(E)-2-Methoxy-4-(3-(4-Methoxyphenyl)Prop-1-en-1-yl)Phenol Induces Apoptosis in HeLa Cervical Cancer Cells via the Extrinsic Apoptotic Pathway

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Introduction

Cervical cancer is the fourth most common cancer affecting women and a common cause of cancer death worldwide [1]. There are many risk factors for cervical cancer; however, the most common cause is infection with human papillomavirus (HPV), especially the high-risk HPV types 16 and 18 [2]. The HPV oncproteins E6 and E7 bind to the tumor suppressor proteins p53 and RB, respectively, and interfere with their functioning [3]. Accordingly, various chemicals have been examined for their ability to target E6 and E7 oncoproteins for potential applications in anticancer therapy [4].

Apoptosis is a process of programmed cell death that plays an important role not only in cancer progression but also in embryonic development and normal cell turnover [5]. There are two major apoptotic signaling pathways: (i) the intrinsic pathway, mediated by mitochondrial factors such as the BCL-2 family of proteins, and (ii) the extrinsic pathway, mediated by death receptors [6]. Caspases are essential factors involved in both the intrinsic and extrinsic apoptotic pathways, and function as central regulators of apoptosis [7].

(E)-2-Methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol (MMPP), derived from butenal, is a recently synthesized Maillard reaction product. Owing to its novelty, little is known about the function of MMPP. In this study, we elucidated the effects of MMPP on apoptosis in cervical cancer by using the HeLa cervical cancer cell line, which is widely used in cancer research. We observed that MMPP was cytotoxic to HeLa cells and induced activation of caspase-3, -8, and -9, without affecting the expression of the viral oncopgenes E6 and E7. In particular, the expression of the death receptors DR5 and FAS was significantly increased by MMPP treatment. There were no significant alterations of mitochondrial intrinsic factors. Taking all these results together, our findings show that MMPP primarily induces apoptosis in HeLa cervical cancer cells via the extrinsic apoptotic signaling pathway, accompanied by an enhanced expression of death receptors.

Keywords: MMPP, (E)-2-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol, apoptosis, cervical cancer, HeLa
similar role, because it is also derived from butenal and has higher chemical stability than BHPB.

In this study, we investigated the anticancer effects of MMPP in HeLa, which is a well-known cervical cancer cell line that harbors HPV 18. We observed that MMPP did not directly suppress the expression of E6 and E7 oncogenes, but decreased the viability of HeLa cells and induced morphological changes and nuclear fragmentation. Moreover, annexin/propidium iodide (PI) staining revealed that MMPP induced early- and late-stage apoptosis in HeLa cells. Finally, both protein and mRNA levels of apoptosis-related factors such as caspases and death receptors were modulated by MMPP treatment. Overall, our findings elaborate the anticancer effects of MMPP and imply that butenal derivatives could be useful as novel strategies for cancer therapy.

Materials and Methods

Cell Culture

Human cervical cancer cell line was obtained from the American Type Culture Collection (ATCC, USA) and normal human keratinocyte cell line HaCaT was used as control cells. Cells were cultured in DMEM (Welgene, Korea) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (FBS; HyClone Laboratories, USA) and incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells used in this study were subjected to no more than 20 passages.

Cell Viability Assays

Cell viability was quantified using the MTS assay (Promega, USA). Cells (0.3 × 10⁵) were seeded into 96-well plates and cultured in 100 μl of DMEM supplemented with 10% FBS, penicillin (100 μg/ml), streptomycin (0.25 μg/ml) for overnight growth. After that, the cells were treated with various concentrations of MMPP for 48 h. Optical absorbance was measured at 492 nm with a microplate reader (Apollo LB 9110; Berthold Technologies GmbH, Germany).

Assessment of Cell Morphology

Cells (2.0 × 10⁴ cells/well) were seeded in 6-well plates and incubated overnight. After 24 h, the cells were treated with various concentrations of MMPP and incubated for a further 48 h. Cell morphology was observed by using an inverted phase-contrast microscope (Eclipse Ts100; Nikon, Japan).

Annexin-PI Staining

Approximately 1.0 × 10⁵ cells were seeded per well in 6-well plates. After incubation for 24 h, the cells treated with various concentrations of MMPP for 48 h. The cells were then washed, harvested by trypsin-EDTA treatment, collected, and stained for 15 min by using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). The proportion of apoptotic cells was determined by flow cytometry on a FACSCalibur instrument, and the data were analyzed by using CellQuest (BD PharMingen, Germany).

PI Staining

Approximately 1.0 × 10⁵ cells/well were seeded in 6-well plates and incubated for 24 h. The cells were then treated with different concentrations of MMPP and incubated for 48 h. The cells were harvested and fixed with ice-cold 70% ethanol at 4°C. After fixation, the cells were washed with PBS and stained with PI (50 μg/ml) and 100 μg/ml RNase A for 30 min. Flow cytometric analysis was performed on a FACSCalibur instrument, and the proportion of apoptotic cells was analyzed by using CellQuest.

Western Blotting

Cells were harvested and lysed in 1 ml of lysis buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 1% NP-40, 1,500 mM sodium chloride, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, and a protease inhibitor cocktail. Lysates were clarified by centrifugation at 32,120 × g for 15 min at 4°C. The protein concentrations were estimated by the Bradford assay (Bio-Rad, USA) with a UV/VIS spectrophotometer (Biorad, Biochrom, UK). Equal amounts of cell lysates were resolved on 12–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the proteins were transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked for 1 h at room temperature in Tris-buffered saline with Tween-20 (TBST; 2.7 M NaCl, 53.65 mM KCl, 1 M Tris-HCl (pH 7.4), 0.1% Tween-20) supplemented with 5% nonfat dry milk. The membranes were incubated overnight at 4°C with primary antibodies specific to each target protein. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies for 1 h at room temperature. After three washes with TBST, the protein bands were visualized by using the WEST-ZOL (plus) Western Blot Detection System (iNIRN Biotechnology, Korea). The following primary antibodies were used: caspase-3, #9662s; caspase-8, #9764s; caspase-9, #9052s; PARP, #9542s; BCL-2, #2876s; BCL-XL, #2762s; BID, #2002s; BAX, #2772s (Cell Signaling, USA); cyclin D1, #sc-25765; cyclin A, #sc-751; cyclin E, #sc-481; and GAPDH, #sc-25778 (Santa Cruz Biotechnology, USA).

RNA Extraction and mRNA Expression Analysis

HeLa cells (1.5 × 10⁴ cells/well) were seeded in 60 mm culture plates. The cells were treated with MMPP (7.5–30 μM) for 48 h. The treated cells were collected and lysed using an easy-BLUE Total RNA Extraction kit (iNIRN Biotechnology) according to the manufacturer’s instructions. The extracted RNA (5 μg) was used to conduct reverse transcription catalyzed by M-MuLV reverse transcriptase (New England Biolabs, USA) and oligo(dT) primers. cDNA was synthesized from the extracted RNA by RT-PCR with ProSTAR (Stratagene, USA). The following primers were used: E6
(HPV 18) F: 5’-GTGTGTATGTGTTGCCGC-3’ and R: 5’-TGATGTGTTGCCGC-TG-3’; E7 (HPV 18) F: 5’-GCCCGGAAATATCCCGG-3’ and R: 5’-GTCGGGCTGGTAAATGTTGA-3’.

Quantitative Real-Time PCR
The mRNA levels of DR5, DR6, FAS, and FASL were analyzed by real-time PCR with relative quantification by using a Rotor-Gene RG 6000 system (Corbett Research, Australia) and SensiFAST (Bioline Inc., USA) for amplification detection. The expression of target genes was normalized to that of GAPDH, which served as the reference housekeeping gene. The gene expression levels were quantified by the \( \Delta \Delta CT \) method with data from three independent trials. The following primers were used: FAS F: 5’-CGGACC CAGAATACCAAGTG-3’ and R: 5’-GCCACCCCGAATGATGCTC-3’; FASL F: 5’-GGGGATGTTTCAGCTCTTCC-3’ and R: 5’-GTGGCCTATTTGCTTCTCCA-3’; DR5 F: 5’-CACCTGGTCAACGATGCTGA-3’ and R: 5’-GCTCAACAAGTGGTCCTCAA-3’; DR6 F: 5’-TGGCTATCCGGAAAAGCTC-3’ and R: 5’-TCTGGGTAGGAGTCTGGAAT-3’.

Statistical Analysis
Statistical analysis was performed by using one-way analysis of variance with Tukey’s honest significant difference test. Three independent experiments were performed. The data are expressed as the mean ± standard deviation (SD, \( n = 3 \)). A value of \( p < 0.05 \) was considered statistically significant.

Results and Discussion

MMPP Exerts Cytotoxic Effects on HeLa Cells
To elucidate the anticancer effects of MMPP, we chose the HeLa cell line, which is the first continuous human cancer cell line [14]. First, the cancer cells were treated with MMPP in a dose- and time-dependent manner (7.5–30 \( \mu \)g/ml) for 24 and 48 h. As expected, the cell viability decreased in a dose-responsive manner. We chose 48-h treatment for the experiments because the cytotoxicity of MMPP was higher after 48 h than after 24 h. Furthermore, in HaCaT, which is a...
non-cancerous normal keratinocyte line, the compound had less toxicity than on HeLa (Fig. 1A). Furthermore, for the 48 h treatment, we varied the MMPP concentration from 7.5 to 30 \( \mu g/ml \) because this concentration is similar to the half maximal inhibitory concentration (IC\(_{50}\)) of typical cancer inhibitors. After treatment of HeLa cells under these conditions, we observed the cellular morphology and nuclear shape by microscopy. The cell number was significantly decreased in the MMPP-treated cells than in the DMSO-treated cells (Fig. 1B). Furthermore, DAPI staining revealed that nuclear fragmentation occurred in MMPP-treated cells (Fig. 1C). Nuclear fragmentation is a characteristic feature of apoptosis [15]. Thus, our data suggest that MMPP decreases the viability of HeLa cells and this probably occurs via the apoptotic mechanism.

**MMPP Induces Apoptosis in HeLa Cells**

Apoptosis is a process of cell death. Upon induction of apoptosis, phosphatidylserine, which is a component of the cell membrane, is flip-flopped from the inner to the outer leaflet of the membrane [16]. Annexin V is most commonly used for detecting apoptosis as it can bind with phosphatidylserine in the outer membrane [17]. Flow cytometry coupled with annexin/PI staining revealed an increase in the proportion of cells in the upper right quadrant with increasing concentration of MMPP, indicating an increase in apoptotic cells (Fig. 2A). Notably, MMPP treatment significantly increased the proportion of cells in the late phase of apoptosis more than DMSO treatment (Fig. 2B). The cell cycle is closely associated with cell death and proliferation. Members of the cyclin family of proteins control the cell cycle; an alteration in the levels of cyclins may result in cellular stress and eventually death [18]. Thus, we performed cell cycle analysis of HeLa cells by PI staining and observed sub-G1 arrest in MMPP-treated cells (Fig. 3). Sub-G1 arrest is an indicator of cell death, as...

**Fig. 2.** Effects of \((E)-2\text{-methoxy-4-(3-(4\text{-methoxyphenyl})prop-1-en-1-yl)phenol (MMPP)} on apoptosis in HeLa cells. (A) HeLa cells were treated with varying doses of MMPP for 48 h. Annexin/PI staining was performed to detect the proportion of cells in different stages of apoptosis. Late apoptotic cells (upper right) increased in MMPP-treated samples. The stained cells were analyzed by FACS. (B) Quantification of the proportion of cells in different stages of apoptosis, as determined by flow cytometry. Late apoptotic cells particularly increased in the final concentration of MMPP-treated sample comparing with control sample (DMSO). Data are presented as the mean ± standard deviation (n = 3). **p < 0.005 versus control cells.
reported previously [19, 20]. We performed the experiment and found sub-G1 arrest. At that moment, we guessed a decrease of cyclin D level, but there were no alterations of cyclins (Fig. S1). We also measured the levels of p53, p21, and Rb, which are cell cycle modulators and DNA damage response factors, but they were not altered (Fig. S1). Taken together, our results indicate that the cell cycle arrest is not a main event of MMPP-induced cell death in HeLa cells as the cyclin protein family was not altered when compared with the control. There are several reports that sub-G1 arrest induces cell death without mention about the cyclin protein family [21–23].

Caspase Family and Death Receptor Proteins Are Activated in HeLa Cells by MMPP Treatment

Before investigating apoptosis signaling pathways in MMPP-treated HeLa cells, we measured the levels of E6 and E7 viral oncogenes. E6 and E7 oncoproteins bind p53 and Rb, respectively, and interfere with their tumor-suppressive functions [3]. Consequently, E6 and E7 levels are closely linked to tumor survival; thus, we investigated whether MMPP could directly inhibit the expression of these oncogenes. However, our experiments revealed that the levels of these oncogenes were not altered upon MMPP treatment (Fig. 4A). Hence, we focused on the effect of MMPP on the apoptotic signaling pathways. Apoptotic pathways are broadly classified into the intrinsic and extrinsic pathways. The intrinsic pathway is closely connected with mitochondrial functions [24]. The BCL-2 protein family plays two distinct roles; the first group consisting of the proteins such as BCL-2 and BCL-XL exhibit anti-apoptotic functions, whereas the second group comprising BID, BAX, and BAD exhibits pro-apoptotic functions [25].

Death receptors and the caspase family of proteins are involved in the extrinsic apoptotic pathway [25]. Caspase-3 is a key protein in the execution of apoptotic signals. It can induce cellular events such as cell shrinkage, DNA fragmentation, and chromatin condensation [26]. To identify

![Figure 3](image)

**Fig. 3.** Cell cycle analysis of (E)-2-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol (MMPP)-treated HeLa cells by flow cytometry coupled with PI staining. Sub-G1 was particularly increased in MMPP-treated samples. (A) Distribution of cell cycle population. MMPP-treated A549 cells were stained with PI and were counted using a flow cytometer (FACS analysis). (B) Histogram of sub-G1 population.
Fig. 4. Effects of \( (E)-2 \)-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol (MMPP) on the markers of programmed cell death. (A) Expression levels of \( E6 \) and \( E7 \) as detected by PCR. Western blot analyses to determine the expression levels of markers of the (B) extrinsic apoptotic pathway, and (C) intrinsic apoptotic pathway. Data are presented as the mean ± standard deviation (\( n = 3 \)).

Fig. 5. Effect of \( (E)-2 \)-methoxy-4-(3-(4-methoxyphenyl)prop-1-en-1-yl)phenol (MMPP) on the expression of death receptors in HeLa. HeLa cells were treated with MMPP and the expression levels of the following death receptors were analyzed by real-time PCR: (A) \( DR5 \), (B) \( DR6 \), (C) \( FASL \), and (D) \( FAS \). Except for \( FASL \), the other death receptors increased in MMPP-treated samples. Data are presented as the mean ± standard deviation (\( n = 3 \)). *\( p < 0.05 \), **\( p < 0.01 \).
the factors regulated by MMPP, western blot analyses were performed to determine the levels of intrinsic and extrinsic apoptotic pathway-related factors such as the caspase family (caspase-3, -8, and -9), and PARP, a DNA repair-related factor. We noticed a decrease in the levels of precursor forms of caspase-3 and -8 and an increase in the levels of their cleaved forms (Fig. 4B). Caspase-8 is regulated via death receptors belonging to the extrinsic apoptotic pathway. The level of the precursor form of caspase-9 was also decreased, but we could not detect the cleaved form of the caspase in our experiments. The level of cleaved PARP was increased as expected. In contrast, the levels of BCL-2 and cyclin family of proteins were not significantly unaltered (Fig. 4C and Fig. S1). Our data revealed that there were no significant alterations of mitochondrial intrinsic factors (Fig. 4C). However, caspase-8 and -3 were processed and the expression levels of death receptors involved in the extrinsic pathway were enhanced (Fig. 5). Hence, this study was focused on apoptosis via the extrinsic apoptotic pathway, not cell cycle arrest. Based on these results, we speculated that MMPP mainly induced the extrinsic apoptotic pathway in HeLa cells, without exerting any direct inhibitory effects on E6/E7 oncoproteins. To confirm this hypothesis, we further measured the expression levels of death receptors by using qPCR. Death receptors are cell membrane receptors that can stimulate apoptosis upon binding to their specific ligands [27]. We found that the well-known death receptors DR5 and FAS were increased by MMPP treatment (Fig. 5A). Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28]. Several reports have indicated an association between apoptosis and increase in death receptor levels [28].

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