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PGV-1 is a Potent Antimitotic Agent

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Abstract

Carcinogenesis may resulted from the malfunctioning of programmed cell death. Most of the anticancer drugs in current use induce apoptosis in susceptible cells. The fact that disparate agent interacting with different targets seem to induce cell death through some common mechanisms suggest that anticancer activity is determined by the ability of inhibiting cell growth. Pentagamavunon-1 (PGV-1) is one of the curcumin analogues which showed to have potency in inhibiting proliferation of T47D human breast carcinoma cells. The effects on T47D cells growth is associated with cell cycle arrest in G2/M phase at the concentration of 2.5 ?M, followed by hyperploidy. The data on polymerization assay, indicated that PGV-1 interact with tubulin in different manner from taxol. PGV-1 inhibit tubulin polymerization on cell culture while taxol stabilized tubulin polymerization. Immunostainning data on PGV-1 treated cells showed slightly tubulin condensation, while taxol treated cells showed tubulin condensation distinctly at 12 minutes after releasing from depolymerizing agent.
In conclusion, PGV-1 represent a new microtubule inhibitor and has the potential to be developed for antimitotic drug

Key words: Pentagamavunon-1, T47D, tubulin

Introduction

Curcumin is one of the major components of turmeric, a phytochemical agent which contains chemopreventive properties against tumors. Many medicinal properties of curcumin are well recognized. Curcumin has been shown to inhibit proliferation of a wide variety of tumour cells. The incubation of T47D breast cancer cells with 10 mM curcumin for 24 hours induced G1 accummulation cell population (Da'i *et al*, 2007). Curcumin was found to induce G0/G1 and/or G2/M arrest, upregulated CDKIs, p21, p27, p53 and slightly down regulate cyclin B1 in umbilical vein endothelial cells (Park *et al*., 2002). Curcumin Research center has found some candidates of curcumin analogues which has the potency of anticancer.

Pentagamavunon-1 (PGV-1) is one of the curcumin analogues which showed to have potency in inhibiting proliferation of T47D human breast carcinoma cell. The previous research found that PGV-1 at the concentration of 2.5 mM modulates cell cycle progression through G2/M arrest, followed by hyperploidy of the cell and *mitotic catarstrophe* on T47D cells (Dai *et al*., 2007). In this effect, PGV-1 induces p21 expression and CDK-1 dephosphorilation. PGV-1 may interact with tubulin leading to the inhibition of microtubule polymerization as indicated by occurrence of tubulin condensation. However the cells may undergo mitosis but it is not followed by cytokinesis resulting in the hyperploidy.

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These findings suggest that PGV-1 may act as microtubule inhibitor and can be categorized as an anti-microtubule agent.

The objective of this research was to observe the mechanisms of PGV-1 as antimicrotubule drug which may interact with tubulin either in polymerizing or depolymerizing of tubulin.

Materials and Methods

Cell culture

T47D cell line was kindly provided by Division of Genetic Function in Animal laboratory, NAIST, Japan. Cells were maintained in DMEM (Sigma) plus 10% fetal bovine serum (Thermo Sci) and 1% penicillin / streptomycin.

Flowcytometric analysis

T47D cells were seeded at 5 x 10^5 cells / well on six wells tissue culture plate. After 24 hours incubation, cells were treated with 2.5 mM PGV-1 and 1 mM Taxol and incubated for 6, 12 and 24 hours. Cells were collected by trypsinized, spin at 1000 rpm for 3 minutes and washed twice with cold PBS. Cells were resuspended in propidium iodide solution (50 mg / ml in PBS contained 1% triton X-100) and then treated with RNAse DNAse free (20 mg / ml) for 10 minutes at 37°C. The treated cells then subjected to Facscalybour flowcytometry. Cell cycle profiles were analysed using cell quest acquisition program.

Immunofluorescence microscopy

T47D cells were grown on glass coverslip at 2.5 X 10^4 cells / well. After 24 hours incubation cells on coverslip were treated with 2.5 mM PGV-1 and 1 mM taxol and incubated for 12 hours and 24 hours. Cells on coverslip were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized with 0.1% triton X-100 in PBS for 5 minutes. After blocking in 1% bovine serum albumine in PBS for 60 minutes, cells were incubated in primary antibody against a-tubulin (1:200) for 1 hour and washed with PBS to remove unbound antibody. Then cells were incubated with anti mouse IgG FITC conjugated secondary antibody (1:100) for 1 hour. Nuclear morphology were observed by staining with DAPI (0.1 mg / ml). cells on coverslip were mounted on permafluor mounting and observed on fluorescence mycroscope.

Microtubule polymerization assay

T47D cells were seeded on 60 mm dish at 8 x 10^5 cells / dish. After 24 hours incubation cells were treated with 2 mg / ml of nocodazol for 2 hours to depolymerize tubulin. Cells then released from nocodazol and lysed at 0, 4, 8 and 12 minutes in 400 ml of microtubule stabilizing lysis buffer containing 4 mM paclitaxel or 4 mM PGV-1, 0.1 M PIPES pH 6.9, 2 M glycerol, 5mM $MgCl_2$, 2 mM EGTA, 0.5% triton x-100, 5 mg / ml leupeptin. Cell lysate were centrifugated at 25.000 rpm for 30 minutes at 22°C for sedimentation of polymeric tubulin. The sediment (containing polymeric tubulin) was subjected to 150 ml solubilization buffer containing 25 mM Tris-HCl pH 7.5, 0.4 M NaCl, 1% NP-40, 0.5% SDS, 0.1% deoxycholate, 5 mg / ml leupeptin. The resulting sample were analyzed on western blot using a-tubulin antibody

Results

The Effect PGV-1 on cell cycle progression of cancer cell

Flowcytometric analysis was used to observe the distribution of cell population on cell cycle. As shown in figure 1, there was an accumulation at G2 / M phase of T47D cells treated with 2.5 mM PGV-1 and 1 mM taxol after 12 hours incubation. After 24 hours incubation, PGV-1 treated cells PGV-1 showed an increase in polyploidy (M5) and decrease in sub G1 phase. The cells treated

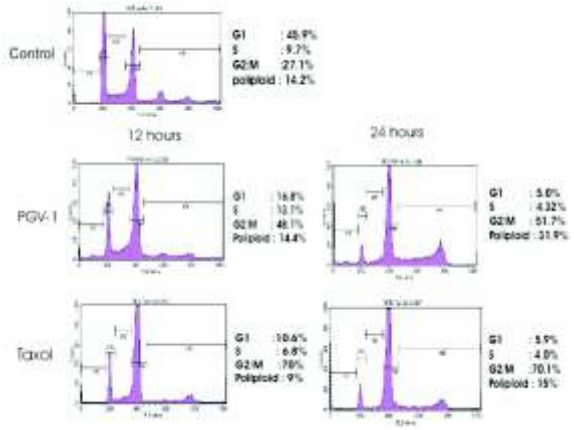

Figure 1. Flowcytometric analysis. Cells were treated with 2.5 mM PGV-1 and 1 mM Taxol, incubated for 12 and 24 hours, and stained with propidium iodide. T47D cells shown an accumulation on G2 / M phase.

with 1 mM taxol showed an increasing in cells with polyploidy DNA content and decreasing in sub G1.

P G V - 1 i n h i b i t s t u b u l i n polymerization

To observe microtubule interaction effect of PGV-1, it was important to observe the polymerization mechanism on PGV-1 treated cells. Nocodazole, a tubulin-depolymerizing agent was used to transiently deploymerize tubulin in cells, followed by releasing in complete medium without nocodazole. Tubulin staining in the cells were determined by fluorescent microscopy and tubulin polymerization were determined by western blot analysis. Tubulin staining showed that cells without treatment exhibit characteristic pattern of individual microtubule as indicated by a fine network mesh of microtubular material and nuclei of these control were intact as visualized by staining with Dapi. Cells treated with PGV-1 showed a slightly fine network mesh, microtubule disruption and multinuclei. Cells treated with taxol showed tubulin condensation distinctly and condensed chromatin (Figure 2). Tubulin polymerization assay displayed that taxol treated cells indicated an accumulation of polymerized tubulin starting at 4 minutes after releasing nocodazole and then striking accumulation at 12 minutes after releasing nocodazole. In contrast, cells treated with PGV-1 showed a decreasing accumulation of polymerized tubulin at 0, 4, 8 and 12 respectively after releasing nocodazole (Figure 3). This findings suggest that PGV-1 and taxol may interact with tubulin in different mechanism.

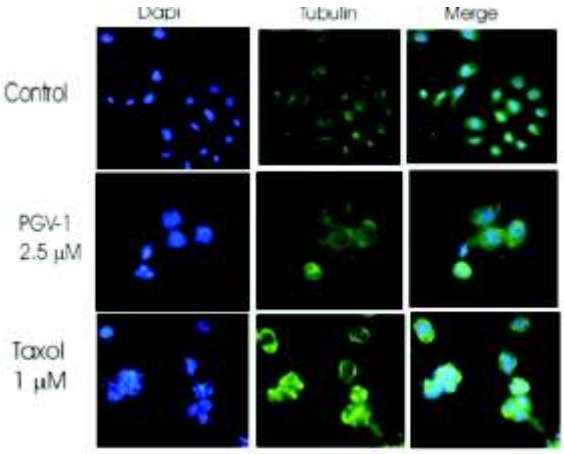

Fig 2. Tubulin polymerization effect of PGV-1. Microtubule were stained with green-FITC conjugated a-tubulin antibody, DNA were stained with DAPI and observed on fluorescent microscope.

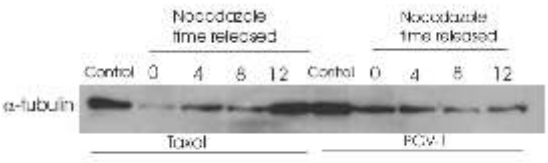

Fig 3. Tubulin polymerization efect of PGV-1 by western blot analysis. T47D cells were treated with ocodazole to depolymerize microtubule and released at 0, 4, 8, 12 minutes, and lysed with buffer containing 4 mM PGV-1 or taxol

Discussion.

Pentagamavunon-1 (PGV-1) is one of curcumin analogues which has the potency of inhibiting cell proliferation. Its IC 50 on T47D cells is 1.5 mM and 2.5 mM on MCF-7 cells which is lower than curcumin (20 mM) (Da'i *et al*, 2007).

The data showed that PGV-1 induced

cell cycle arrest in G2/M phase significantlyat 2.5 mM concentration which was followed by hyperploidy of the cells and seemed to induce mitotic catarstrophe. The previous study showed that PGV-1 induced p21 expression and cdc-2 activation (Da'1, 2007). Activation of cdc-2 may cause cell accumulation at hiperploidi area (M5) in flowcytometric analysis. A compound which has the potency of inducing G2/M arrest followed by hiperploidi, cdc-2 and p21 activation indicates the characteristic of antimicrotubule agent (Okada and Mak, 2004, Wang *et al*, 2000).

Microtubule is a major component of the mitotic spindle, which pulls the chromosome apart at mitosis and then splits the dividing cell into two. The majority of antimitotic agents induce mitotic arrest by interacting with tubulin (Zhang *et al*, 2006). Several antimitotic agent have been reported to induce P53 and inhibit cyclin-dependent kinase, p21 and activate/inactivate several protein kinase including raf/Ras, mitogen activated protein kinase and p34cdc2. The previous study on PGV-1 showed that PGV-1 induced apoptosis by p53-independent apoptosis in T47D cells (Da'i *et al*, 2007).

The data on mycroscopic analysis indicated that PGV-1 disrupt microtubule assembly. Cell treated with taxol showed a distinct microtubule condensation and condensed chromatin. This finding indicated that PGV-1 had different mechanism from taxol in interacting with tubulin. Taxol stabilized tubulin polymerization while PGV-1 inhibit tubulin polymerization. However, the antimitotic effect of PGV-1 need to be confirmed further by fact that the treatment of cells with this compound triggered several molecular event involved in mitotic signaling cascade. This includes of some important mitotic regulatory proteins such as Bcl2, cdc25C and cyclin B accumulation.

As the conclusion of this research, PGV-1 modulates cell cycle progression through G2/M arrest and inhibits tubulin polymerization. PGV-1 can be categorized as antimitotic agent.

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