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T47D cells arrested at G2M and Hyperploidy Formation Induced by a Curcumin's Analogue PGV-1

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Abstract

Pentagamavunon-1 (PGV-1) is a curcumin analogue, which is more stable in organic solution and more rigid in its chemical structure than curcumin. As a curcumin analogue, PGV-1 was considered to have anticancer activities. This research was conducted to study the effect of PGV-1 on the cycle progression of T47D cells. Cytotoxic effects of PGV-1 on T47D cells were determined using MTT assay, and the the effect on cell cycle progression was carried out using flowcytometry. Western blot analysis was used to analyze protein expression corresponding to cell cycle progression. The result showed that at the concentration of 2.5 μM PGV-1 inhibited cell cycle progression through G2/M arrest and induced of cells hyperploidy formation. The hyperploidy formation induced by PGV-1 was related to the increase of cdc-2 expression. PGV-1 2.5 μM elevated the level of p21 CIP/KIP through p53- independent manner. Apoptosis was also induced by PGV-1 at early phase of treatment indicated by PARP cleavage due to activation of caspase-3/7 after 12 h treatment. The results above suggest that PGV-1 inhibits the growth of T47D cells targeted on microtubules.

Keywords: PGV-1, G2/M arrest, apoptosis, p21

Introduction

Curcumin (Figure 1) is a β-diketone constituent of turmeric obtained from the powdered root of *Curcuma longa*, Linn. Curcumin performed anti-cancer properties through cell growth inhibition and apoptosis induction. Curcumin inhibit cell proliferation by inducing a growth arrest in the G2/M phase on breast cancer cell line, as well as in other stages (Mehta *et al*., 1997; Ramachandran and You, 1999, Simon *et al*., 1990, Choudury *et al*., 1999). Arresting cells at G2/M phase by curcumin is coused by the mitotic spindle checkpoint (Holy *et al*., 2002).

Curcumin is unstable at pH above 6.5 and on the presence of light (Tonnesen and Karlsen, 1995; van der Goot, 1997). The instability is attributed to the active methylene group. Omission of this group results in the more stabile curcumin analogue. A preliminary pharmacophore model divided curcumin into three regions (Robinson, 2003). Region A requires an aromatic ring, region B is composed of a symmetrical diendione linker, and region C also requires an aromatic ring (Figure 1). Examination of curcumin suggested that the two aromatic regions might be critical for potential ligand-receptor binding. A reasonable approach

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was to explore compounds with differences in the carbon chain connecting of the aromatics region. Pentagamavunon-1 (2,5-bis-(4'-hydroxy-3',5'-dimethyl)-benzillidine-siclopentanone, known as PGV-1, Figure 1), which has been developed and patented by Samhoedi *et al.* (2004) from Gadjah Mada University, is one of the candidates to be developed as antiinflamatory and anticancer agent. This experiment was to evaluate the effect of the curcumin's analogue on the cell cycle progression.

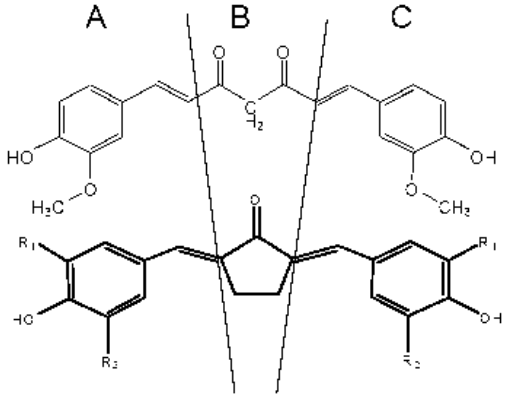


Figure 1. Curcumin and its analogues pentagamavunon (PGV) pharmacophore division. Part A and C, both are aromatic ring, which could be heteroaromatic, symmetrical or not symmetrical. Part B of the structure is b-diketone group, which has –CH2- active methylene group responsible to the curcumin instabilization (PGV-1: R_1=R_3= H) (Robinson, 2003).

Suppresion of the cell proliferation has been related to the biological activity of the protein p53. This protein elicits tumor suppression activity by inducing cell cycle arrest and apoptosis (Somasundaram and Deiry, 2000; Volgelstein *et al*., 2000). Mutations of p53 gene induce the tumorigenesis. T47D cells has been reported for the missense mutation at the residue of 194 of p53. This mutation causes the p53 not functional in T47D cells, diminish or abolish the cell cycle regulation (O'Connor *et al*., 1997). P21 WAF1/CIP1 is the downstream effector of p53 that mediates both G1 and G2/M phase arrest (Niculescu *et al*., 1998). P21 protein levels can occur transcriptionally by p53 independent mechanism (Ding *et al.,* 2001; Sato *et al*., 2002).

Hyperploidy cells can be induced in mammalian cells by inhibitor microtubule organization (Verdoodt et al., 1999). Prolonged exposure to antimicrotubule drugs (AMDs) induces cell to exit mitosis and enter G1 phase without complete cell division known as mitotic slippage (El Hajouji *et al*., 1998). Those cells undergo premature DNA replication and form hyperploidy cells known as endoreduplication (Di Leonardo *et al*., 1997). Biochemical features of cell death induced by AMDs indicates abnormal cdc-2/cyclin B activation and multiple micronuclei known as mitotic catastrophe (Okada and Mak, 2004). Normally, Cyclin B is expressed during late S and G2 and bind to Cdc-2. This complex then requires phosphorylation of T161 on Cdc-2 by Cdc-2 activating kinase (CAK). The complex is remain inactive due to its inactivation by inhibitory phosphorylation of Cdc-2 at Threonine 14 (T14) by Myt1 and Tyrosin 15 by Wee1 (T15) (Poon *et al*., 1997; Weinert, 1997; Mueller *et al.,* 1995; Parker and Piwnica-Worms, 1992). This experiment exhibited that curcumin analogue (PGV-1) is able to act specifically, similar with the AMDs action on the breast cancer cell line (T47D). This compound induced G2/M arrest, hyperploidy, increase p21 expression, activated cdc-2 protein, and may induce mitotic catastrope.

Materials and Methods

Cell line and culture

T47D cells were provided by Prof. Masashi Kawaichi (Nara Institute Science and Technology, Japan). Cells were cultured in DMEM medium supplemented with 5% fetal bovine serum (Gibco), 100U/ml penicil-

lin and 100 µg/ml streptomycin.

Chemicals

PGV-1 and PGV-0 was synthesized and purified at Gadjah Mada University. Curcumin was purchased from E-Merck.

Cytotoxicity test

Cells were harvested by trypsinization and, subsequently, collected in a 15 ml conical tube and centrifuge at 1000 rpm for 5-10 minutes. The supernatant was discarded and the cells were washed once with 1 ml PBS and spinned at 1000 rpm for 5 minutes. The supernatant was again discarded and medium was added up to 1 ml. Cells were seeded onto 96 well plate (15.000 cells in each well), and incubated for 24 hours. The sample was added with the final concentration as indicated. The cells were incubated for 24 hours. The medium was discarded and replaced with the new one, added with MTT (5 mg/ml) and followed by four hours incubation. The formazan was diluted in 10% SDS HCl (0.1%). The result was measured using ELISA reader (570 nm).

Flowcytometry

The harvested cells (7.5 X 10^5) were seeded onto 5 cm dish and incubated for 24 h at 37 ^oC in 5% CO_2. Cells were treated using the sample at final concentration of PGV-1 2.5 mM and incubated for 24 h. Cells were harvested by trypsinization and collected in 15 ml tube, followed by centrifugation at 1500 rpm for 5 minute. The supernatant was discarded and 500 µl of flow reagent was added, followed by incubation for 2 minutes at room temperature. Subsequently, RNa-se solution was added at final concentration 1 mg/ml and Run FACS using Facs Calibur and Cellquest software (Becton and Dickinson).

Western blott analysis

Equal amount of proteins from cell lysates were solubilized in 5X sample buffer and electrophoresed in 10-15% sodium dodecyl sulfate-polyacrilamide gels and transferred to PVDF membrane. The membranes were first incubated with either primer antibody against p21, cdc-2, caspase-3, caspase-7, phosporylated cdc-2 at tyrosine 15 or cleavage PARP (all were purchased from Cell Signaling Technology) and then with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Enhanced chemiluminescence (ECL) system (Amersham) was used to detect the proteins.

Results

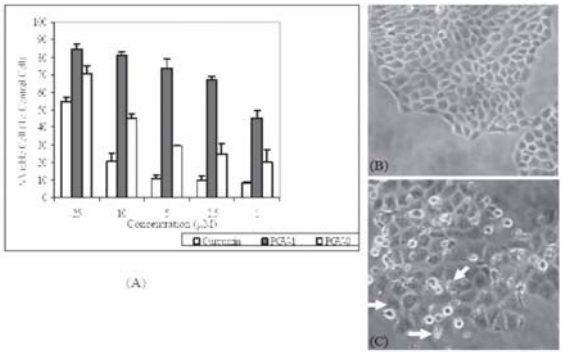
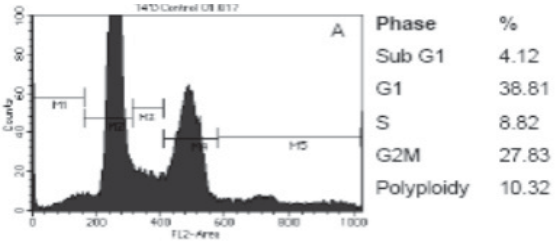


Figure 2. (A) Cytotoxic effect of PGV-1, PGV-0 and curcumin on T47D cells detected by MTT assay. The absorbances of formazan converted to % viable cells were plotted against concentrations. The IC-50s of PGV-1, PGV-0, and curcumin were 1.74, 9,39, and 24,97 mM respectively. (B) Normal cell morphology exhibited by control cells. (C) Multinucleate cell death resulting from the treatment of PGV-1 2.5 µM (arrow, C).

Cytotoxic test showed that PGV-1 has higher potency than the other curcumin analogue (PGV-0) and its parent compound curcumin. These results suggested that PGV-1 is the best candidate of curcumin analogue to be developed as anticancer agent with IC_{50} against T47D cells 1,73 mM. The cells morphology induced by PGV-1 showed significant inhibition, and exhibited multinucleate cell death (Figure 2.)



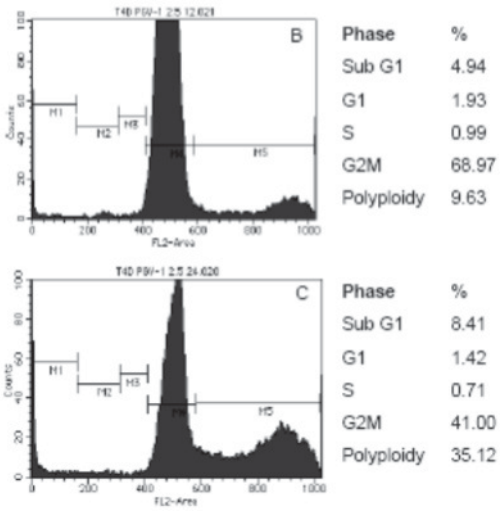


Figure 3. Effect PGV-1 on cell cycle progression. Flowcytometry analysis on T47D cells induced with PGV-1 2,5 μM, cells became arrested rapidly at G2M (4n DNA) after 12 h treatment (B) and cells became hyperploidy after 24 h treatment (C) compare to the control cells (A).

Cell cycle progression T47D cells induced by PGV-1 2,5 μM

As shown in Figure 3 (A,B,C), the majority of cells became rapidly arrested at 4N DNA after treatment for 12 hours (68,97%), and formed polyploidy cells after 24 hours treatment (41,00%). Sub G1 population increased 4,91% at 12 h incubation and 8,41% after 24 hours incubation.

Western blott analysis was run to find out the proteins involved in the apoptosis and cell cycle progression (especially in G2/M phase). Apoptosis was indicated by caspase-3,7 activation started at 12 hours incubation. These results were consistent with the cleavage of PARP as apoptosis marker (Figure 4A). Apoptosis did not involved extrinsic pathway as indicated no caspase-8-clevage. Apoptosis also was p53-independent pathway. There were not protein level change of Bax (Figure 4B) and PUMA (Figure 4A). Both are proapoptotic-protein p53-dependent.

The p21 protein levels increased after 24 hours incubation, together with cdc-2 activation (Figure 4A). Collectively, these results clearly indicated that T47D cells growth were inhibited by PGV-1 and apoptosis occurred at the early phase of treatment. The polyploidy (hyperploidy) formation indicated that PGV-1 inhibited the T47D cells, in which the T47D cells were unable to divide themselves completely. The uncompleted division was due to premature DNA replication which was triggered by abnormal cdc-2 activation and increased p21 expression.

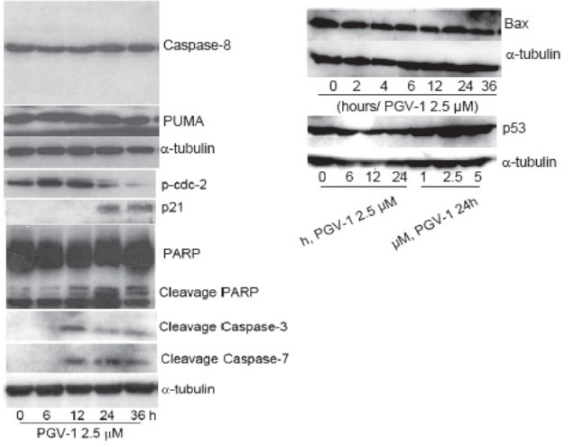


Figure 4. Effect PGV-1 on apoptotic gene expression. Western blot analysis from the cell lysates showing PGV-1 induced the caspase-3/7 activation after 12 h treatment, which was consistent with the PARP cleavage. This compound induced the cdc-2 activation after 24 h treatment and the p21 protein was elevated at the same time. Together with flowcytometry data, these result clearly indicated that PGV-1 inhibited the cells growth and induced the apoptosis at the early time of incubation.

Discussion

PGV-1 inhibited the T47D cells proliferation with similar mechanism to AMDs, i.e. by inducing the hyperploidy (polyploidy) cell formation and cells commit to apoptosis (Wang *et al*., 2000). PGV-1 also activated the cdc-2 kinase protein, one of the biochemical features of antimicrotubule action on the cells (Okada and Mak, 2004).

Generaly apoptosis is related to cytochrome c release from mytocondria and in-

duce Apaf1 and procaspase-9 complex formation leading to caspase-9 activation (Kasibhata and Tseng, 2003; Herr and Debatin, 2001) or without clevage caspase-9 (Stennicke *et al*., 1999). PGV-1 induced apoptosis on T47D cells seems to be regardless on the caspase-8 pathway (Figure 4). This result indicated that PGV-1 induce apoptosis via intrinsic pathway. Apoptosis induced by PGV-1 also seems via p53-independent pathway (Figure 4).

Previous experiment resulted AMDs treatment induced Bcl-2 hiperphosphorylation. The phosphorylated form causes Bcl-2 can not form heterodimer with BAX and causes release cytcocrome c from mithocondria and induce apoptosis by caspases activation through p53-independent (Blagosklonny *et al;*., 1997; Haldar *et al*., 1996; Srivastava *et al*., 1998). Caspase-3 activation responsible for the Poly (ADP-ribose) polymerase (PARP) clavege as the apoptosis marker to be 89 and 24 kda protein (Kaufman *et al*., 1993; Nicholson *et al*., 1995; Cryns and Yuan, 1998).

PGV-1 activated caspase-3/7 started at 12 h after incubation followed by PARP cleavage (Figure 4). This result was concomitant with apoptosis evidence of MCF-7 cells induced by AMD's as shown the Bcl-2 hiperphosphorylation at 3h after incubation (Wang *et al*., 1999). Similiar result also resulted by Srivastava *et al*., (1998), their experiment on cells treatment with AMDs resulted in AMDs hyperphosphorylation of Bcl-2 after 6h incubation followed caspase-3 activation after 12h incubation. Those experiments indicate Bcl-2 phosphorylation initiate the apoptosis.

It is noteworthy that the roles of Bcl-2 phosphorylation in apoptosis still remain controversial. The other experiment resulted in contrast, Bcl-2 phosphorylation inhibit the apoptosis process (Horiuchi *et al*., 1997; Ito *et al*., 1997). Bcl-2 phosphorylation in the regulation AMDs-initiated apoptosis apparently remains to be clarified.

Generaly Bcl-2 phosphorylation involve kinase proteins like: a) AMDs treatment induces c-raf-1 activation and simultanously followed by Bcl-2 phosphorylation (Blagosklonny *et al*., 1996; 1997), b) antimicrotubule agent induces PKA activation and phosphorylates Bcl-2 lead to apoptosis. PKA activation also related to microtubule disruption by AMDs (Srivastava *et al*., 1998), c) The other pathway involved MAP kinase. AMDs induced apoptosis related with caspase-3 activation by JNK/SAPK dependent or independent Bcl-2 phosphorylation. JNK/SAPK activation via Ras pathway and apoptosis signal regulating kinase (ASK-1). This activation started at early phase AMD's-induced apoptosis (up to 16 h treatment) (Wang *et al*., 1999; Chen *et al*., 1998; Seimiya *et al*., 1997). This activation also related with microtubule disruption by AMDs.

Wee1 and Myt1 are kinase proteins that inactivate the cdc-2 kinase protein (Molinari, 2000). Wee1 and Myt1 are caspase-3 substrate (Chang and Yang, 2000). Caspase-3 activation inactivate both protein. Activation of cdc-2 kinase protein induce the cells entry mitotic phase and caused abberant mitotic. Previous experiments with AMDs resulted in mitotic exit by AMDs without chromosom segregation and divided cells completly (Di Leonardo *et al*., 1997; Elhajouji *et al*., 1998). This evidence causes cells to have 4N DNA content and still underogoing mitosis but not cytokinesis (endoreduplication) and form hyperploidy (Di Leonardo *et al*., 1997) and may cause mitotic catastrope incidence.

Cdc-2 activation may be trigered by the other kinase as the AMDs treatment consequency. Microtubule disruption also induce mitotic checkpoint activation, which was indicated by the elevation of p21 protein level through p53 independent, due to p53 mutation in the T47D cells. This eleva-

tion caused the cells blocked at G2/M phase and become hyperploidy. This hyperploidy cells may be the G1 like arrested cells or mitotic catastrope that possibly become the pathway to apoptosis of the cells.

As the conclusion of this research, PGV-1 inhibit T47D cells proliferation by inducing apoptosis and cell cycle arrest at G2/M phase and hyperploidy cells through p21 expression. PGV-1 action may be mimic AMDs inhibit cell proliferation.

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