

Andrographolide Induces Apoptosis of HPV16-positive Cervical Cancer Cells**via Downregulation of c-fos, a Host Transcriptional Protein****แอนโดรกราโฟไลด์กระตุ้นอะพอพโทซิสของเซลล์มะเร็งปากมดลูกที่มี HPV16****ผ่านการลดการแสดงออกของ c-fos ซึ่งเป็นโปรตีนควบคุมการถอดรหัสของโฮสต์**

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ABSTRACT

Andrographolide (androg) induces apoptosis of HPV16-infected cervical cells by suppressing E6 oncogene and p53 protein restoration. To understand this, we investigated effects of androg on expression of transcriptional factors AP-1 (c-fos and c-Jun) that might affect expression of HPV16 E6/E7 in SiHa cells (HPV16-positive) and C33A cells (HPV-negative). Apoptosis induction was evaluated by EB/AO staining and flow cytometry then confirmed by detecting HDAC6, p53, and Bak using western blot. The results showed that androg strongly induced apoptosis of SiHa cells with significant difference from C33A cells corresponding with apoptotic marker (Bak and HDAC6) level. The c-fos expression was suppressed in apoptotic SiHa cells that showed low expression of HPV16 E6/E7 and restoration of p53 protein. This result suggested that androg might affect downregulation of c-fos transcripts that influence on the expression of HPV16 E6 and cell apoptosis.

บทคัดย่อ

โปรตีน E6 ของ HR-HPV ทำลายโปรตีน p53 ทำให้เกิดมะเร็งปากมดลูก แอนโดรกราโฟไลด์ (แอนดรอกริด) ทำให้เซลล์มะเร็งปากมดลูกที่มี HPV16 เกิดอะพอพโทซิส โดยลดการแสดงออกของโปรตีน E6 ส่งผลให้โปรตีน p53 มีการแสดงออกเพิ่มขึ้น เพื่อเข้าใจถึงปรากฏการณ์นี้ ผู้วิจัยได้ตรวจสอบผลของแอนดรอกริดต่อแสดงออกของโปรตีน Ap-1 (c-fos และ c-Jun) ในเซลล์เพาะเลี้ยง SiHa ซึ่งมี HPV16 และ C33A ซึ่งไม่มี HPV16 โดยตรวจวัดการเกิดอะพอพโทซิส การแสดงออกของ mRNA ของ E6 E7, HDAC6, AP-1 และ Bak และการแสดงออกของโปรตีน p53 ผลการศึกษาพบว่า แอนดรอกริดทำให้เซลล์เพาะเลี้ยงชนิด SiHa ตายมากกว่า C33A มีการแสดงออกที่ลดลงของโปรตีน E6 และ E7 และ c-fos ในขณะที่การแสดงออกของ p53 และ Bak เพิ่มขึ้น สรุปได้ว่าแอนดรอกริดลดการแสดงออกของ c-fos transcripts ซึ่งส่งผลมีการแสดงออกของ HPV16 E6 ลดลง แล้วเกิดอะพอพโทซิส

Keywords: Andrographolide (androg), Cervical cancer, Human papillomavirus (HPV)**คำสำคัญ:** แอนโดรกราโฟไลด์ (แอนดรอกริด) มะเร็งปากมดลูก Human papillomavirus (HPV)

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Introduction

Cervical cancer is the second most common cancer in women worldwide and also in Thailand. It is a consequence of a long-term infection with high risk human papillomavirus (HR-HPVs) particular HPV16 and HPV18 via viral oncoprotein (E6/E7) overexpression which targets on p53, a tumor suppressor protein, and retinoblastoma (Rb) protein, respectively (Moody, Laimins, 2010). The overexpression of HPV E6 and E7 genes mainly depend on the availability of host-transcription factors (Mishra et al., 2015) such as activator protein-1 (AP-1), nuclear factor 1 (NF1), octamer-binding transcription factor 1 (Oct1), and specificity protein 1 (SP1) which have been reported to activate HPV E6/E7 promoter (p97) (Chong et al., 1991; Hebner, Laimins, 2006) and epigenetic control (Mascolo et al., 2012). In cervical cancer cell lines, AP-1 is activated by homo- and/or heterodimer of c-fos and c-Jun protein during cancer progression (Liang et al., 2011). Evasion of apoptosis is a hallmark of cervical cancer and induction of apoptosis by the cytotoxic anticancer agents is one of the strategies adopted for developing chemotherapeutics treatment of cervical cancer (Jaudan et al., 2018).

Andrographolide (androg: $C_{20}H_{30}O_5$), a diterpenoid lactone, is isolated from *Andrographis paniculata* which is a traditional medicinal herb observed in many areas in Asia as Thailand (Chong et al., 1991; Shi et al., 2009). Androg has been reported to have anticancer properties (Ekalaksananan et al., 2015 ; Matsuda et al., 1994) via inhibition of cell cycle progression (Shi et al., 2008) and induction of apoptosis in various cancer cell lines such as colorectal cancer (Shi et al., 2008), breast cancer (Satyanarayana et al., 2004), gastric cancer (Lim et al., 2017) and cervical cancer (Ekalaksananan et al., 2015). Androg triggers intrinsic and extrinsic apoptotic pathways in different cancer cells via mechanisms involving activation of p53, reactive oxygen species (Mascolo et al., 2012) and topoisomerase II (Jaudan et al., 2018).

Our previous study found that androg induced apoptosis of HPV-associated cervical cancer through suppression of HPV oncoproteins and modulating p53 expression (Ekalaksananan et al., 2015). To understand about the reduction process of HPV16 E6 oncogene, this study aimed to investigate the effects of androg on expression of AP-1 (c-fos and c-Jun), a host transcriptional protein, in HPV16 positive SiHa cells. The expression of apoptosis-related proteins and E6/E7 oncoprotein were also determined.

Objectives of the study

This study aimed to investigate the effects of androg on expression of AP-1 (c-fos and c-Jun), a host transcriptional protein, in HPV16 positive SiHa cells.

Methodology

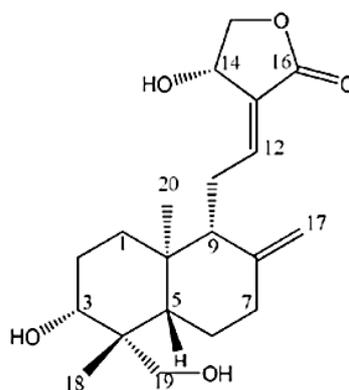
Cell lines

Cervical cancer cell lines: SiHa (HPV16-positive) and C33A (HPV16-negative) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO, USA) and antibiotics (100 μ g/mL streptomycin and 100 U/mL penicillin) in a humidified atmosphere with 5% CO_2 at 37°C.

Compound

Andrographolide (androg: clear and colorless tetragonal (MeOH); m.p.231-232°C; 100% purity) provided by Associate Professor Dr. Chantana Aromdee, Faculty of Pharmaceutical Sciences, Khon Kaen University were used in

this study. Chemical structure and its pharmacological properties were identified (Fig. 1), as previously described (Aromdee et al., 2011). The working solutions of this compound was freshly prepared with optimal concentrations in culture medium.



Andrographolide (Androg)

Figure 1 Andrographolide chemical structure.

Determination of subtoxic concentration of androg in cervical cancer cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) by cell viability assay

The viability of androg-treated cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were seeded at 2×10^4 cells/well in a 96-well tissue culture plate and incubated at 37°C , 5% CO_2 for 24 h. The cells were treated with different concentrations (0-200 $\mu\text{g}/\text{mL}$) of androg and incubated for 72 h. Each dilution was performed in a triplicate manner. After incubation, 20 μL of 5 mg/ml of MTT (PanReac AppliChem, USA) were added to each well and measured the absorbance at 540 nm wavelengths by a microplate reader (TECAN; P. Intertrade equipments Co, LTD., Männedorf, Switzerland). Cells treated with various concentrations of DMSO (0-100%) and untreated cells were used as solvent controls and a negative control. The percentages of the surviving cell were calculated as follows:

$$\text{The ratio of cell survival (\%)} = \frac{(\text{mean absorbance in test wells}) \times 100}{(\text{mean absorbance in control wells})}$$

The average of cell survival (%) and the different concentrations (0-200 $\mu\text{g}/\text{mL}$) of androg was plotted to construct a standard curve. Subsequently, cytotoxic concentration (CC_{50}), subtoxic concentration (Mascolo et al.) (5) and 2-fold subtoxic concentration ($2 \times \text{SC}$) of androg were calculated by constructing a standard curve between each concentration of compound and the average of cell survival (%).

Detection of apoptosis in cervical cancer cell lines: SiHa and C33A by using ethidium bromide/acridine orange (EB/AO) staining and flow cytometry

The effects of androg on cell apoptosis were determined by using EB/AO staining. Cells were seeded at 1×10^4 cells/well in a 96-well tissue culture plate and incubated at 37°C , 5% CO_2 for 24 h. The cells were treated in triplicate with subtoxic concentration (Mascolo et al.) and 2-fold subtoxic concentration ($2 \times \text{SC}$) of androg and incubated for 48 h. The apoptotic cells with condensed or fragmented chromatin were determined under an Eclipse NiU

microscope (Nikon Corporation, Tokyo, Japan) after adding 14 μL of 100 $\mu\text{g}/\text{mL}$ EB/AO mixture (Merck Millipore, Germany). The apoptotic cells were analyzed by counting the stained cells in random fields in each treatment conditions. Cells treated with 50 $\mu\text{g}/\text{mL}$ cycloheximide (CHX) and 0.3%-0.6% dimethyl sulfoxide (DMSO) were used as positive control and negative control, respectively.

Effects of compounds on cell apoptosis were analyzed by flow cytometry. Cells were seeded at 4×10^5 cells/well in a 24-well tissue culture plate and incubated at 37°C , 5% CO_2 for 24 h. The cells were treated with subtoxic concentration (Mascolo et al.) and 2-fold subtoxic concentration (2x SC) of compounds and incubated for 48 h. The apoptotic cells were detected by FITC Annexin V Apoptosis Detection Kit II (Invitrogen, USA). The treated cells were harvested by trypsinization and washed with ice-cold phosphate buffered saline (PBS). Then, cells were resuspended in 100 μL 1X binding buffer and transferred to a 5 mL falcon round-bottom tube. 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI) were added and gently vortexed the cells and incubated for 15 min at room temperature in dark condition. After incubation, 400 μL of 1X binding buffer were added to each tube and analyzed by flow cytometry within 1 h. Cells treated with 50 $\mu\text{g}/\text{mL}$ CHX and 0.3%-0.6% DMSO were used as a positive and a negative control, respectively.

Detection of mRNA and protein expression in Androg-treated SiHa cells by quantitative real time polymerase chain reaction (qRT-PCR) and western blot

The mRNA expression of HPV16 E6/E7, Bak, c-fos, c-Jun and HDAC6 was detected by qRT-PCR. Total RNA was extracted from androg-treated SiHa cells using TRIzol® reagent (Invitrogen, USA) and quantified using the NanoDrop™ 2000/2000c spectrophotometer (Thermo-Fisher, USA). Approximately 1 μg of extracted RNA was reverse-transcribed to complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit® (Thermo-Fisher, USA) following the manufacturer's instructions. Before reverse transcription, all RNA samples were treated with DNase I (Promega, Wisconsin, USA) to remove any contaminating genomic/viral DNA and tested for each target using PCR. The cDNA was stored at -20°C for the next experiments.

Quantification of HPV16 E6/E7, Bak, c-fos, c-Jun and HDAC6 mRNA was done using a SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in a 10 μL final volume, according to the manufacturer's instructions. All samples were analyzed in duplicate using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA). The untreated and DMSO-treated cells were used as a negative control, 50 $\mu\text{g}/\text{mL}$ cycloheximide (CHX)-treated SiHa was used as a positive control and *GAPDH* gene was used as an internal control. The oligonucleotide sequences of specific primers and the optimized conditions were shown in Table 1

Table 1 Nucleotide sequences of specific primers and their qRT-PCR conditions.

Targets	Sequences (5'-3')	Annealing	Product size
GAPDH	F: TCATCAGCAATGCCTCCTGCA R: TGGGTGGCAGTGATGGCA	60°C	117 bp
E6	F: GTTACTGCG ACGTGAGGTATATG R: CATTATCACATACAGCATATGGATTC	54°C	90 bp
E7	F: CCGGAAAGAGCCCATTACAA R: GCTTTGTACGCACAACCGAA	55°C	75 bp
Bak	F: GGGTCTATGTTCCCCAGGAT R: GCAGGGGTAGAGTTGAGCA	65°C	158 bp
c-Fos	F: AGAATCCGAAGGGAAAGGAA R: CTTCTCCTTCAGCAGGTTGG	65°C	150 bp
c-Jun	F: CAGGTGGCACAGCTTAAACA R: GTTTGCAACTGCTGCGTTAG	65°C	80 bp
HDAC6	F: TGGCTATTGCATGTTCAACCA R: GTCGAAGGTGAACTGTGTTTCCT	65°C	127 bp

The expression of apoptosis-related protein p53 affected by androg was selected to confirm by western blot. The expression of p53 was measured in terms of protein levels using western blot assay. Proteins were extracted from the androg-treated SiHa cells using TRIzol® reagent (Invitrogen, USA) as recommended by the manufacturer. The total protein concentration of cell lysate was determined by the Bradford protein assay using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA). The pattern of each protein in samples was analyzed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Anti-p53 polyclonal antibody (Santa Cruz, USA) was used for p53 target.

Statistical analysis

All experiments were repeated at least twice. Statistical significance for each experiment was determined by Student's t-test or one-way analysis of variance (ANOVA) as appropriate with an alpha of 5%. Calculations were performed and graphs produced using Prism 5.0 software for Windows (GraphPad Software, San Diego, USA). Graphs of results show the mean and error bars depict the standard deviation (\pm SD).

Results

Determination of subtoxicity concentration (Mascolo et al.) and CC_{50} of androg by cell viability assay

To determine the subtoxic (Mascolo et al.) and 50% cytotoxic (CC_{50}) concentrations of androg in cervical cancer cell lines (C33A and SiHa), MTT assay was performed. The CC_{50} and SC of androg in each cell line were showed in Fig 2 and Table 2. Therefore, these concentrations were used for further experiments.

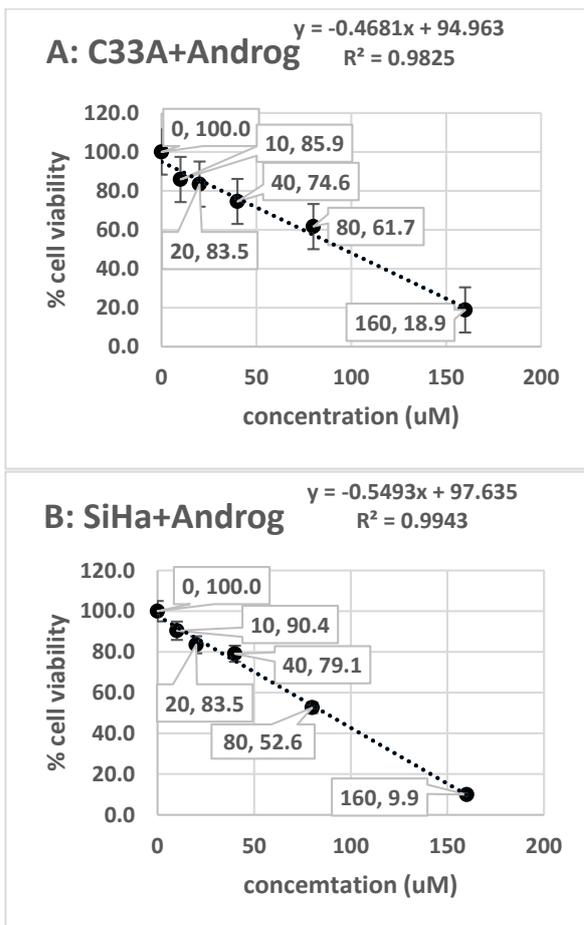


Figure 2 CC_{50} and subtoxic concentration (Mascolo et al.) of Androg in C33A (A) and SiHa (B) cell lines. Cells were treated with androg for 72 h at various concentrations (0-200 $\mu\text{g/mL}$) and cell viability was determined by MTT assay.

Table 2 The CC_{50} and subtoxic concentration (Mascolo et al.) of androg in C33A and SiHa cell lines.

	C33A		SiHa	
	CC_{50} (μM)	SC (μM)	CC_{50} (μM)	SC (μM)
Androg	96.05	21.28	85.59	21.44

Androg induced apoptosis of HPV-positive cells (SiHa) but not HPV-negative cells (C33A)

To investigate effects of androg to induce apoptosis in cervical cancer cell lines (C33A and SiHa), EB/AO staining and flow cytometry were performed. EB/AO staining showed that androg induced apoptosis of SiHa cell which is HPV-positive cell line when compared with DMSO-treated and untreated cells (Fig 3). This effect was confirmed by flow cytometry. The result showed that androg induced cell death in SiHa cells more than C33A cells when compared with DMSO-treated and untreated cells (Fig. 4). This result demonstrated that androg significantly induced apoptosis of HPV-positive cell line (SiHa) but low effect on HPV-negative cell line (C33A).

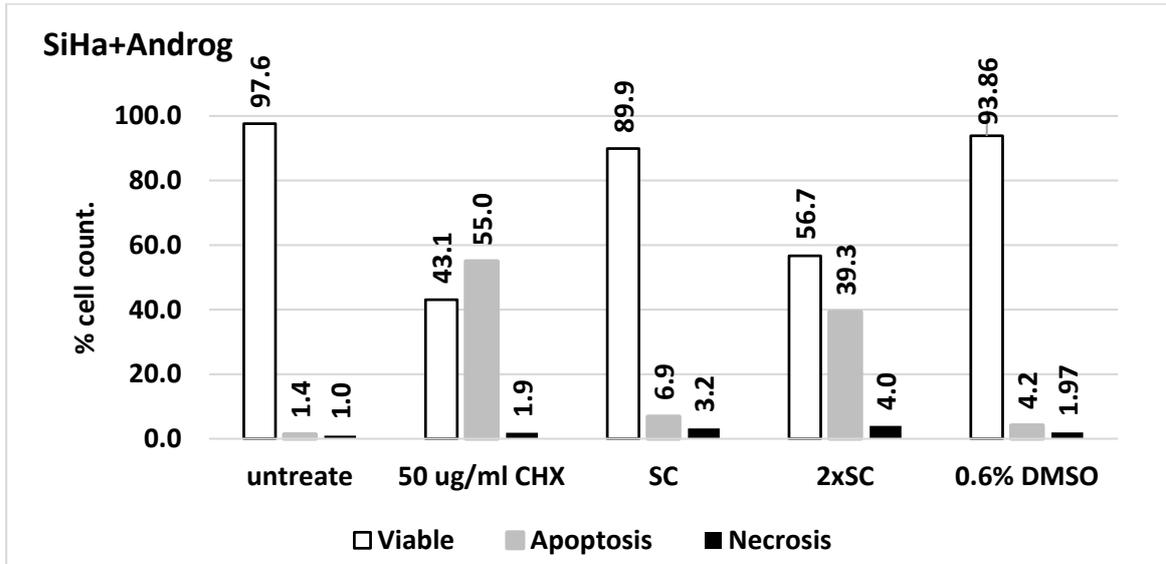


Figure 3 Effect of androg on apoptosis of SiHa cells. SiHa cells were treated with various concentrations of androg, incubated for 48 h and stained by EB/AO. CHX: Cyclohexamide, SC: sub-toxic concentration and 2xSC: 2-fold subtoxic concentration.

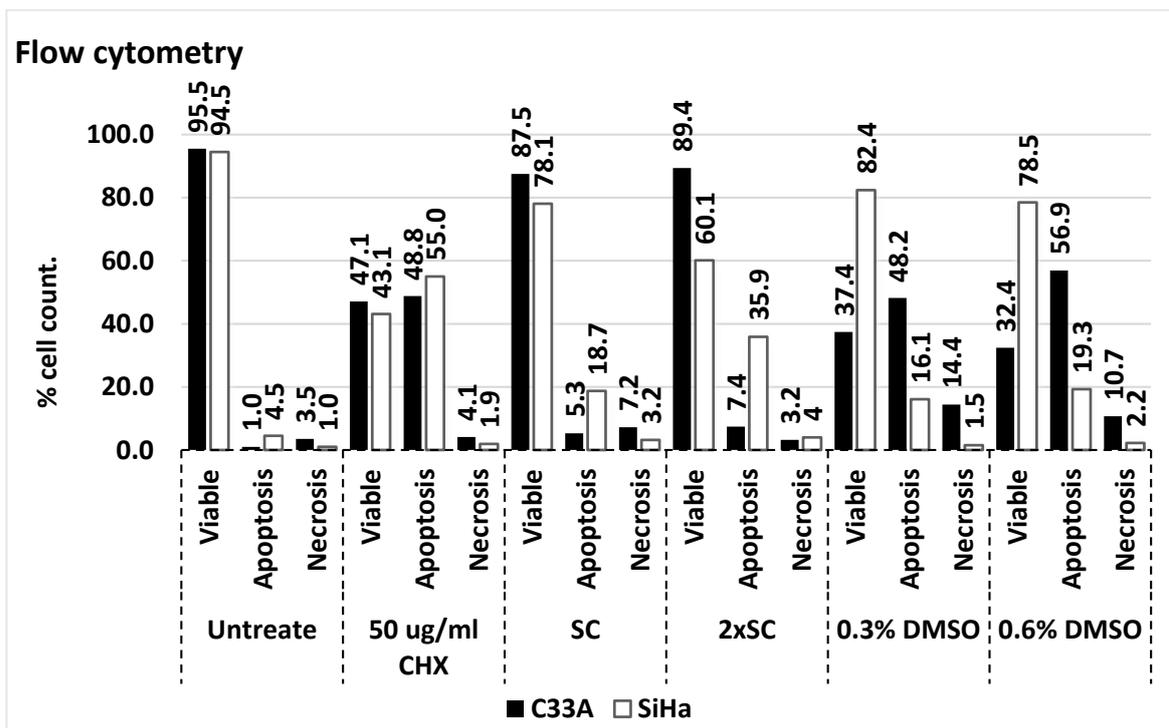


Figure 4 Effect of androg on apoptosis of C33A and SiHa cells. C33A and SiHa cells were treated with various concentrations of androg, incubated for 48 h and investigated by flow cytometry. CHX: Cyclohexamide, SC: subtoxic concentration and 2xSC: 2-fold subtoxic concentration.

Androg suppressed mRNA expression of HPV16 E6 and E7 in SiHa cells.

To investigate the effect of androg on HPV16 E6 and/or E7 mRNA, SiHa cells were treated with androg at SC and 2xSC. After 48 h of treatment, the mRNA level of HPV16 E6 was significantly decreased in 2xSC concentration of androg when compared to control group (Fig. 5A). The expression level of HPV16 E7 was also downregulated when SiHa cells were treated with 2xSC of androg (Fig. 6B).

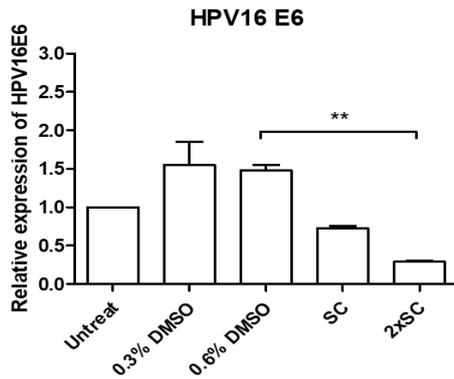
Androg affected HPV16 E6/E7 promotor activity in SiHa cells.

Since several transcription factors including AP-1 (c-fos and c-Jun) were associated with an activation of HPV 16 E6/E7 promotor. Therefore, we hypothesized that androg can suppress the expression of AP-1 lead to HPV E6 and E7 downregulation in SiHa cells. To test our assumptions with respect to the androg-suppressed AP-1, the level of c-fos and c-Jun were estimated by qRT-PCR. As demonstrated in Fig. 5C and 5D, the mRNA level of both c-fos and c-Jun were significantly decreased in 2xSC of androg. However, the expression level of c-Jun was similar to the control in SC of Androg (Fig. 5C and 5D). Our results indicated the androg effects of on expression of c-fos that might influence to transcriptional activity of HPV E6 and E7 promoter.

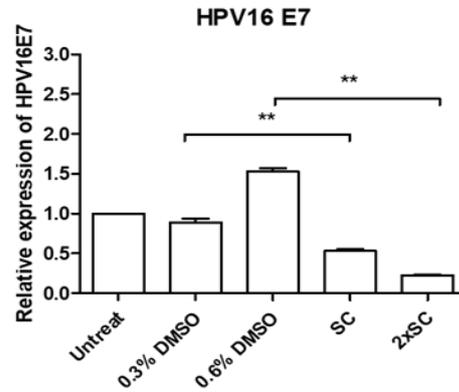
Androg-dependent HPV E6 and E7 suppression potentiate apoptosis-related proteins and p53 in SiHa cells

To investigated the effects of androg to induce apoptosis via reduction of HPV16 E6/E7 mRNA expression, p53 protein level in androg-treated SiHa cells was detected by using western blotting assay. The result showed that p53 level was significantly increased in 2xSC of androg-treated SiHa cells in contrast to 50 µg/mL CHX which is a positive control and untreated cells (Fig. 6). To support the effect of androg to induce apoptosis via reduction of HPV16 E6/E7 mRNA expression and modulating p53 expression, Bak which is pro- apoptotic protein and HDAC6 which is anti- apoptotic protein and downstream target of p53 protein, mRNA expression in androg-treated SiHa cells were detected by using qRT-PCR. The result showed that the mRNA level of Bak was gradually increased (Fig. 5E) but HDAC6 was decreased (Fig. 5F) in androg-treated SiHa cells when compared with untreated and DMSO-treated cells.

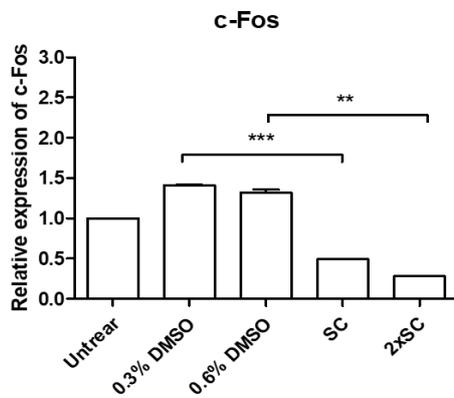
A: HPV16 E6



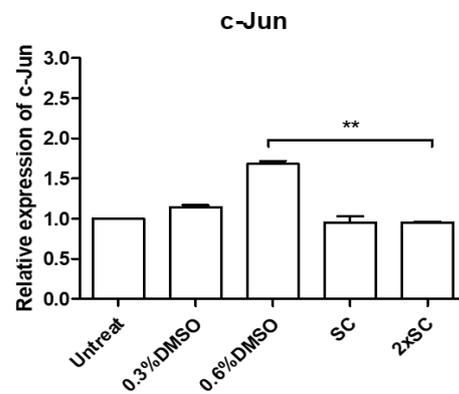
B: HPV16 E7



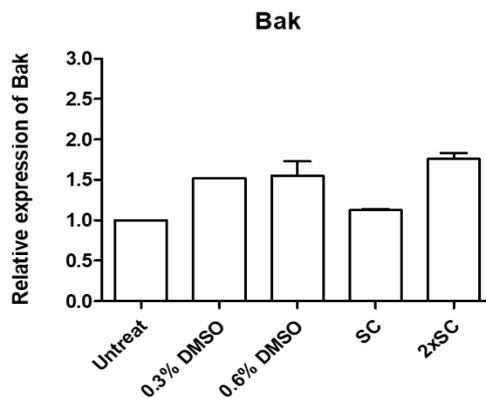
C: c-Fos



D: c-Jun



E: Bak



F: HDAC6

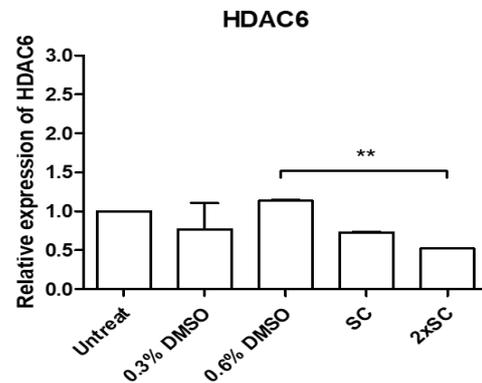


Figure 5 The effect of androg on HPV16 E6/E7, apoptotic related protein: Bak, transcription factor (Ap-1): c-fos and c-Jun and histone deacetylase 6 (HDAC6) mRNA expression in SiHa cells. Cells were treated with androg at 2xSC and SC for 48 h. HPV16 E6 (A), HPV 16 E7 (B), c-fos (C), c-Jun (D), Bak (E) and HDAC6 (F) mRNA expression were determined by qRT-PCR. The mean and error bars represent \pm SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

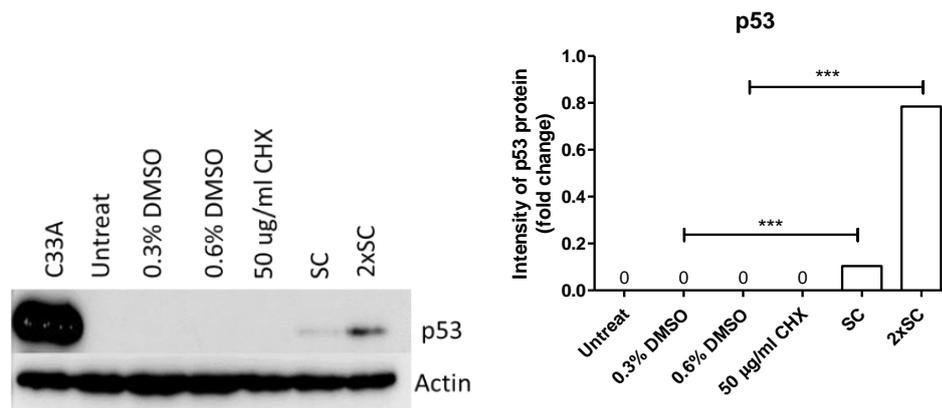


Figure 6 The effect of androg on p53 level in SiHa cells. Cells were treated with androg at SC and 2xSC for 48 h and p53 protein level was determined by western blot. CHX: Cyclohexamide, SC: subtoxic concentration and 2xSC: 2-fold subtoxic concentration. The mean and error bars represent \pm SD. *** $P < 0.001$.

Discussion and Conclusions

The result of EB/AO staining and flow cytometry showed that androg induced apoptosis of HPV-positive cervical cancer cells (SiHa). We investigated HPV16 E6/E7 mRNA expression in androg-treated SiHa cells by qRT-PCR and the result showed that the HPV16 E6/E7 mRNA expression were decreased when compared with DMSO-treated cells and untreated cells. The HPV16 E6/E7 expressions in DMSO-treated cells were not statistically significantly difference from untreated cells. This result demonstrated that androg induced cervical cancer cell apoptosis via reduction of HPV16 E6/E7 oncoprotein.

In our previous study, we found that androg had an activity to suppress transcriptional activity of early oncogene promoter of HPV16. Host transcription factor such as active protein-1 (AP-1) including c-fos and c-Jun which associates with HPV16 E6/E7 promoter activation consequently. The c-fos and c-Jun mRNA expressions were investigated in androg-treated SiHa cells. The result showed that c-fos mRNA level was clearly decreased while c-Jun mRNA level was graphically decreased particularly at 2xSC concentration. In control apoptotic cells (the CHX-treated cells), c-fos and c-Jun was downregulated and upregulated, respectively. This result suggested the effect of androg on HPV promoter transcriptional activity. However, AP-1 transcription factor will be activated by homo- and/ or heterodimer formation of Fos and Jun family proteins especially c-fos and c-Jun that have been reported in many cancers. We suggested that androg may affect other host transcription factors that are associated with HPV 16 E6/E7 promoter activity, so protein expression pattern in androg-treated HPV-positive cervical cancer cells should be further investigated

Apoptosis-related protein p53 which is a target of HPV16 E6 oncoprotein and its downstream targets (Bak and HDAC6) were investigated in androg-treated SiHa cells. The result showed that pro-apoptotic protein as Bak mRNA level was gradually increased but statistically not significant. HDAC6 which is anti-apoptosis and downstream target of p53 protein was significantly decreased at 2xSC while Bak was slightly increased but not significantly difference in DMSO-treated and untreated cells. The p53 level was clearly increased at 2xSC in androg-treated SiHa cells when compared with the CHX-treated

and untreated cells. This result confirmed our previous study that androg induced p53 protein restoration (Ekalaksananan et al., 2015). The result also showed that androg increased accumulation of Bax protein which is directly downstream target of p53. This data demonstrated that androg could induce p53 restoration and its downstream targets.

Taken together, this study indicated that androg reduced HPV16 E6/E7 oncogenes expression via decreasing of host transcription factor (c-fos) that associated with HPV16 E6/E7 promotor activity leading to apoptosis

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