

## Apoptosis Mediated Cytotoxicity of Curcumin Analogues PGV-0 and PGV-1 in WiDr Cell Line

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### Abstract

Previous study has reported that colon cancer can be blocked by anti-inflammatory drugs through the down regulation of cyclooxygenase 2 (COX 2) expression by which they capable of inducing apoptosis. Two analogues of curcumin, PGV-0 (2,5-bis-(4'-hydroxy-3'-methoxy)-benzilidene-cyclopentanone) and PGV-1 (2,5-bis-(4'-hydroxy,3',5'-dimethyl)-benzilidene-cyclopentanone) have the better potencies of anti-inflammatory effects than curcumin. Both PGV-0 and PGV-1 also show the much stronger inhibition of COX 2 activities than curcumin and aspirin *in vivo*. In this study, the COX 2 inhibition and apoptosis induction effects of PGV-0 and PGV-1 in WiDr colon cancer cell line were explored to discover potent chemopreventive agents for cancer colon treatment. Induction of pro-apoptotic protein Bax expression was also examined to understand the role of another mechanism of apoptosis induction. The result showed that IC<sub>50</sub> values of PGV-0 and PGV-1 in WiDr cell determined by MTT assay were 45  $\mu$ M and 8  $\mu$ M, respectively. Moreover, evidence of apoptosis examined by double staining with ethidium bromide-acrydine orange indicated that both PGV-0 and PGV-1 caused apoptotic bodies at inhibitory concentrations. On the other hand, immunocytochemistry assay indicated that PGV-0 significantly decreased COX 2 expression, but contradictory PGV-1 did not show any inhibition. PGV-0 also induced higher Bax expression than PGV-1. These findings suggest that PGV-1 is the most prospective chemopreventive agent although it acted via independent inhibition of COX 2 expression pathway. Therefore, further mechanism of growth inhibition and apoptosis induction of PGV-1 is important to be explored.

**Key words:** PGV-0, PGV-1, WiDr cell line, apoptosis, Bax, Cyclooxygenase 2

### Introduction

Previous study has reported that colon cancer can be blocked by anti-inflammatory drugs through the down regulation of cyclooxygenase 2 (COX 2) expression by which they capable of inducing apoptosis (Chao *et al.*, 2005). Curcumin, the yellow pigment

in turmeric, shows the anti-inflammatory effect and also exhibits the suppression of COX 2 expression in different cancer cells including colon cancer cells (Aggarwal *et al.*, 2005). Moreover, two analogues of curcumin, PGV-0 (2,5-*bis*-(4'-hydroxy-3'-methoxy)-benzilidene-cyclopentanone) and PGV-1 (2,5-*bis*-(4'-hydroxy,3',5'-dimethyl)-benzilidene-cyclopentanone) have the better potencies of anti-inflammatory effects than curcumin. Both PGV-0 and PGV-1 also showed the much stronger inhibition of COX 2 activities than curcumin and aspirin *in vivo* (Molnas Team, 2001). Therefore, in present study the COX 2 inhibition effects of PGV-0 and PGV-1 in WiDr colon cancer cell line were explored to discover potent chemopreventive agents for colon cancer treatment.

Up regulation of pro-apoptotic protein i.e. Bax is another way to induce apoptosis occurrence. Bax pore formation is important for the release of cytochrome C, one of apoptosis mediator. On the other hand, Bax activity is inhibited by anti-apoptotic protein such as Bcl-2 and Bcl-xL which could form heterodimer with Bax. Thus, suppression of anti-apoptotic protein expression mediates the formation of Bax pore and induces apoptosis (King, 2000).

Inhibition of COX 2 expression and induction of pro-apoptotic protein Bax could induce the cancer cells to undergo apoptosis. Cancer cells are able to hinder apoptosis because of mutation or change in normal physiological properties. Consequently, cell cycle progression would be actively occurs and uncontrollable (Hanahan and Weinberg, 2000). Moreover, compounds that have good potency to sensitize tumor cells to induce apoptosis are considered to be prospective chemopreventive agents (Chao *et al.*, 2005).

## Materials and Method

### Chemicals and Cells

Curcumin was obtained from Sigma, PGV-0 and PGV-1 were obtained from Curcumin Research Center (CRC), Faculty of Pharmacy, GMU. Dimethylsulfoxide (DMSO), MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide], and other chemicals were purchased from Sigma. Dulbecco's modified Eagle medium (DMEM), RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA 1x were purchased from Gibco. The colon cancer cell line, WiDr, was obtained from Parasitological Department of Medical Faculty and the breast cancer cell line, T47D, was obtained from NAIIST, Japan. COX 2 antibody was obtained from DACO and Bax antibody was obtained from Cell Signalling. All of reagents for luciferase assay were provided by Laboratory of Animal Science, NAIIST, Japan.

### **Plasmid**

The NF- $\kappa$ B-responsive luciferase reporter construct and the NF- $\kappa$ B expression vector, pCMX-*lacZ*, was a kind gift from Prof. Kawaichi, NAIST, Japan.

### **Sample preparation**

Test sample solutions of curcumin, PGV-0, and PGV-1 (50.000 mM) were prepared by dissolving each compound in DMSO. Serial dilutions of sample solutions with culture medium were prepared immediately before *in vitro* assays were performed.

### **Cell viability**

Cell viability was determined by using MTT assay. WiDr cells ( $3 \times 10^3$  cells/ well) were distributed into 96 well-plates and incubated for 48 hours. After rinsed the cells using phosphate buffer saline (PBS), cells were treated with the samples and after 24 hours incubation MTT reagent was added. Stopper reagent was added after formazan formation prior to MTT reduction. The absorbance of each well was measured using ELISA reader at 595 nm.

### **Apoptosis assay**

After 48 hours incubation, WiDr cells ( $5 \times 10^4$  cells/ well) were treated with 30 mM curcumin, 45 mM PGV-0, and 10 mM PGV-1 for 15 hours. After incubation, cells were stained for 5 minutes with acrydine orange – ethyidium bromide staining solution (each 5  $\mu$ g/ml in PBS) and viewed immediately by fluorescence microscope. Apoptotic cells which had lost their membrane integrity appeared orange and showed morphological features of apoptosis. Cells were identified as apoptotic on the basis of specific morphological criteria, including condensation and fragmentation of chromatin, and formation of apoptotic bodies.

### **Immunocytochemistry assay**

WiDr cells ( $5 \times 10^4$  cells/ well) were seeded into coverslip in 24 wells-plate. Following 48 hours incubation, cells were treated with 30 mM curcumin, 45 mM PGV-0, and 10 mM PGV-1 for 15 hours. The cells attached on coverslip's surface were been treated with immunocytochemistry reagent according to the manufacturer's protocol.

### **Transfection and luciferase assay**

T47D cell were seeded at a concentration of  $2.5 \times 10^4$  cells/well in 24-well plate. After 21 hours incubation, refresh the medium with serum-free medium and continue incubation for 4 hours, and then cells were transfected with 0.8  $\mu$ g containing pNF $\kappa$ B-TAL-Luc and pCMX-*lac Z* and 2  $\mu$ l of lypofectamine 2000 (Invitrogen) each well.

After 5 hours incubation medium were refreshed with culture medium containing 10% FBS and continue incubation. At 24 hours after transfection cell were treated with medium containing 1.5  $\mu\text{M}$  PGV-1, 20  $\mu\text{M}$  curcumin, 15  $\mu\text{M}$  PGV-0 and 1 ng/ml of TNF- $\alpha$ . 24 hours after treatment cells were lysate using picagene lysis buffer and luciferase activity was measured using luminometer

## Results

### Effect of PGV-0 and PGV-1 on cell growth

Cell viability assay was done to determine the inhibitory concentration ( $\text{IC}_{50}$ ) of PGV-0, PGV-1, and also the reference curcumin in WiDr cells. All of these compounds showed growth inhibitory effect in dose dependent manner. The  $\text{IC}_{50}$  values of PGV-0, PGV-1, and curcumin were 45, 8, and 27  $\mu\text{M}$ , respectively. PGV-1 exhibited the strongest growth inhibitory effect among the compounds (Fig.1).

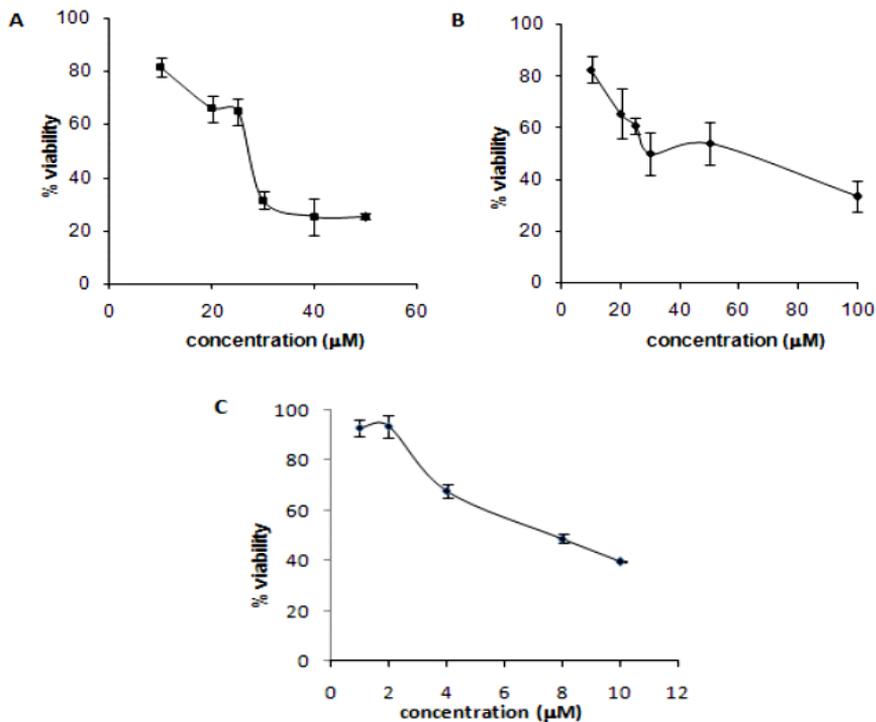


Figure 1. Cell viability assay of (A) Curcumin, (B) PGV-0, (C) PGV-1 in WiDr cells. Result showed that curcumin, PGV-0, and PGV-1 had the  $\text{IC}_{50}$  values of 27  $\mu\text{M}$ , 45  $\mu\text{M}$ , and 8  $\mu\text{M}$ , respectively.

### Effect of PGV-0 and PGV-1 on apoptosis

All of curcumin, PGV-0, and PGV-1 were capable of inducing apoptosis at inhibitory concentration (Fig. 2). The green fluorescence indicates the viable cells while the orange-red fluorescence indicates the death cells. Apoptotic cells show the occurrence of chromatin condensation and the orange-red apoptotic bodies. PGV-1 appeared to have the strongest apoptotic effect.

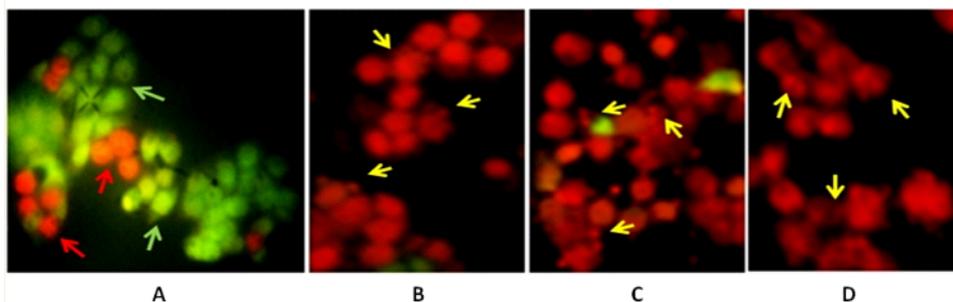


Figure 2. Apoptosis induction of curcumin and pentagamavunons in WiDr cell. (A) control cells, (B), (C), and (D) cell treated with 30  $\mu$ M curcumin, 50  $\mu$ M PGV-0, and 10  $\mu$ M PGV-1 at incubation time 15 h.

### Inhibition of NF- $\kappa$ B activation

This study was preliminary study to predict the probability of inhibition of NF- $\kappa$ B activation before we do the same experiment in WiDr cells. With respect to apoptosis induction, the luciferase assay also showed the similar result where PGV-1 showed the strongest inhibition on NF- $\kappa$ B activation (Fig. 3). Curcumin showed the inter-

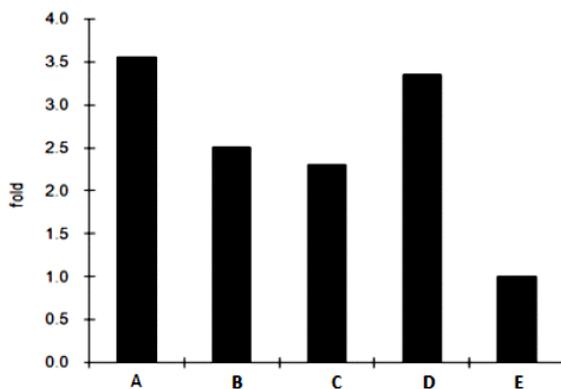


Figure 3. Suppression on NF- $\kappa$ B activation in T47D cells. (A) control cell induced by TNF- $\alpha$ , (B), (C), (D) cells treated with 20  $\mu$ M curcumin, 1.5  $\mu$ M PGV-1, 10  $\mu$ M PGV-0, (E) control cell without TNF- $\alpha$ . PGV-1 showed the strongest suppression on NF- $\kappa$ B activation induced by TNF- $\alpha$ .

mediate effect, while PGV-0 did not show any significant inhibition. Verification of this result should be done by the real examination in WiDr cells.

#### **Induction of Bax expression**

To understand the further mechanism of apoptosis induction, immunocytochemical assay was done to determine the change of Bax expression. The result showed that curcumin induced Bax expression on highest level followed by PGV-0. In contrast, PGV-1 didn't show significant induction (Fig. 4). PGV-1 might be act trough the suppression of antiapoptosis protein such as Bcl-2 and Bcl-xl.

#### **Inhibition of COX 2 expression**

Immunocytochemical analysis on COX 2 indicated the high level of COX 2 expression in control cells, showed by darken brown colour. Curcumin and PGV-0 showed significant inhibition on COX 2 expression, showed by bluish color. Interestingly, PGV-1 didn't showed similar response although it gave the strongest growth inhibitory effect and showed the better anti-inflammatory effect than curcumin *in vivo* (Fig. 5) (Molnas Team, 2001).

#### **Discussions**

Previous study showed that curcumin inhibited p65/ Rel A expression, NF- $\kappa$ B dependent transcriptional activity, and expression of NF- $\kappa$ B-dependent anti-apoptotic genes. However, curcumin also induced apoptosis in p65 over-expressed HCT116 human colon cancer cells. These findings indicate that curcumin induces apoptosis trough NF- $\kappa$ B independent mechanism (Collet and Campbell, 2006). We hypothesized that the curcumin analogue PGV-0 induces apoptosis trough independent NF- $\kappa$ B /Rel A transcriptional activity since PGV-0 did not inhibit the NF- $\kappa$ B activation in T47D cells. However, this result should be confirmed by experiment in WiDr cells.

The mechanism of apoptosis induction of PGV-0 also involves up regulation of pro-apoptotic protein Bax, although this effect is not better than curcumin. Moreover, PGV-0 also induces apoptosis by suppression of COX 2 expression by which it up regulates p53 transcription factor. Mechanisms mediated this response need to be explored. Interestingly, PGV-1 did not show similar response compare to curcumin and PGV-0 although it gave the strongest growth inhibitory effect and showed the better anti-inflammatory effect than curcumin *in vivo* (Molnas Team, 2001). PGV-1 seems to induce apoptosis trough the inhibition of NF- $\kappa$ B-dependent anti-apoptotic genes. The present study showed that PGV-1 significantly inhibit the activation of NF- $\kappa$ B in T47D cells. Since PGV-1 did not induce the expression of pro-apoptotic Bax, its pro-apoptotic effect could be

through the inhibition of NF- $\kappa$ B/ Rel A anti-apoptotic downstream such as Bcl-xL, Bcl-2, or X-linked inhibitor of apoptosis (XIAP).

In T47D cells, PGV-1 treatment inhibits cell cycle progression at G2/M and mitosis phase (Da'i, 2007). Disturbance on microtubule and failure of mitosis completion result in activation of mitotic checkpoint indicated by the expression of p21 by p53-independent in T47D cells. It caused accumulation of cells at G2/M phase as well as hyperploid cells. These accumulations subsequently induce cells to undergo apoptosis (Wang *et al.*, 2000). Furthermore, it needs to be examined whether PGV-1 also inhibits cell growth through cell cycle arrest in WiDr cells by accumulation of cells in G2/M phase.

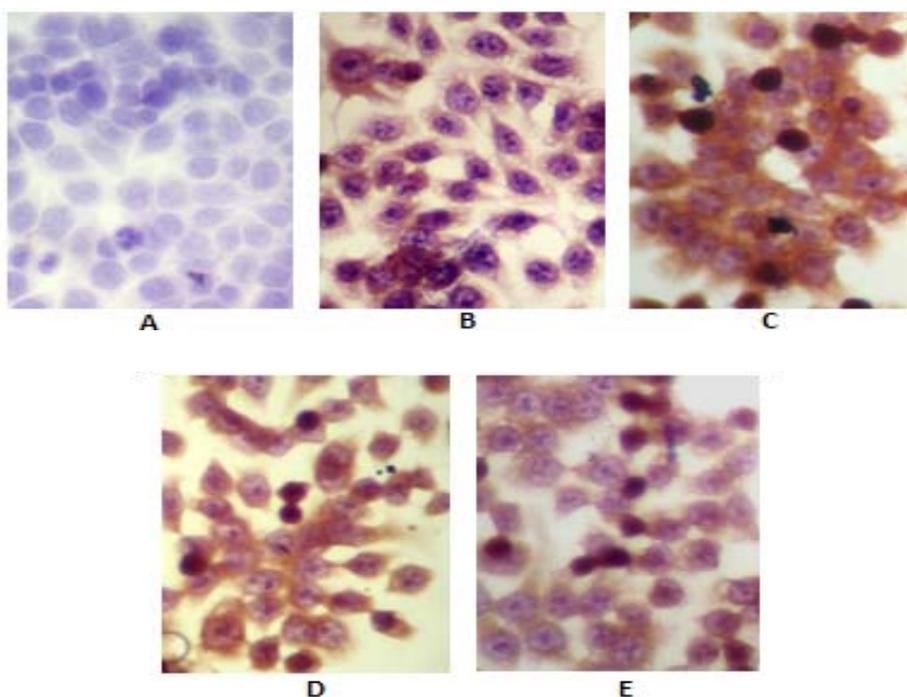


Figure 4. Induction of Bax expression in WiDr cells. (A) Control cell without Bax antibody, (B) control cells, (C) cells treated with 30  $\mu$ M curcumin, (D) cells treated with 50  $\mu$ M PGV-0, and (E) cells treated with 10  $\mu$ M PGV-1. The intensity of brown color of cytoplasm indicates the level of proapoptotic protein Bax expression, the more the intensity the higher Bax expression.

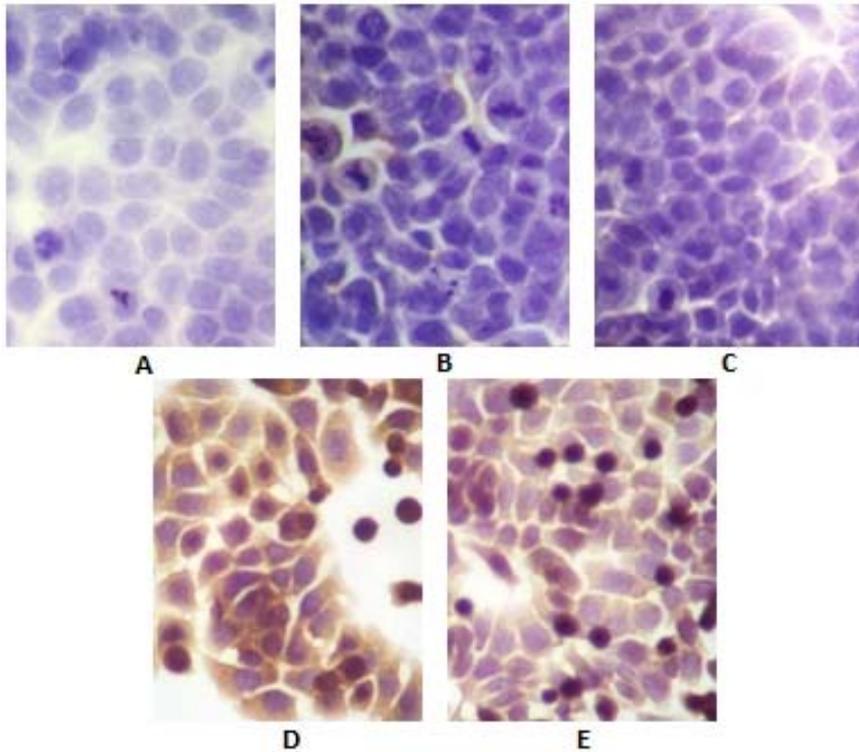


Figure 5. Inhibition of COX 2 expression in WiDr cells. (A) Control cells without COX 2 antibody, (B) cells treated with 30  $\mu$ M curcumin, (C) cells treated with 50  $\mu$ M PGV-0, (D) control cell, (E) cells treated with 10  $\mu$ M PGV-1. Blue colour of cytoplasm indicates inhibition of COX 2 expression and brown color of cytoplasm indicates COX 2 expression, the more the intensity the higher COX 2 expression

## Conclusion

Taken together, all of those results indicate that PGV-1 is the most prospective chemopreventive agent although PGV-1 acts via independent inhibition of COX 2 expression pathway. PGV-1 has the strongest effect on apoptosis induction in WiDr cells. Moreover, the molecular mechanism of growth inhibition and apoptosis induction of PGV-1 is important to be explored.

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