessment can be carried out under fluorescence microscope. Viable and dead cells will give green and red fluorescences respectively.

DNA fragmentation assay

Cells 2 X 10^5 were cultured with medium containing curcumin or PGV-0 at concentration as indicated above. Cells were incubated for 24 h in CO2 5% incubator at 37oC. Cells were lysed in (50 mM tris HCl pH 8, 100 mM EDTA, 100 mM NaCl) for 700 μl and added proteinase K 1 μl (20 mg/ml) and incubated in room temperature for 24 jam. The lysate was added with phenol 700 μl and shaked slowly for 2 h. Upper solution wash

relocated to the other tube and added with isoprophanol 500 μl with Na Asetat, than added with absolute alcohol and shaked slowly, and frozen at -20^{o}C for 1 h. The solution was centrifuged at 12.000 rpm for 10 min. The DNA were washed with 70% ethanol and diluted with TE 1X.

Immunocytochemistry

Cells were plated at 1,5 X 10^{4} cells/well and cultured in 24-wells plate until 80 % confluent. At time 0, medium was replaced by fresh complete medium with PGV-0 10 μM and curcumin 20 μM and incubated in CO-2 5% incubator at 37oC. Then, cells were harvested and were washed and adhered for 30 min to poly-L-lysine-coated slides. Fixed with acetone for 30 min. Cells washed, and blocked in 10% normal mouse serum (1:50)Triton X/PBS for 1 h at room temperature. Cells were stained for 1 h at room temperature with primary Ab (p53, BAX and Bcl-2 in 2% normal goat serum/0.4% Triton X/PBS. After washing three times in PBS/ 0,2% tween, secondary antibody were applied for 15-30 min, 1 : 2 in PBS and added 5% AB serum then washed with PBS three times. The slide was incubated with streptavidin-biotin-complex for 15 min, 1 : 2 in PBS and added 5% AB serum and washed three times in PBS. Slides were incubated in DAB (3,3 diaminobenzidin) solution for 3-8 min and washed with aquadest. Cells were counterstained for 3-4 min with H&E Protein expression was assessed under light microscope. Positive expression will give a dark brown colour in nucleus and cells with no expreesion will give purple.

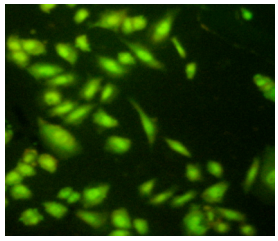
Western blot

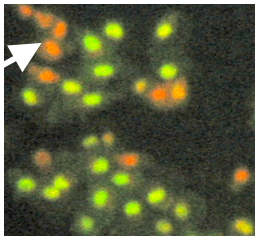
Cell were washed twice with cold Phosphate-buffered saline (PBS) and lysed in phosphate/ SDS sample buffer (20mM sodium phosphate, pH 7.2, 2% SDS, 0.001% bromphenol blue, 0.3 M dithithreitol and 2% glycerol) containing 3% 2-mercaptoethanol. 10 μg the cell lysate were then separated on SDS PAGE 7,5% SDS-PAGE and electrophoretically transferred to an imobilon-P membrane (Milipore). Following this step, the membrane was proceeded as follow ; blocking with 5% bovine serum albumine in PBS for 1 h, incubating with Caspase-3 primary antibody for 2 h, blocking with 5% skim milk in PBS for 30 minutes, incubating with a horseradish peroxidase-labeled

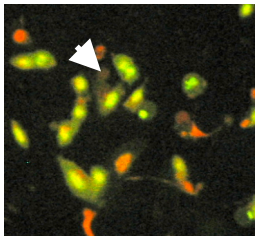
secondary antibody (1 :3000 to 1 :5000 dilution in 5% skim milk containing PBS) for 45 nin, and washed by PBS-T for 30 min. All steps were done at room temperature. After reacting with an Enhanced Chemiluminescence (ECL) reagent (Pharmacia-Amersham-Biotech), the blotting membrane then visualized to X-ray Fuji Film (Fuji RX).

Results

Citotoxicity test concluded that PGV-0 and curcumin inhibited the T47D cells with IC50 values were 11 μM and 22 μM respectively (Meiyanto, 2006). From the result above it is clear that PGV-O could inhibit cellular proliferation in T47D cells through apoptotic induction.



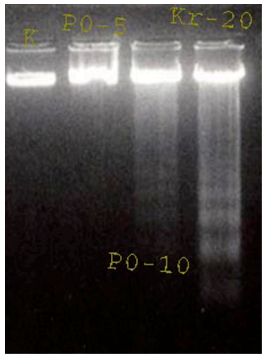




1 2 3

(A)

1 2 3 4



(B)

1 2

(C)

Fig 1. (A) Cells were doublestained with ethidium bromide and acrydine orange (1) Control cells (2) cells with PGV-0 10 μM and (3) cells with curcumin 20 μM, green fluorescence indicates viables cells and red one indicate dead cells (B) DNA electrohoresis of T47D cells (1) Control cell (2) Cell with 5 μM of PGV-0 (3) Cell with PGV-0 of 10 μM and (4) with curcumin 20 μM (C) Western Blott analysis targeted for Caspase 3 (1) Control cell (2) with PGV-0 10 μM ➔ = apoptotic bodies.

Viable cells and dead cells could be distinguished by intercalation between ethidium bromide and DNA that indicated the apoptotic cells (Fig 1(A)). The apoptotic activity confirmed as well by DNA laddering that indicated activity of DNA-se due to the DNA fragmentation (Fig 1(B)). The result of this study summarized that PGV-0 induced apoptosis process started at 10 μM on T47D breast cancer cell line.

DNA-se activation was demonstrated by the activation of Caspase-3 that cleaved into two part of proteins. Pro- Caspase-3 (inactive Caspase-3) has 32 KD in size, and after cleavage (activation) produced proteins with 17 and 12 KD. PGV-0 at the concentration of 10 μM induced Caspase-3 activation (Fig 1,B and C)).

Caspase 3 can be activated by mitochondrial pathway by releasing the cytochrome C. Cytochrome C is released from mitochondria due to the caspase 9 activation to form a complex with Apaf-1 (apoptosis activating factor). This complex is called as apoptosome to activate Caspase-3 (King, 2000). The release of cytochrome C from mitocondrium is facilitated by Bax which is expressed through p53 induction that play a role as transcription factor. Bax protein can be inhibited by Bcl-2 protein by heterodimeric formation (Hanahan and Weinberg, 2000).

To confirm the mechanism of PGV-0 induced apoptosis, this research observed the effect of PGV-0 or curcumin on the expression of p53, Bax, and BCl-2 by using immunocytochemistry method. Interestingly, the expression of p53 on the PGV-0 treated cells was increased compare to the control cells and the increasing level of p53 expression on the PGV-0 treated cells was higher than on the curcumin treated cells (Fig. 2, lane-1). Moreover, PGV-0 was stronger to decrease the expression of BCl-2 than curcumin did (Fig. 2, lane-3). However, the increasing level of Bax expression on the both treated cells not significantly different, but still higher than the control cells (Fig. 2, lane-2). These data show that PGV-0 more potent to induce apoptosis on the T47D cells than curcumin.

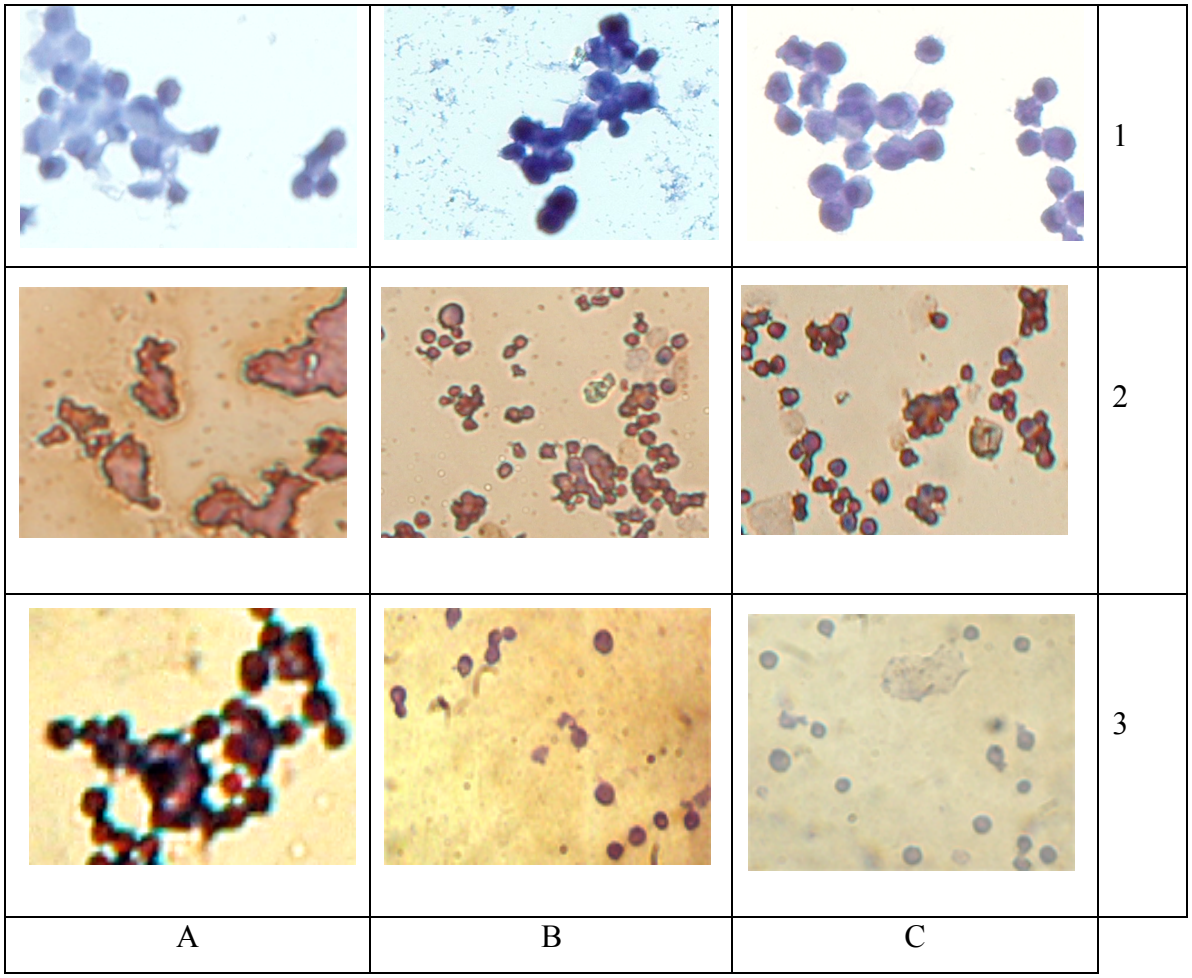


Fig 2. Protein involves in apoptotic process on T47D cells (1) p53, (2) BAX and (3) Bcl-2) examined by immunocytochemistry methods (A) Control cells (B) induced by PGV-0 at 10 μM dan (C) induced by curcumin at 20 μM. This experiment used DAB as the chromogen, dark brown staining in nucleus indicated the positive expression of the protein, cells were counterstained with H&E showed purple color.

Discussion

Curcumin well known to inhibit IκBα phosphorilation leading to inhibition of a transcription factor, NF-kB (Shishodia *et al.* (2003). The inhibition of NF-kB activation causes decreasing of CycD1, COX-2 and MMP-9 expression leading to proliferation inhibition. On the other pathway, the inhibition of NF-kB also causes decreasing of Bcl-2 and Bcl-xL expression, two main antiapoptotic proteins. T47D cells express Bcl-xL at high level (Hahm and Davidson, 1998). Curcumin inhibit Bcl-2 and Bcl-xL expression in myeloma cells (Bharti *et al*., 2003). PGV-0 and curcumin show decreasing of BCl-2 level on T47D, and may also decrease the Bcl-xL level.

PGV-0 and curcumin also induce apoptosis by p53 dependent manner. On MCF-7 (another breast cancer cell line), curcumin induces apoptosis by increasing the p53 expression and followed by increasing the BAX protein level leading to apoptotic process via mitochondrial pathway (Choudhuri *et al*., 2002). The result of the present study shows that PGV-0 and curcumin increased the p53 protein (Fig 2). *P53* mutation at L2 occurs in T47D cells causes lost of function of p53 as cell cycle regulator (Schafer *et al.*, 2000). p53 as a transcription factor is inhibited by MDM2. p53 and MDM2 interaction induces p53 degradation. Mutated p53 protein can not interact with MDM2 causing acumulation of p53 in the cells (Sigal and Rotter, 2000). This mutation should not influence to increase BAX protein expression. However the present result showed that PGV-0 and curcumin could increase the BAX protein level, although it was at a low level. Huang *et al.* (1999) stated that missense mutation at DNA binding region of *p53* did not influence the Bcl-2 level but it should influence the BAX level. The result of this present study showed lowering Bcl 2 level significantly due to PGV-0 and curcumin induction, but it was not significant for the BAX protein(Huang *et al*., 1999). These data suggested that the apoptosis may occur by increasing the other p53's downstream effectors like Noxa, p53AIP1, KILLER/DR5, and Puma (Nakamura, 2003; Villunger *et al.*, 2003). Noxa and Puma are members of Bcl-2 family that contain BH3 domain and could form dimmer with Bcl-2 or Bcl-xL. Overexpression of these protein could induce apoptosis by activating the caspases protein.

Conclusion

PGV-0 induced apoptosis of T47D breast cancer cell line at 10 μM by Caspase-3 activation due to the decreasing of the Bcl-2 protein.

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