



Different Mechanisms of Androg and IPAD on Apoptosis Induction in Cervical Cancer Cells

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ABSTRACT

Androg and IPAD are recently known to possess potent anti-cancer potential that can induce apoptosis in various cancer cells. Indeed, apoptosis can be initiated by different mechanisms in different cell types. Previously, we found that Androg and IPAD can suppress HPV16 E6 oncogene expression and restore p53 in HPV16 positive cervical cancer, SiHa and Caski cell with different activity. To investigate the different effects of Androg and IPAD in HPV16 positive cervical cancer cells, in this present study, SiHa and C33A (HPV16 negative cervical cancer cell with p53 mutation) were treated with Androg and IPAD, at various concentrations for 48 h, subsequently detected cell apoptosis and DNA degradation by Annexin V assay and gel electrophoresis. P53 targeting Bax protein level was determined using western blot. The results showed that IPAD at subcytotoxic concentration induced apoptosis in both SiHa and C33A cells, whereas treated with Androg, apoptosis was detected only in SiHa cells. In addition, DNA degradation assay demonstrated DNA ladder in both SiHa and C33A cell treated with IPAD, in contrast to treatment with Androg, DNA ladder was detected only in SiHa cells which associated with HPV oncogene suppression. By western blot, Bax protein level from SiHa cells treated with IPAD was higher than Androg corresponding with p53 restoring, contrast to C33A with p53 mutation, Bax was not detected. This result showed that IPAD contains different activity from Androg to induce apoptosis in both HPV positive and HPV negative cervical cancer cells and suggested that IPAD might associate to apoptosis pathway more than Androg, and these apoptosis inductions are needed to be more elucidated by further assay.

Keywords: HPV16 E6 oncogene, IPAD, Apoptosis

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Introduction

Cervical cancer is a major cause of cancer-related deaths in females and is caused by infections with high risk human papillomaviruses (HR-HPVs), HPV types 16 and 18 which are together responsible for about 70% of cervical cancers (Franco et al., 2001). The mechanisms of HPVs to cause cancer are only partially elucidated. In most cases, HPV E6 and E7 oncogenes are over expressed and then have the effect on cell transformation, increasing cell proliferation and decreasing apoptosis by alteration of multiple intracellular signaling pathways (Zur Hausen, 2002). The treatment approaches in cervical cancer and HPV induced abnormal cervical cells have not achieved satisfactory outcomes despite rapid progress in understanding the biology of HPVs and cervical cancer cells.

Andrographis paniculata is a medicinal plant found dispersedly in Asia such as Thailand, It is used to cure fever, inflammation, common cold, diarrhea and other infectious diseases. Andrographolide (Androg), a bicyclic diterpenoid lactone, is originated from *A. paniculata*. Our previous study showed that Androg and its derivative, namely 3, 19-isopropylidene andrographolide (IPAD) have different activities to suppress transcriptional activity of early oncogene promoter HPV16 and E6 oncogene expression which subsequently affects cervical cancer cell apoptosis. A two-fold subcytotoxic concentration of IPAD exhibited an inhibitory effect on E6 oncogene expression at 48-h post treatment. Interestingly, p53 protein was restored in a downstream process and was detected earlier by IPAD treatment than by Androg treatment. This result corresponded to the level of cell apoptosis and cell cycle arrest at the G2/M phase. They may be effective agents for HPV prevention and cervical cancer

treatment. However, the molecular mechanisms are not elucidated. Therefore, this study aims to investigate the different effects of Androg and IPAD on apoptosis induction in HPV positive and HPV negative cervical cancer cell lines according to the level of p53 protein expression. DNA fragmentation assay and western blot analysis were performed.

Objective

To investigate the different effects of Androg and IPAD on apoptosis induction in HPV positive and HPV negative cervical cancer cell lines

Materials and methods

Cell lines

SiHa cells (HPV16 positive cervical cancer cells) and C33A (HPV negative cervical cancer cells with p53 gene mutation) were maintained in Dulbecco's modified Eagle's medium or DMEM (Gibco®Life Technologies, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 100 µg/mL streptomycin, 100 IU/mL penicillin, 40 µg/mL gentamycin, 2.5 µg/mL fungizone, and 10% (v/v) heat-inactivated fetal bovine serum. All cell line cultures were performed at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. For cell cultures with indicated treatments, DMEM with 2% FBS was used as a maintenance medium.

Medicinal compounds

All compounds were dissolved in dimethyl sulfoxide (DMSO) as stock solution (2 mg/ml) and stored at -20°C. The stock solutions were freshly diluted to the indicated concentrations with culture medium before using in experiments. Compounds of *A. paniculata* used in this study consisted of andrographolide (Androg) and 3, 19-isopropylidene andrographolide (IPAD).

The structure of *A. paniculata* is shown in Figure 1.

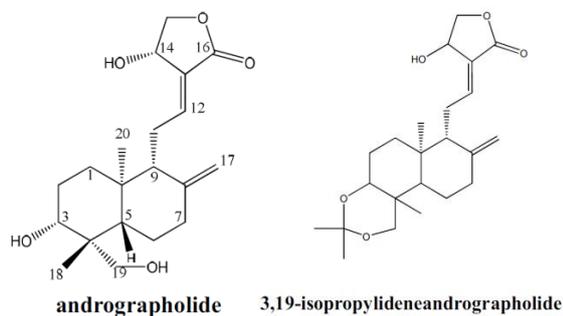


Figure 1 The structure of andrographolide (Androg) and 3, 19-isopropylidene andrographolide (IPAD) (Yan et al., 2012)

Apoptosis detection by Annexin V assay

Cells were treated with Androg and IPAD for 48 h. Cells were collected and stained with Annexin V-FITC apoptosis detection kit II (BD Biosciences, California, U.S.A.), after which were analyzed by observing under the fluorescent microscope. Data were calculated by counting stained cells obtained from 3 fields independently.

Agarose-gel analysis for DNA fragmentation assay

Cells were treated with various doses of Androg and IPAD for 48 h. DNA was extracted by the use of apoptotic DNA ladder (Invitrogen, CA) according to the manufacturer's instructions. Samples (3 µg/ lane) were loaded and separated on a 2% agarose gel.

Western blot analysis

To confirm the different effects of Androg and IPAD on p53 expression, the p53 targeting, Bax protein expression was determined. The cell lysate of treated SiHa and C33A cells was subjected to SDS-PAGE, and Bax protein was detected by Western blot analysis using an anti-human Bax rabbit polyclonal IgG

(diluted 1:200; SantaCruz Biotechnology, Santa Cruz, CA) as a primary antibody and a horseradish peroxidase-conjugated goat-anti rabbit IgG as a secondary antibody. The Bax protein signal was detected with a chemiluminescence solution (Super Signal West Pico, Thermo Scientific, Waltham, MA). Beta-actin was used as the internal control by detecting it on the previous membrane using an anti beta-actin mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA) and a horseradish peroxidase conjugated goat-anti mouse IgG as the primary and secondary antibodies, respectively.

Statistical Analysis

The results were reported as the mean ± SD of at least 3 experiments. Statistical analysis was performed using an unpaired Student's t-test by SPSS software. The results were considered statistically significant if $P \leq 0.05$.

Results

Effects of Androg and IPAD on cell morphology changes

Morphological changes were observed in SiHa and C33A cells on treatment with subcytotoxic concentration (13.88 and 9.71 µM), CC50 (142.26 and 152.34 µM) of Androg and subcytotoxic concentration (12.22 and 11.33 µM), CC50 (72.67 and 69.70 µM) of IPAD, and Cyclohexamide (CHX) (60 µg/µl), respectively, with rounding of the cytoplasmic periphery along with gradual detachment of cells from substrates. Features consisted cell membrane blebbing and cell shrinkage in treated cells. The treated cells were counted for Annexin V stained apoptotic cells as shown with green color points which were established for early apoptotic cells (Fig. 2). Then the total green

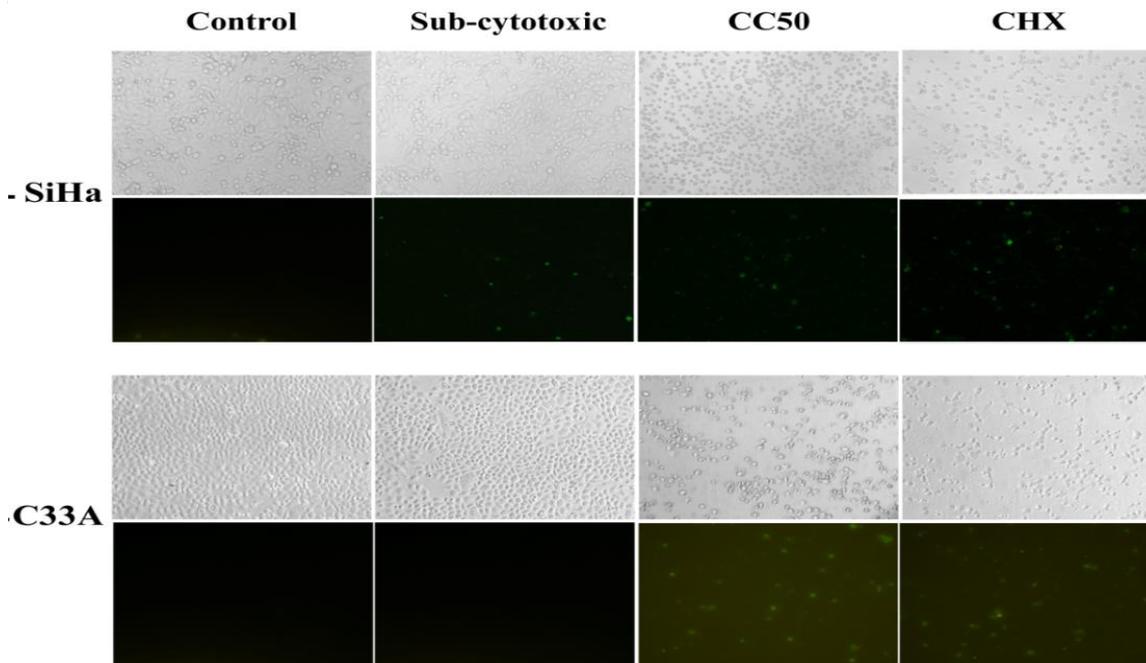


Figure 2 Representative of cell morphological changes: SiHa and C33A cells were treated with Androg at sub-cytotoxic concentration (13.88 and 9.71 μM), CC_{50} (142.26 and 152.34 μM), respectively and Cyclohexamide (CHX) (60 $\mu\text{g}/\mu\text{l}$), and incubated for 48 h. After incubation, morphological changes were examined under bright microscope (Top) and fluorescent microscope (Bottom) in which cell membrane blebbing and cell shrinkage were found in treated cells.

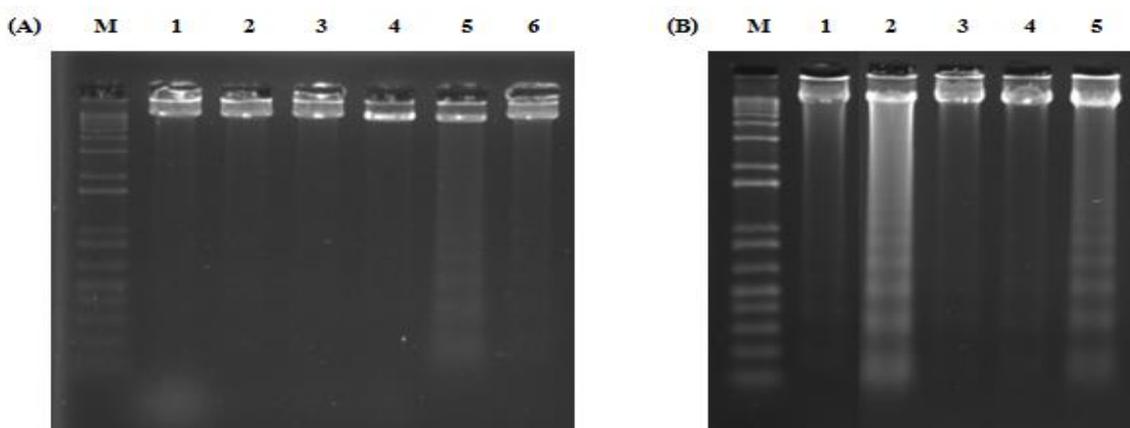


Figure 3 DNA fragmentation results after treatment of C33A and SiHa cells with Androg. Cells were incubated in the absence (control) or presence of Androg at subcytotoxic and CC_{50} concentration for 48 h, and CHX (60 $\mu\text{g}/\mu\text{l}$) as a positive control. Genomic DNA was extracted, electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under UV illuminator. **(A)** C33A cell treated with Androg at various concentrations, lane M was 100 bp marker. Lane 1 and 2 were 0.1% and 1% DMSO. Lane 3-4 were sub-cytotoxic and CC_{50} . Lane 5 and 6 were CHX and untreated cells, respectively. **(B)** SiHa cells treated with Androg at various concentrations, lane M was 100 bp marker. Lane 1 was untreated cell. Lane 2 was CHX. Lane 3-4 were sub-cytotoxic and 2-times of sub-cytotoxic concentrations, respectively. Lane 5 was CC_{50}

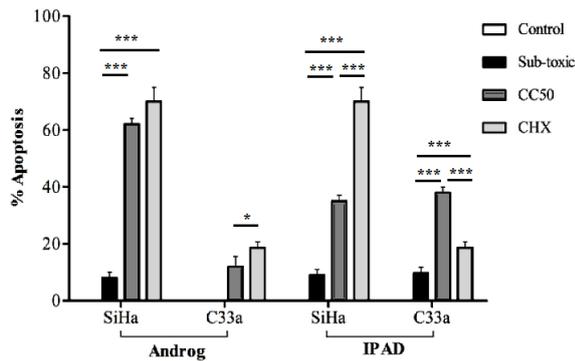


Figure 4 The total counted Annexin V-stained apoptotic cells. * indicates significant difference ($P < 0.05$), ** $P < 0.05$, *** $P < 0.001$ between control and compounds

cells were counted for 3 independent fields in each condition under fluorescent microscope (Fig. 4).

Effects of Androg and IPAD on DNA fragmentation

DNA degradation results showed that Androg could induce only SiHa cell (Fig. 3B) to undergo apoptosis, but not C33A cell (Fig. 3A) with a dose-dependent manner. In contrast, IPAD could significantly induce SiHa and C33A cells (Fig. 5) to undergo apoptosis as shown by ladder bands.

Effects of Androg and IPAD on Bax, a pro-apoptotic marker expression

SiHa and C33A cells were treated with Androg and IPAD at 1x and 2x subcytotoxic concentration for 48 h followed by Bax detection using Western blot analysis. The result showed that compounds had the effect on treated SiHa cells by inducing Bax expression as compared to control SiHa cells. Interestingly, IPAD could induce Bax expression gradually increasing at 2x subcytotoxic concentration

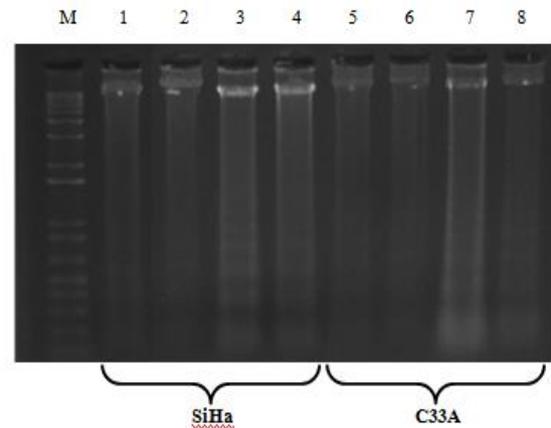


Figure 5 DNA fragmentation results after exposure of SiHa and C33A cells to IPAD. Lane M was 100 bp marker. Lane 4 and 8 were CHX. Lane 1 and 5 were untreated SiHa and C33A cells, respectively. Lane 2 and 6 were subcytotoxic concentration, Lane 3 and 7 were CC_{50} .

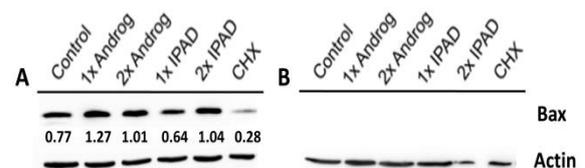


Figure 6 Effect of compounds at various concentrations on Bax protein expression in human papillomavirus 16 (HPV16) positive SiHa cells (A) and HPV negative C33A cells (B).

(Fig. 6A). On the other hand, Androg and IPAD could not induce Bax expression in C33A cells (Fig. 6B).

Discussion

During the past several years, the evidence was gradually accumulated that many cancer chemotherapeutic agents could kill the cancer cell by inducing apoptosis. Thus, identifying mode of cell death has been recognized as a novel strategy for the screening of anti-cancer drugs. Androg and IPAD the

natural compounds which exhibit anti-cancer effect on various types of cancer, including cervical cancer, which may be at least in part, responsible for apoptosis induction. In this present study, we investigated the different effects of Androg and IPAD on restoration of p53. HPV positive and HPV negative cervical cancer cells were used to induce apoptosis. DNA degradation assay and western blot analysis were used to investigate DNA ladder and p53 targeting Bax protein, respectively. The result showed IPAD activity on apoptosis induction not only in HPV positive cervical cancer cells (SiHa cells), but also in HPV negative cervical cancer cells. Higher level of Bax protein supports more apoptosis pathway for p53 restoration of IPAD than Androg in treated cells.

Previously, we found that Androg and IPAD have different activity to suppress transcriptional activity of early oncogene promoter HPV16 E6 oncogene expression and subsequently affected on cervical cancer cell apoptosis (Ekalaksananan et al., 2015). Additionally, our result showed that Androg could induce apoptosis only in SiHa cells (Fig. 4), in which DNA degradation was also confirmed (Fig. 3B), but not effect to C33A cells.

In this study, at 48 h after treatment with Androg and IPAD in C33A cells, only IPAD exhibits the apoptotic induction and DNA degradation. Because C33A is HPV negative cervical cancer, therefore, this result suggested that IPAD may induce apoptosis without association with HPV16 E6 oncogene suppression. The mitochondrial apoptotic pathway is regulated by the Bcl-2 family of proteins, including the anti-apoptotic members Bcl-2 and Bcl-xL, and the pro-apoptotic members Bax and Bak. The balance between the levels of pro-apoptotic and anti-apoptotic protein is important for cell survival and death (Liang et al.,

2011). These proteins might be regulated by IPAD in the treated cells and affect on apoptosis induction in these cells. A study in prostate cancer cells (PC-3) has found that Androg induces apoptosis via the activation of caspase 3, upregulation of bax, and downregulation of bcl-2 (Zhao et al., 2008). In this present study, IPAD exhibited apoptotic induction in different pathways more than Androg which showed apoptosis in the treated C33A cells for 48 h with sub-cytotoxic concentration by Annexin V assay (Fig. 4), then both 1x and 2x-subcytotoxic concentrations by DNA degradation assay (Fig. 5), but showed no appearance of Bax protein expression (Fig. 6B). This effect may correspond to p53 mutation in C33A (Waggoner et al., 1994). The result was also confirmed by increment of the protein expression of Bax after treatment with Androg and IPAD (Fig. 5a) in HPV16-positive SiHa cells. Bax is the downstream effector of p53. The previous report showed that curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction (Choudhuri et al., 2002). Our recent study showed that the p53 tumor suppressor protein could be restored by the decreased HPV E6 expression leading to induce HPV positive cervical cancer cell apoptosis after treatment with Androg and IPAD compounds (Ekalaksananan et al., 2015). The induction of cell apoptosis by Androg has also been found in several cancer cell lines, including breast cancer cells (Harjotaruno et al., 2008), human leukemic HL-60 cells (Cheung et al., 2005), and human rheumatoid arthritis fibroblast-like synoviocytes (Yan et al., 2012). Therefore, IPAD might contain activity of apoptosis induction in several pathways. The specific molecular mechanisms of these compounds, especially IPAD for HPV16 E6 oncogene expression correlation and



apoptosis induction in these cell lines are needed to be further clarified.

Conclusion

This study demonstrated that IPAD have different effect on apoptosis induction from Androg, due to its ability in inducing HPV positive SiHa cells and HPV negative C33A cells to apoptosis at dose-dependent manner. Therefore, effects of Androg and IPAD are needed to be more elucidated for their different specific targets on apoptosis induction which may specifically associate to HPV oncogene expression.

Acknowledgement

This study was kindly supported by Human papillomavirus, Epstein-Barr virus and Carcinogenesis research group, Khon Kaen University, Thailand.

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