Anti-invasion activity of *trans*-4-methoxy-cinnamaldehyde (4-MCA) in cervical cancer cells

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Abstract

Cervical cancer is still the common cause of cancer death in women worldwide. Human papillomavirus (HPV), particularly HPV16, is the most important etiological agent for this cancer. In Asia including Thailand, high incidence of infection by HPV16E6D25E/E7N29S variant or Asian variant having amino acid variations at residue 25 of E6 and residue 29 of E7 has been reported. Even though screening of cervical cancer and HPV detection could be successfully performed, scientists are still searching for new anticancer agents with anti-invasion ability for use with metastatic and recurrent patients. 4 methoxycinnamaldehyde (4-MCA) purified from the rhizome of *E. pavieana* (Zingiberaceae) has recently been shown to exhibit promising anti-tumor activity on several cancer cells. In this study, we aimed to investigate the cytotoxic effect and anti-invasion property of 4-MCA in C-33A human cervical cancer cells stably expressing HPV16 Asian variant oncoproteins. Results from MTT assay revealed dose-dependent effects of 4-MCA on these C-33A cells. This activity, as shown by Flow cytometry, was mediated by induction of apoptosis. In addition, at a non-toxic concentration, 4-MCA significantly inhibited invasion ability of C-33A cells. A marked decrease of MMP14, but not MMP9, expression as assessed by real time PCR suggested that 4-MCA partly inhibited cell invasion property of C-33A cervical cancer cells expressing HPV16 oncoproteins through down-regulation of MMP14 gene.

Introduction

Cervical cancer is still the leading cause of death in women in developing countries. In Thailand, an estimated 8,000 cervical cancer cases and 4,500 deaths was reported in 2017[1]. Infection with high-risk human papillomaviruses (HPV) is widely accepted to be crucial for cancer development. HPV oncoproteins E6 and E7 are the two viral proteins produced after integration of viral genome into host chromosome and are shown to be responsible for cancer initiation via the inactivation of key host tumor suppressor proteins, p53 and pRb[2]. In addition, E6E7 oncoproteins could also increase invasion ability of cervical cancer cells by upregulating MMP2, MMP9 and MMP14[2-4]. Among approximately 200 HPV types, HPV16 is the most prevalent type detected in cervical cancers worldwide[5]. Interestingly, HPV16 E6 isolated from different geographical areas showed different amino acid variations with a variation at residue 25 changing from aspartic acid to glutamic acid (D25E) being the most common type found in Asia including Thailand[6-10]. This amino acid variation seemed to be associated with oncogenic potentials[11]. Likewise, some E7 variations have been isolated and the most common variant in E7 of HPV16 identified in clinical samples from Europe, Africa and Asia was N29S changing from asparagine to serine at residue 29[12]. These results suggested that HPV16

E6D25E/E7N29S or HPV16 Asian variant might possess different impact on infected cells as compared to prototype oncogenes.

Recently, the 4-methoxycinnamaldehyde or 4-MCA a bioactive compound isolated from the rhizome of *E. pavieana* (Zingiberaceae)[13], was shown to have cytotoxic effects on several cancer cells[14, 15]. It also showed cytoprotective activity against human respiratory syncytial virus or RSV in human larynx epidermoid carcinoma cells, HEp-2[16]. In this study, we examined anticancer and anti-invasion activities of 4-MCA on cervical cancer cells expressing HPV16 Asian variant oncogenes. The molecular targets of 4-MCA contributing to anti-invasion activity were also investigated.

Methodology

Cell lines and cell culture

HaCaT (human immortalized keratinocyte) and C-33A (HPV-negative human cervical cancer) were used in this study. C-33A cells were transfected with pcDNA3 expression plasmid containing HPV16 E6D25E/E7N29S oncogenes and pcDNA3 plasmid control. Cells stably expressing Asian variant oncogene and vector control were selected by 1 mg/ml G418 (Geneticin). All cell lines were cultured in DMEM supplemented with 10% (V/V) heat inactivated FBS, 100 U/ml penicillin G, 100 U/ml streptomycin and 20-25 mM HEPES. Cells were incubated at 37ºC under humidified atmosphere in 5% CO_2 incubator.

Cell viability assay by MTT

Cells (25,000 cells/ml) were plated into 96-well plate and incubated at 37ºC overnight. The culture medium was replaced with fresh medium containing with various concentrations of 4-MCA (25, 50, 100, 200 µM) and cells were incubated for another 48 hr. To determine cell viability, the medium was discarded and 100 µl of 0.5 mg/ml MTT serum-free DMEM were added into each well. After 3 hr of incubation, the medium was gently discarded and 200 µl of DMSO were added, then the absorbance 540 nm was measured by a microplate reader. 4-MCA was purchased from Sigma-Aldrich.

Annexin V apoptosis assay

Cells were plated into 6-well plate and incubated overnight. The fresh medium containing IC_{50} concentration of 4-MCA was added and cells were incubated under the same condition for 24 hr. After that cells were gently trypsinized and stained with Annexin V FITC and PI for 5 min. The stained cells were monitored for fluorescence signal using BD FACS Canto Flow Cytometer.

Matrigel invasion assay

In vitro cell invasion assay was performed in a 24 well Transwell chamber with a 8.0 µM pore polycarbonate membrane. Twenty ng of Matrigel diluted in 100 µl fresh DMEM was added into the upper chamber. Matrigel and incubated overnight. After Matrigel polymerization, the medium was discarded and the fresh DMEM was added into upper chamber and incubated for 15 min. Cells were suspended in serum-free DMEM containing IC_{50} concentration of 4-MCA and added into the upper chamber. DMEM supplemented with 10%FBS was added into the lower chamber. After 24 hr of incubation, the membrane of transwell was fixed with 4% paraformaldehyde for 45 min. then membrane was stained with 0.5% crystal violet overnight. After washing with distilled water, numbers of invading cells on stained membranes were counted under the microscope.

Determination of gene expression by real-time PCR

Cells were plated into 6-wells plate and incubated overnight. The old media was replaced with the fresh medium containing various concentrations ($^1/_{10}$, $^1/_4$, $^1/_2$ IC_{50}) of 4-MCA diluted in DMEM. Cells were incubated under the same condition for 24 hr then RNA was extracted using Trizol reagent RNA extraction assay. The RNA was converted into cDNA using reverse transcriptase. Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix. Fold increase of gene expression was normalized to GAPDH and calculated using $2^{\wedge-\Delta\Delta Ct}$ method. Specific primers were designed, including MMP9 (Fw: 5'-TGCGCTACC ACCTCGAACTT-3', Rw: 5'-GATGCCATTGACGTCGTCCT-3'), MMP14 (Fw: 5'-CCTGCCTGCGTCCATCA-3', Rw: 5'-TCCAGGGACGCCTCATCA-3'), GAPDH (Fw: 5'-CCATGGAGAAGGCTGGGG-3', Rw: 5'-CAAAGTTGTCATGGATGACC-3') and were used to detect gene expression. Thermal profile used in this experiment is 95°C 30 sec, 60°C 30 sec, and 72°C 30 sec for 40 cycles.

Results and Discussion

4-MCA was toxic to C-33A cells expressing HPV16 E6E7 Asian variant oncogenes.

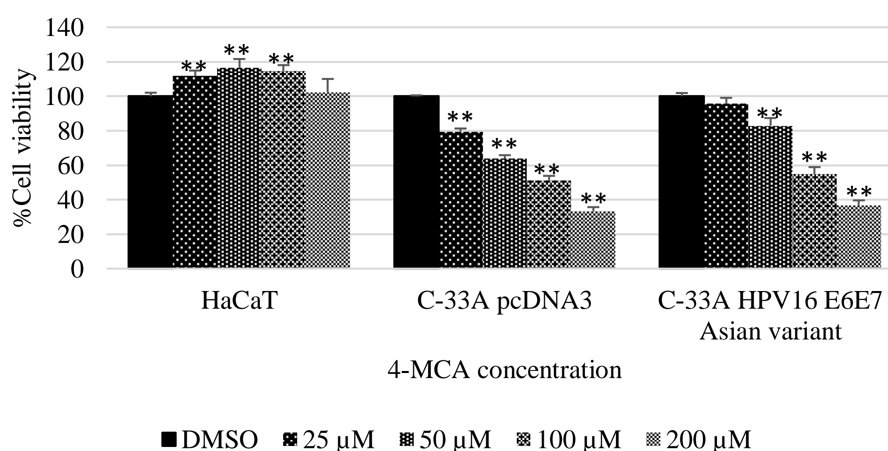

Figure 1. Effects of 4-MCA on cell viability as determined by MTT assay. Cells were treated with various concentrations of 4-MCA (25, 50, 100, 200 µM) for 48 hr. Numbers of cells were counted in comparison with those treated with 0.1% DMSO which was set to 100%. Data are presented as mean ± S.E. of three independent experiments. The significant difference was accepted at $p < 0.01$ (** when compared between 4-MCA and DMSO treated cells).

At 48 hr., 4-MCA significantly reduced viability of C-33A cells, both with and without HPV16E6E7 Asian variant oncogenes, in dose-dependent manners. At low concentration, 4-MCA showed a slight proliferation effect only on HaCaT cells (Figure 1). According to their IC_{50} values, C-33A cells were more sensitive to 4-MCA when compared to HaCaT. C-33A containing HPV16E6E7 Asian variant were similarly sensitive to 4-MCA as compared to C-33A pcDNA3. The IC_{50} values of cisplatin, which was used as a positive control, were also not significantly different in all cell lines (Table 1). Unlike cisplatin, 4-MCA was likely to be selectively toxic to cancer cells.

Our results were similar to Panata's study which showed that 4-MCA was 4 times more toxic to C-33A and MDA-MB-231 as compared to Vero cells [15].

Table 1. IC_{50} values of 4-MCA compared to Cisplatin on cell lines at 48 hr.

Cell line	IC_{50} (mean ± SD, µM)	
	4-MCA	**Cisplatin**
HaCaT	>200	2.0 ± 1.3
C-33A pcDNA3	79.2 ± 15.9	7.0 ± 2.1
C-33A HPV16 E6E7 Asian variant	111.5 ± 11.5	7.0 ± 1

4-MCA induced cell death through apoptosis pathway

C-33A cells expressing HPV16E6E7 Asian variant were exposed to 110 µM (IC_{50}) of 4-MCA for 24 hr. After staining with Annexin V-FITC and PI for cell death observation, the results from flow cytometry showed that 4-MCA treatment significantly increased numbers of dead cells in both early and late state apoptosis quadrants (19%) when compared to DMSO treatment (4.2%) (Figure 2). This indicated that 4-MCA could induce cell death through apoptosis process in C-33A cells.

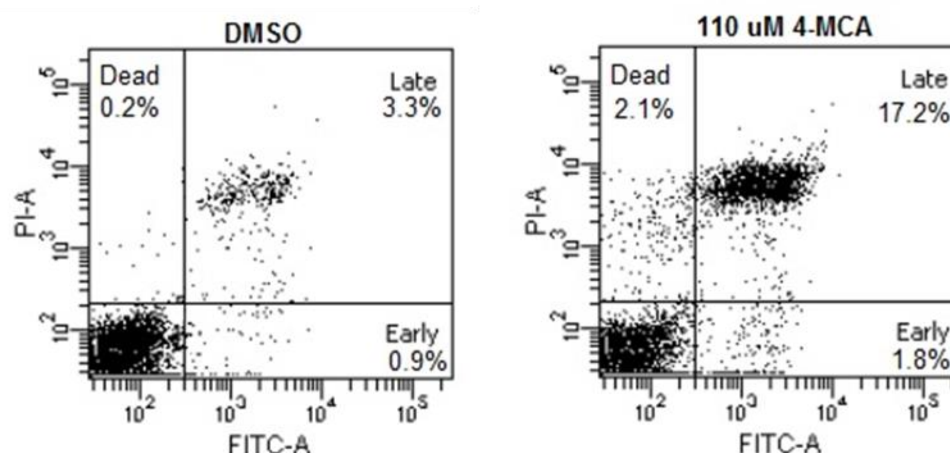

Figure 2. Effect of 4-MCA on apoptosis induction in C-33A expressing HPV16 E6E7 Asian variant oncogenes. After 24 hr incubation in DMSO or 4-MCA (at IC_{50} concentration, 110 uM), cells were stained with Annexin V-FITC and PI and percentage of apoptotic cells (early and late quadrant represent early apoptosis and late apoptosis, respectively) were assessed by Flow cytometer. Dead quadrant represents cell death by rupture or necrosis.

4-MCA inhibited invasion of C- 33A both with and without HPV16 E6E7 Asian variant

In Matrigel invasion assay, both C-33A with pcDNA3 and C-33A with HPV16 E6E7 Asian variant were treated with ¼ IC_{50} concentration of 4-MCA or DMSO (as negative control). In DMSO treatment, the numbers of invading C-33A cells expressing HPV16 E6E7 Asian variant were higher than those with pcDNA3 indicating that viral oncoproteins promoted cell invasion. Treatment with 4-MCA significantly reduced the numbers of invading cells in both C-33A cells (Figure 3), indicating that 4-MCA inhibited invasion ability of C-33A cells.

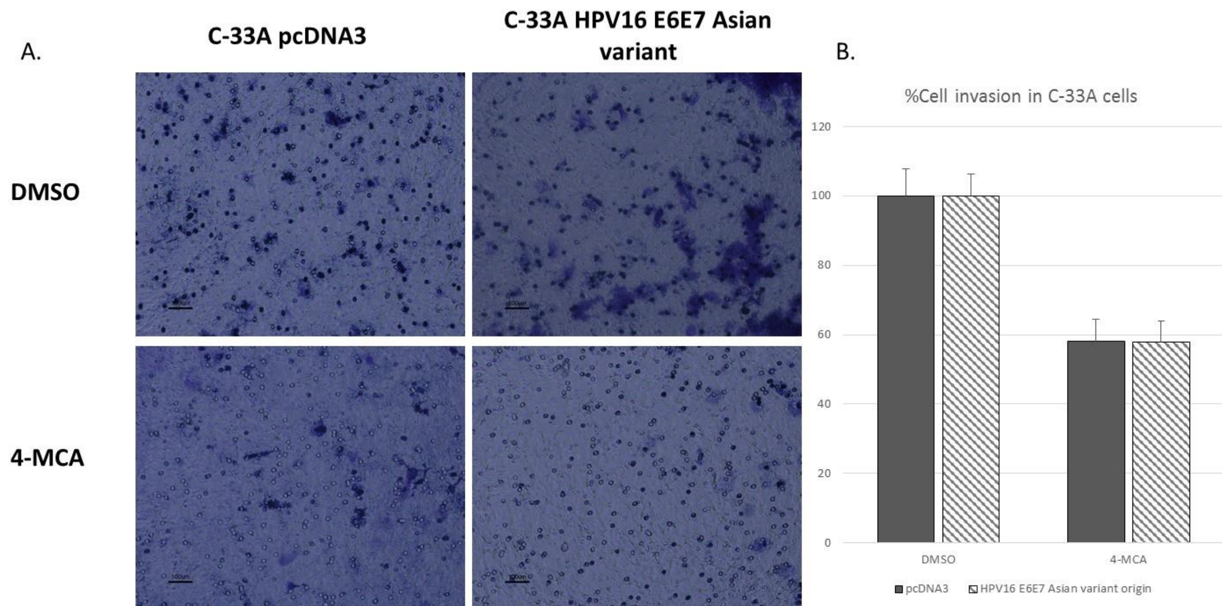

Figure 3. Effect of 4-MCA on invasion ability of C-33A cells as assessed by Matrigel invasion assay. Both C-33A with pcDNA3 and C-33A with HPV16 E6E7 Asian variant were treated with ¼ of IC_{50} concentration 4-MCA or DSMO. Representative pictures of invading cells observed under the microscope (A) and calculated percent of cell invasion (B) were shown. Our results showed that 4-MCA inhibited invasion of both C-33A cells at almost 50 percent at ¼ IC_{50} concentration,

4-MCA downregulated MMP14 transcription.

Several studies reported that MMP2, MMP9 and MMP14 were up-regulated in cervical cancer tissues and HPV positive cells[3, 4, 17]. We then hypothesized that 4-MCA might inhibit cell invasion by down-regulating expression of these MMP genes. To test this assumption, both C-33A pcDNA3 and C-33A HPV16 E6E7 Asian variant were treated with $^{1}/_{10}$, ¼, or ½ IC_{50} concentrations of 4-MCA for 24 hr. Cells were harvested and RNA was extracted and quantified by real-time PCR. Expression of MMP9 and MMP14 genes in 4-MCA treated cells was compared to DMSO treatment control (Figure 4).

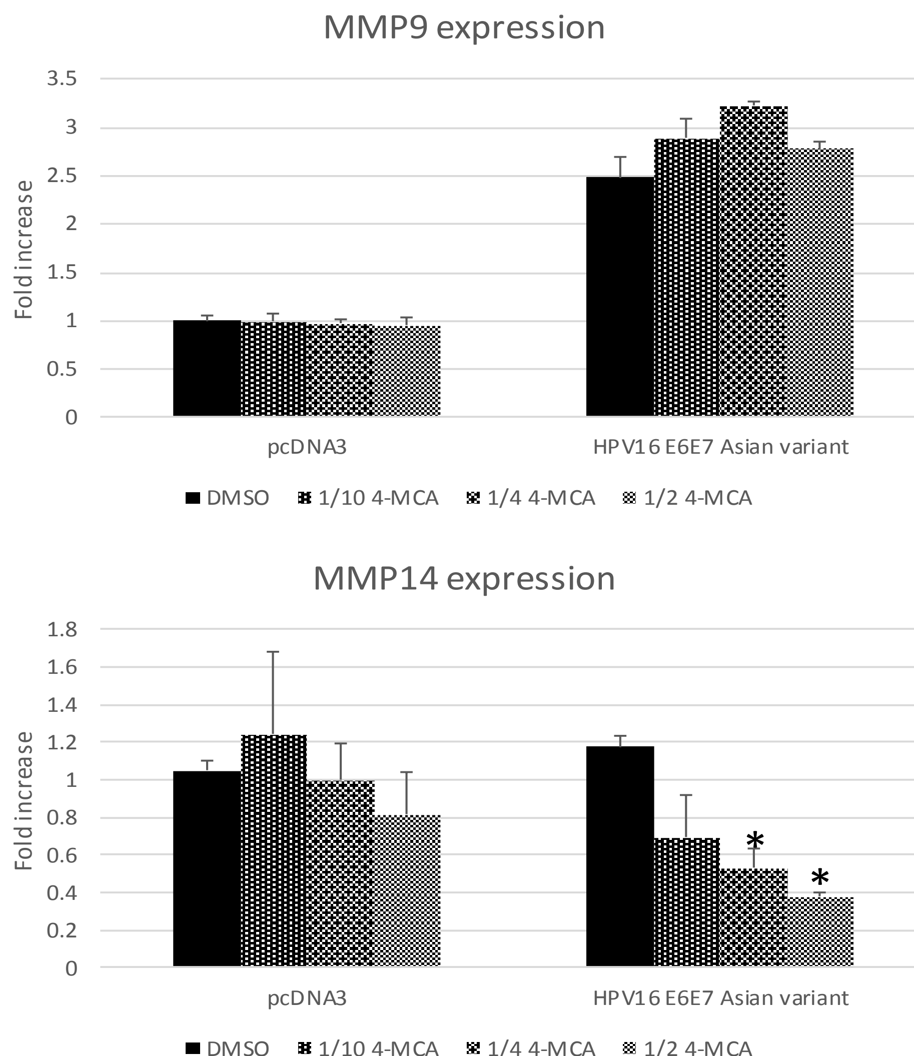

Figure 4. Effect of 4-MCA on MMP expression in C-33A pcDNA3 and C-33A HPV16 E6E7 Asian variant. Cells were treated with $^{1}/_{10}$, ¼ and ½ IC_{50} concentrations of 4-MCA for 24 hr. Gene expression was determined by real-time PCR using GAPDH as an internal control. Data are present as mean ± S.E. of two independent experiments.

Fold increase of gene expression after normalization to GAPDH was determined as compared to C-33A pcDNA3 treated with solvent DMSO control which was set as 1. Results have shown that HPV16 Asian variant expressing cells showed a significant increase of MMP9 expression when compared to DMSO control cells. However, increasing concentrations of 4-MCA had no effect on MMP9 expression in both cells. On the contrary, there was a dose-dependent effect of 4-MCA on MMP14 gene expression in C-33A HPV16 Asian variant cells as lower levels of MMP14 transcripts were detected at higher concentrations of 4-MCA. No clear effect of 4-MCA on MMP14 gene expression was observed in C-33A without HPV oncoproteins. These results suggested that HPV16 Asian variant oncoproteins increased invasion ability of cervical cancer cells by up-regulating MMP9 and with lower extent MMP14 expression and this activity could be inhibited by 4-MCA. We proposed that the invasion inhibitory activity of 4-MCA was partly due to inhibition of MMP14 transcription. The molecular mechanism underlying specific action of 4-MCA on MMP14 transcription awaits further investigation.

Conclusion

In this study, we have shown that 4-MCA exhibited similar toxicity to C-33A cervical cancer cells expressing HPV16E6E7 Asian variant oncogenes and those without oncogenes. However, it showed no toxicity to immortalized keratinocyte HaCaT cells. The cytotoxic effects of 4-MCA on cells with HPV16 Asian variant oncogenes was shown to be mediated by activation of apoptosis. Moreover, 4-MCA could significantly inhibit invasion ability of C-33A cells, both with and without HPV16 Asian variant oncogenes. Its anti-invasion activity was shown to be partly involve suppression of MMP14, but not of MMP9, expression. This study is the first report on anti-invasion activity of 4-MCA in HPV16 oncogene expressing C-33A cells.

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