치의과학석사학위논문

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2019년 07월

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ABSTRACT

A study on the apoptotic effect and molecular mechanism of oridonin in mucoepidermoid carcinoma cell lines

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Oridonin, an active diterpenoid isolated from *Rabdosia rubescens*, has been reported to have anti-cancer activities on several tumors. The purpose of this study is to investigate the anti-cancer effect and molecular mechanisms of oridonin in mucoepidermoid carcinoma

(MEC). To investigate the anti-cancer effect and molecular mechanisms of oridonin, trypan blue exclusion assay, Live/Dead cell viability assay, 4′-6-diamidino-2-phenylindole (DAPI) staining, Western blotting analysis, flow cytometry, reverse transcription-polymerase chain reaction (PCR), quantitative PCR, mitochondrial membrane potential assay, construction of overexpression vector, and transient transfection were performed. Oridonin treatment induced apoptosis and the inhibited the expression of myeloid cell leukemia-1 (Mcl-1) protein through a post-translational regulation in MC-3 and YD-15 cell lines. Oridonin significantly increased the expression level of truncated Bid (t-Bid) as a downstream target of Mcl-1 and subsequently decreased the mitochondria membrane potential. Ectopic expression of Mcl-1 protein was sufficient to reverse the apoptosis and t-Bid expression by oridonin in both cell lines. Taken together, these results suggest that oridonin has an apoptotic effect through the

modulation of Mcl-1 and t-Bid in human MEC cell lines and may be a potential anti-cancer drug candidate for the treatment of human MEC.

Keywords: Mucoepidermoid carcinoma, Oridonin, Apoptosis, Myeloid cell leukemia-1, truncated-Bid

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1. Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignant tumor of salivary gland, and is well known for having a considerable cellular heterogeneity, including epidermoid, intermediate and mucin producing cells [1, 2]. Patients at high grade and advanced stages are reported to be associated with poor survival rates [3]. Although radiotherapy and chemotherapy with surgery are the main treatment modalities in MEC[4, 5], their various side effects have been reported [6]. To overcome these limitations, it is important to continue to identify new tumor-specific molecular target and explore new, efficient and less toxic drug candidates for MEC treatment.

Bcl-2 family member is highly conserved across the species and is known to be a promising target for chemotherapy [7]. Myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic member of the Bcl-2 family, has

been shown to play an anti-apoptotic role for cell survival [8, 9]. Mcl-1 is also overexpressed and is associated with poor outcomes of various malignant tumors including hepatocellular carcinoma [10], breast cancer [11], and esophageal squamous cell carcinoma [12]. It sequesters pro-apoptotic members of Bcl-2 family such as Bax, Bak, Bim, and t-Bid through its direct binding to them, followed by blocking their oligomerization for the formation of protein-permeable pores on the mitochondrial outer membrane [13, 14]. Finally, Mcl-1 allows the release of cytochrome c into cytoplasm, leading to the activation of the caspase cascade and ultimately induction of apoptosis [15, 16]. It is therefore important to consider Mcl-1 as a chemotherapeutic target for a variety of cancers.

Oridonin is a diterpenoid extracted from the medicinal herb *Rabdosia rubescens*, which has attracted much research interest because it exhibits anti-cancer effects in various cancer cells [17, 18].

The anti-cancer mechanisms of oridonin include the Fas/FasL-mediated extrinsic apoptotic pathway, PI3K/Akt or MAPK signaling pathway-related intrinsic apoptotic pathway [19-22]. In addition, recent studies have shown that oridonin has mitochondria-mediated apoptotic effects on a variety of cancer cells through Bcl-2 family [23, 24]. However, the precise effects of oridonin on MEC cells and the underlying mechanism have not been studied yet.

In this study, I investigated the anti-cancer effect of oridonin of oridonin and the apparent underlying mechanism in MC-3 and YD-15 human MEC cell lines.

2. Materials and Methods

2.1 Cell culture and chemical treatment

MC-3 and YD-15 cell lines were obtained from Forth Military Medical University (Xi'an, China) and Yonsei University (Seoul, Korea), respectively. Both cell lines were maintained in either DMEM/F12 or RPMI1640 supplemented with 10% fetal bovine serum and 100U/ml each penicillin in a humidified atmosphere containing 5% CO_2 at 37℃. All experiments were performed with cells cultured at 50~60% confluence. Oridonin (Figure 1A) was purchased from Abcam (Cambridge, UK) and cycloheximide (CHX) were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Each chemical was dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at -20°C.

2.2 Trypan blue exclusion assay

The effect of oridonin on cell viability was investigated using trypan blue exclusion assay. Cells were incubated with oridonin for 48hr, stained with 0.4% trypan blue solution (Gibco, Paisley, UK), and viable cells counted using a hemocytometer.

2.3 Live/Dead assay

Live/Dead & Viability/Cytotoxicity assay kit (Invitrogen, Carlsbad, CA, USA) was used to determine the cytotoxicity of oridonin in MEC cell lines. Calcein-AM is retained in living cells, producing intense green fluorescence through intracellular esterase activity. Ethidium homodimer-1 enters dead cells with damaged membranes and binds to nucleic acids, producing bright red fluorescence. Briefly, cells were stained with 2 μM calcein-AM and 4 μM ethidium homodimer-1 and incubated for 30 min at room temperature (RT). Cells were analyzed

under a fluorescence microscope (Leica DM5000B, Leica Microsystems, Wetzlar, Germany) with a suitable excitation and emission filter.

2.4 Western blot analysis

Whole-cell lysates were extracted with RIPA lysis buffer (EMD Millipore, Billerica, CA, USA) containing phosphatase inhibitors (Thermo Fisher Scientific, Rockford, IL, USA) and protease inhibitor cocktails (Roche, Mannheim, Germany). Protein concentrations were measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins at the same concentration were separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY, USA). The membranes were blocked with 5% skim milk dissolved in Tris-buffered saline-Tween-20 buffer (T-BST) for 1hr

at RT. The membranes were then washed with T-BST and incubated with primary antibodies overnight at 4℃. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at RT for 2hr. Rabbit anti-human polyclonal antibodies against cleaved caspase-3, cleaved PARP, Bcl-xL, active-Bak, active-Bax, Bak, Bax, Bim, t-Bid, and Mcl-1 were obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). Mouse anti-human monoclonal antibodies against β-actin antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Antibody-bound proteins were detected using enhanced chemiluminescence Western blotting Luminol reagent (Santa Cruz Biotechnology, Inc) and visualized using a LAS-500 imaging system (GE Healthcare Life Sciences, Chicago, IL, USA). The densitometric analysis of Western blotting was quantified using ImageJ software (version 1.51k, NIH, Bethesda, MD, USA).

2.5 Flow cytometry analysis

Flow cytometry was performed to analyze cell cycle and apoptosis of the MEC cell lines. Cells were harvested after oridonin treatment for 48 hr, washed twice with PBS, and fixed with 70% ethanol at -20℃ for overnight. Cells were then re-suspended in PBS containing 20 μg/ml RNase A and propidium iodide (P4170, Sigma-Aldrich) for 15 min at 37˚C. DNA contents were detected using a fluorescence-activated cell sorter (FACS) caliber (Becton-Dickinson) and relative DNA content was calculated with Cell Quest software (BD Biosciences, San Jose, CA, USA).

2.6 4′-6-diamidino-2-phenylindole (DAPI) staining

DAPI solution (Sigma-Aldrich) was used to investigate the nuclear morphological changes of apoptotic cells. Cells seeded on 60 mm^2 plates were treated with different concentrations of oridonin for 48hr.

After the treatment, the cells were harvested, washed twice with PBS, and fixed with 100% methanol at RT for 10 min. The cells were washed again with PBS, plated on coated glass slides, and stained with 2 μg/ml of DAPI solution. The morphological changes of the cells were observed under a fluorescence microscope.

2.7 Annexin V/ Propidium iodide (PI) staining

Apoptosis were measured using a FITC Annexin V apoptosis detection kit (BD Pharmingen). Harvested cells were washed twice with PBS and stained with Annexin V-FITC and PI dye at RT for 15min. The stained cells were then analyzed using a FACS caliber and calculated with Cell Quest software (BD Biosciences).

2.8 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Easy-BLUE total RNA extraction kit

(iNtRON Biotechnology, Daejeon, Korea) and cDNA was synthesized using a cDNA synthesis kit (Enzo Life Sciences, Inc, Lausen, Switzerland). The resulting target cDNA was subjected to PCR using *HiPi* PCR PreMix (ELPISBIOTECH, Inc, Daejeon, Korea) and amplified using the following primers: sense 5'-GAG GAG GAG GAC GAG TTG TA-3', antisense 5'-CCT TAC GAG AAC GTC TGT TGT GAT AC-3' for Mcl-1, and sense 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3', antisense 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3' for GAPDH. The amplification of Mcl-1 and GAPDH was performed for 28 cycles (30 sec at 95℃, 35 sec at 60℃, and 45 sec at 72℃). The amplified PCR products were detected using 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.9 Quantitative real time PCR (qPCR)

The resulting target cDNA was subjected to PCR using

AMPIGENE qPCR Green Mix Hi-Rox (Enzo Life Sciences, Inc). qPCR was performed by a StepOne Plus Real-Time PCR System (Applied Biosystems, CA, USA) and the resulting target cDNA was amplified using the following primers: sense 5'-GTA TCA CAG ACG TTC TCG TAA GG-3', antisense 5'-CCA CCT TCT AGG TCC TCT ACA T-3' for Mcl-1 and sense 5'- GTG GTC TCC TCT GAC TTC AAC-3', antisense 5'- CCT GTT GCT GTA GCC AAA TTC-3'. The amplification of Mcl-1and GAPDH was performed for 40 cycles (2 min at 95℃, 10 sec at 95℃, and 30 sec at 60℃).

2.10 Mitochondrial membrane potential (ΔΨm) assay

Changes in ΔΨm was determined using a MitoScreen kit (BD Pharmingen). Harvested cells were washed twice with PBS and incubated with JC-1 solution at 37℃ for 30 min. The cells washed twice using 1X Assay Buffer and JC-1 fluorescence was analyzed by

flow cytometry.

2.11 Construction of Mcl-1 over-expression vector and transient transfection

The open reading frame of human Mcl-1(NM_021960) genes was amplified from cDNA using the following primers: Mcl□1 sense 5′□ GAA TTC ATG TTT GGC CTC AAA AGA□3′, with an included *Eco*RI site, Mcl□1 antisense 5′□GAA TTC CTA TCT TAT TAG ATA TGC□3′, with an included *Eco*RI site and cloned into a pGEM®□T Easy Vector System (Promega, Madison, WI, USA). The genes were cloned into the multiple cloning site of pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA). MC3 and YD15 cells were transfected with empty pcDNA3.1 or a pcDNA3.1□Mcl□1 vector construct using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

2.12 Nude mouse xenograft assay

Seven-week-old BALB/c nu/nu male mice were purchased from NARA Biotech (Pyeongtaek, Korea). All mice were handled in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines approved by CHA university (IACUC approval number: 180154). MC-3 cells were subcutaneously injected into the flanks of the mice. Vehicle control or oridonin (15 mg/kg/day) were administered intraperitoneally to the tumor-bearing mice every other day for 24 days from approximately 10 days after incubation (day 0). Tumor volume, tumor weight, and body weight were measured every 4 days. The tumor volume was measured along the two diameter axes with calipers and calculated using the following formula: $V = \pi/6\{(D + d)/2\}^3$, where D and d were the larger and smaller diameters, respectively.

2.13 Statistical analysis

Statistical analysis was performed using a one-way ANOVA with SPSS 22 (SPSS, Chicago, IL, USA) and statistical significance was defined as a $P < 0.05$.

3. Results

3.1 Oridonin inhibits cell viability and induces cell death in human MEC cell lines

To investigate the growth-inhibitory effect of oridonin, MC-3 and YD-15 cells were incubated with various concentration of oridonin (0-30 μM) for 48hr. The results showed that statistical significance was observed at 15 and 30 μM of oridonin for MC-3 cells and 6.25, 12.5, and 25 μM for YD-15 cells, respectively (Figure 1B). To determine the cytotoxic effects of oridonin, Live/Dead assay was performed under the same conditions as trypan blue exclusion assay in MEC cell lines. As illustrated in Figure 1C, oridonin led to an increase in the ratio of dead cells (red fluorescence) in a concentration-dependent manner. These results indicate that oridonin can inhibit cell viability and induce cell death in MEC cell lines.

3.2 Oridonin induces apoptosis in MEC cell lines

To determine the type of cell death induced by oridonin, PARP and caspase 3 as markers of apoptosis were detected by Western blotting. In response to oridonin treatment, the cleavages of caspase 3 and PARP were markedly increased compared with the vehicle control (Figure 2A). To test the effect of oridonin on sub-G_1 population, flow cytometry was performed. The cell population in the Sub G_1 phase increased from 8.96 to 13.31-fold in both cell lines treated with oridonin (Figure 2B). When visualizing the apoptosis by DAPI staining, oridonin significantly increased the number of apoptotic nuclei with condensation or fragmentation (white arrows) in MEC cell lines (Figure 2C). To further verify the apoptotic activity of oridonin, Annexin V/PI double staining was performed. The results showed that Annexin-positive cells were significantly increased in a concentration-dependent manner (Figure 2D). Taken together, these results suggest that oridonin

enhances the apoptotic cell death in human MEC cell lines.

3.3 Oridonin down-regulates the Mcl-1 protein through post-translation modification

To elucidate the fundamental mechanism of oridonin-induced apoptosis, I have identified the expression of Bcl-2 family as a key mediator of apoptosis. Western blotting showed that oridonin treatment significantly reduced the expression level of Mcl-1 protein and increased PARP cleavage in a concentration- and time-dependent manner (Figures 3A and 3D), but oridonin did not affect the expression of other anti-apoptotic protein (Bcl-xL) in both cell lines (Figure 4). To determine if Mcl-1 is regulated at the transcriptional level, RT-PCR and qPCR were performed. Mcl-1 mRNA levels were not affected by oridonin in both cell lines (Figures 3B, 3C, and 3E). To further determine the effect of oridonin on Mcl-1 protein turnover, chasing

analysis was performed using the cycloheximide (CHX), an inhibitor of protein synthesis, followed by Western blotting. As a result, Mcl-1 protein levels were significantly decreased by simultaneous treatment with oridonin and CHX compared to CHX treatment only (Figure 5 Top panel). The half-life of Mcl-1 protein in the oridonin and CHX-cotreated group was about 11.5 and 10.8 hr compared with 23.3 and 44.0 hr in the CHX-treated group in MC3 and YD-15 cells, respectively (Figure 5 bottom panel). These results suggest that the mode of action of oridonin is through inhibition of Mcl-1 protein level through post-translational modification.

3.4 The depletion of Mcl-1 protein is associated with oridonin-induced apoptosis in MEC cell lines

To verify whether the effect of oridonin-mediated apoptosis is dependent on Mcl-1, two cell lines were transfected with an empty

vector or an Mcl-1 expression vector. Mcl-1 overexpression significantly restored cell viability and PARP cleavage previously affected by oridonin in both cell lines (Figures 6A and 6B). This observation was further confirmed by showing that the ratio of apoptotic nuclei in Mcl-1 overexpressing cells was reduced compared with the control (Figure 6C). These results suggest that the principal mechanism of oridonin-mediated apoptosis may involve the targeting of Mcl-1 protein in human MEC cell lines.

3.5 Oridonin induces mitochondrial apoptosis by regulating the Mcl-1/t-Bid signaling axis

Most apoptotic stimuli require mitochondrial outer membrane permeabilization (MOMP), by pro-apoptotic proteins such as Bax, Bak, Bim, and t-Bid [25]. JC-1 staining was performed to determine whether oridonin induced apoptosis through the mitochondria dysfunction.

Oridonin showed a significant reduction in red fluorescence compared to vehicle control, confirming that the loss of ΔΨm was induced in both cell lines (Figure 7A). Next, to further study mitochondrial-dependent pathway in oridonin-mediated apoptosis, I analyzed the expression levels of pro-apoptotic proteins. As shown in Figure 7B, the expression of t-Bid was dramatically increased by oridonin in a concentration-dependent manner, whereas other pro-apoptotic proteins such as Bak, Bax, and Bim were not commonly affected in both cell lines (Figure 4). To ascertain the involvement of Mcl-1 in t-Bid expression during oridonin-induced apoptosis, a Mcl-1 expression vector was used. As expected, ectopic expression of Mcl-1 protein significantly reduced the expression of t-Bid in oridonin-treated cells (Figure 7C). These results suggest that these results suggest that t-Bid may be an essential downstream molecule of Mcl-1 during oridonin-induced apoptosis in MEC cell lines.

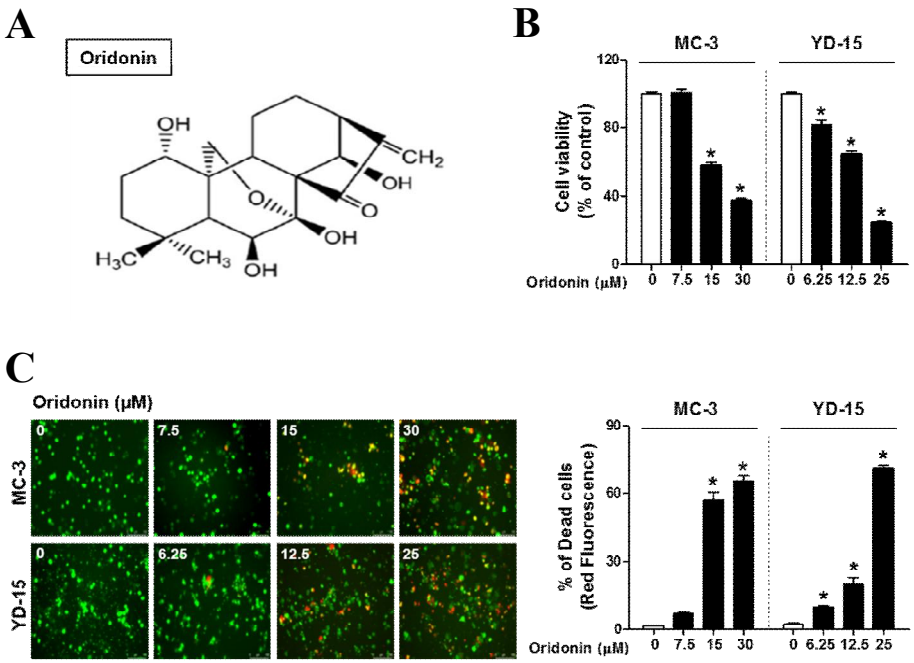

Figure 1. Effect of oridonin on cell viability in MEC cell lines (A) Chemical structure of oridonin. **(B)** MC-3 and YD-15 cells were treated with DMSO or oridonin (0 to 30 μM) for 48hr and cell viability was determined using a trypan blue exclusion assay. **(C)** Cytotoxic effect of oridonin was estimated using Live/Dead assay. The percentage of dead cells was quantified. Graph shows the means ± SD of three independent experiments. $P<0.05$, significance compared with control group (*).

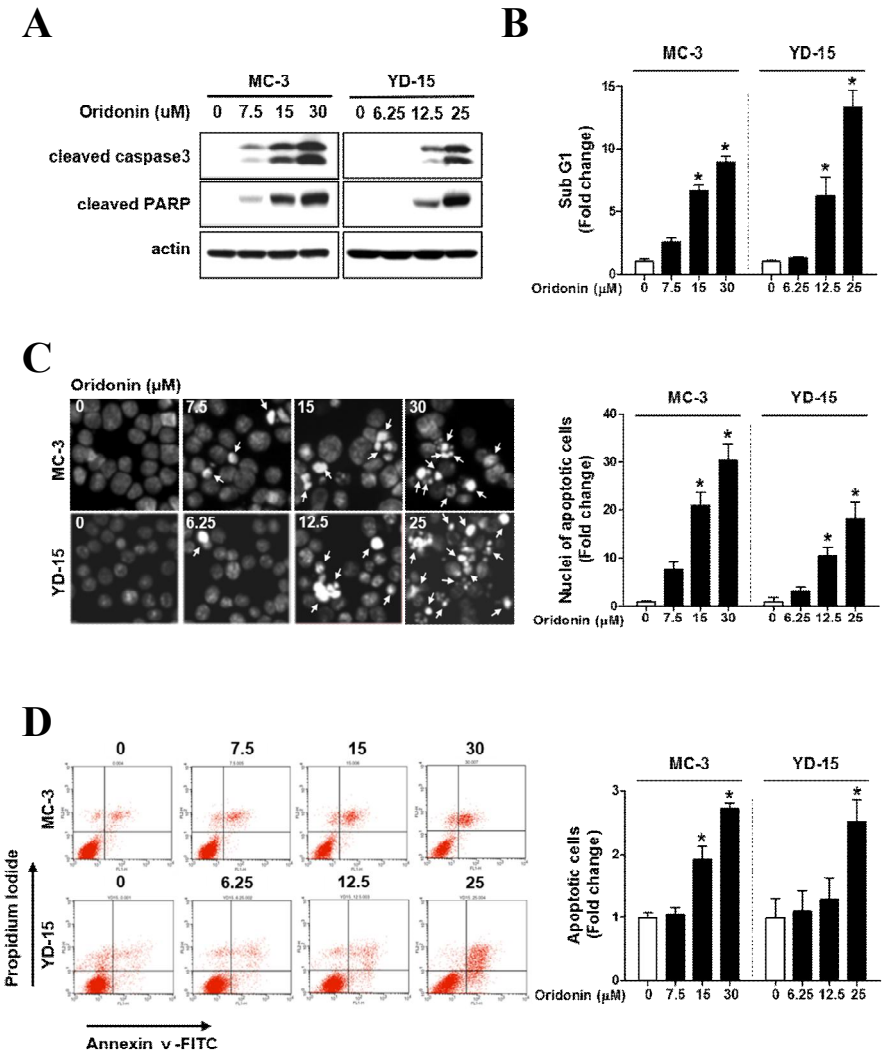

Figure 2. Effect of oridonin on apoptosis in MEC cell lines (A) The apoptotic effects of oridonin were determined using a Western blotting for cleaved caspase-3 and PARP. β–actin was used as control for

normalization. **(B)** Sub-G_1 population was analyzed by PI staining **(C)** Apoptotic features such as chromatin condensation and nuclear fragmentation were detected by DAPI staining (indicated by white arrows). **(D)** Annexin V/PI staining in MC-3 and YD-15 cells treated with DMSO or oridonin for 48hr was performed to detect apoptosis. Graph shows the means ± SD of three independent experiments. $P<0.05$, significance compared with control group (*).

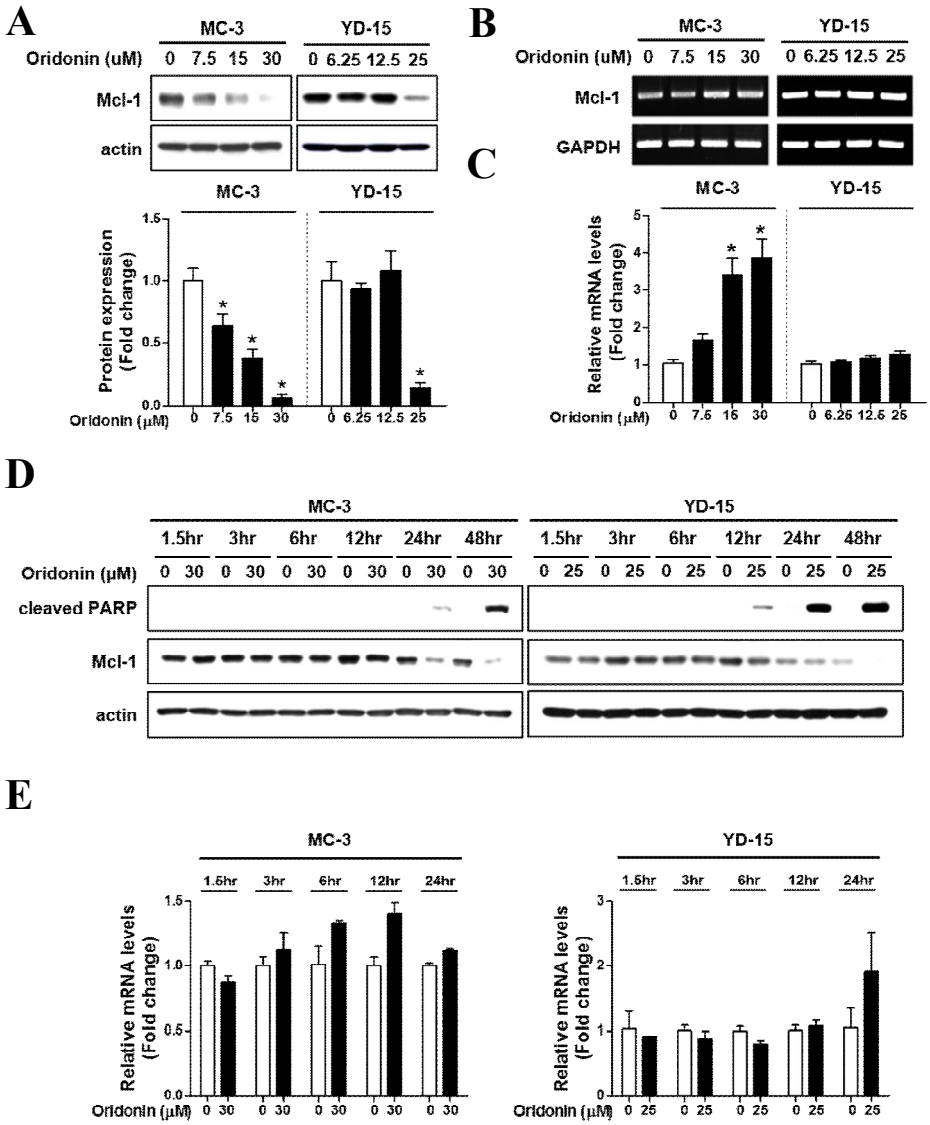

Figure 3. Inhibitory effects of oridonin on Mcl-1 protein expression in MEC cell lines (A) The effects of DMSO or oridonin for 48hr on

Mcl-1 protein were determined using a Western blotting. mRNA level of Mcl-1 was detected by RT-PCR **(B)** and qPCR **(C)**. Expression levels of Mcl-1 protein or mRNA were determined in a time-dependent manner using Western blotting **(D)** and **(E)** qPCR, respectively. Graph shows the means ± SD of three independent experiments. $P<0.05$, significance compared with control group (*).

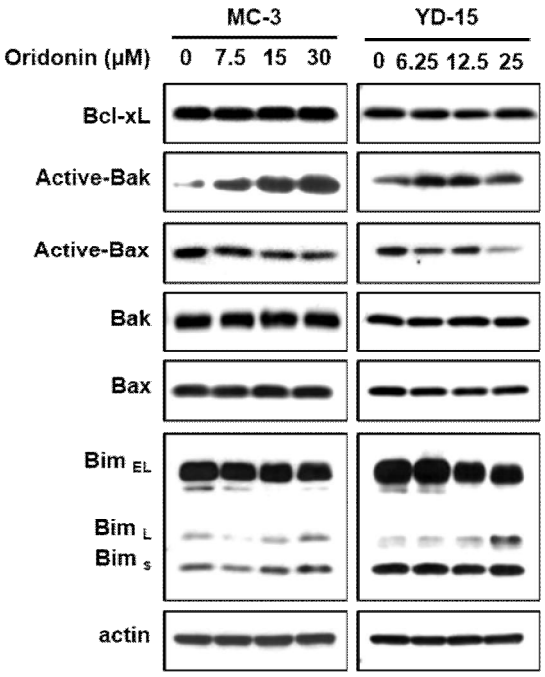

Figure 4. The effects of oridonin on the protein expression of Bcl-2 family in MEC cell lines MC-3 and YD-15 cells were treated with DMSO or various concentrations of oridonin for 48hr. The expression levels of Bcl-2 family proteins (Bcl-xL, active-Bak, active-Bax, Bak, Bax, and Bim) were determined by Western blotting. The results represent two independent experiments.

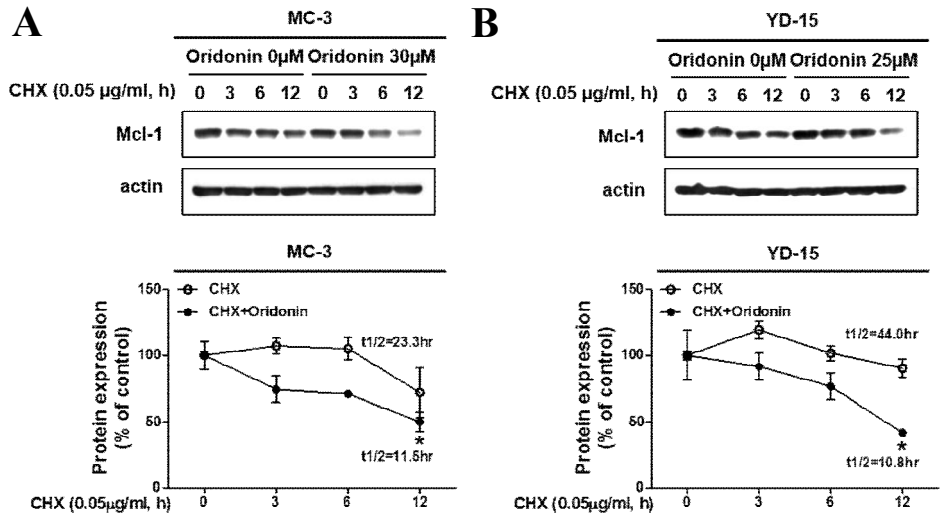

Figure 5. The effect of oridonin on Mcl-1 protein turnover in MEC cell lines (A, B) Effect of oridonin on Mcl-1 protein turnover was determined by Western blotting in MC-3 and YD-15 cells pretreated with cycloheximide (CHX; 0.05 μg/ml) with or without oridonin (30 μM) for 3, 6, and 12 hr. Graph shows the means ± SD of three independent experiments. $P<0.05$, significance compared with control group (*).

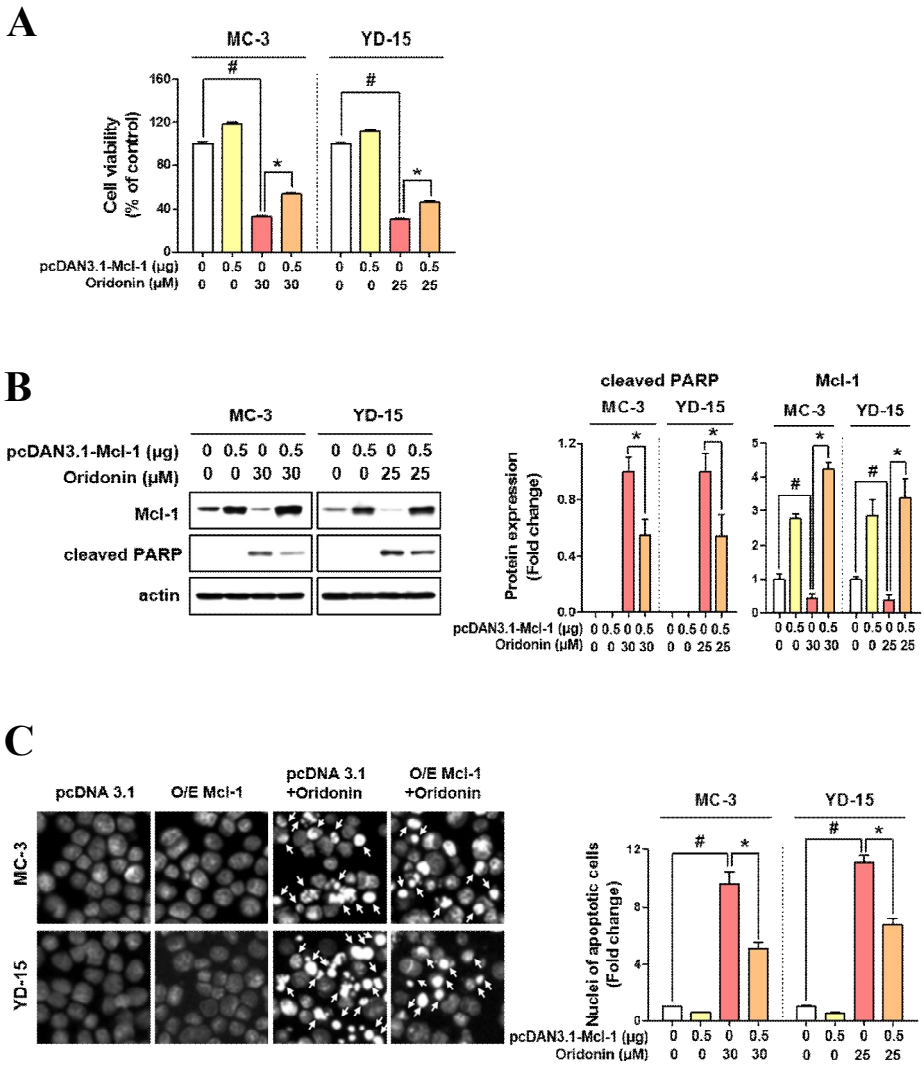

Figure 6. The apoptotic effect of oridonin through depletion of Mcl-1 protein in MEC cell lines Cells were transiently transfected with 0.5μg of empty pcDNA3.1 or pcDNA3.1-Mcl-1. After 6hr, both cell

lines were treated with oridonin for 48hr. **(A)** Cell viability was determined by a trypan blue exclusion assay. **(B)** Expression levels of Mcl-1 and cleaved PARP were determined by Western blotting. **(C)** Apoptotic features were detected by DAPI staining. Graph shows the means ± SD of three independent experiments. $P<0.05$, significance compared with vehicle control group ($^{\#}$) and $P<0.05$, significance compared with control group (*).

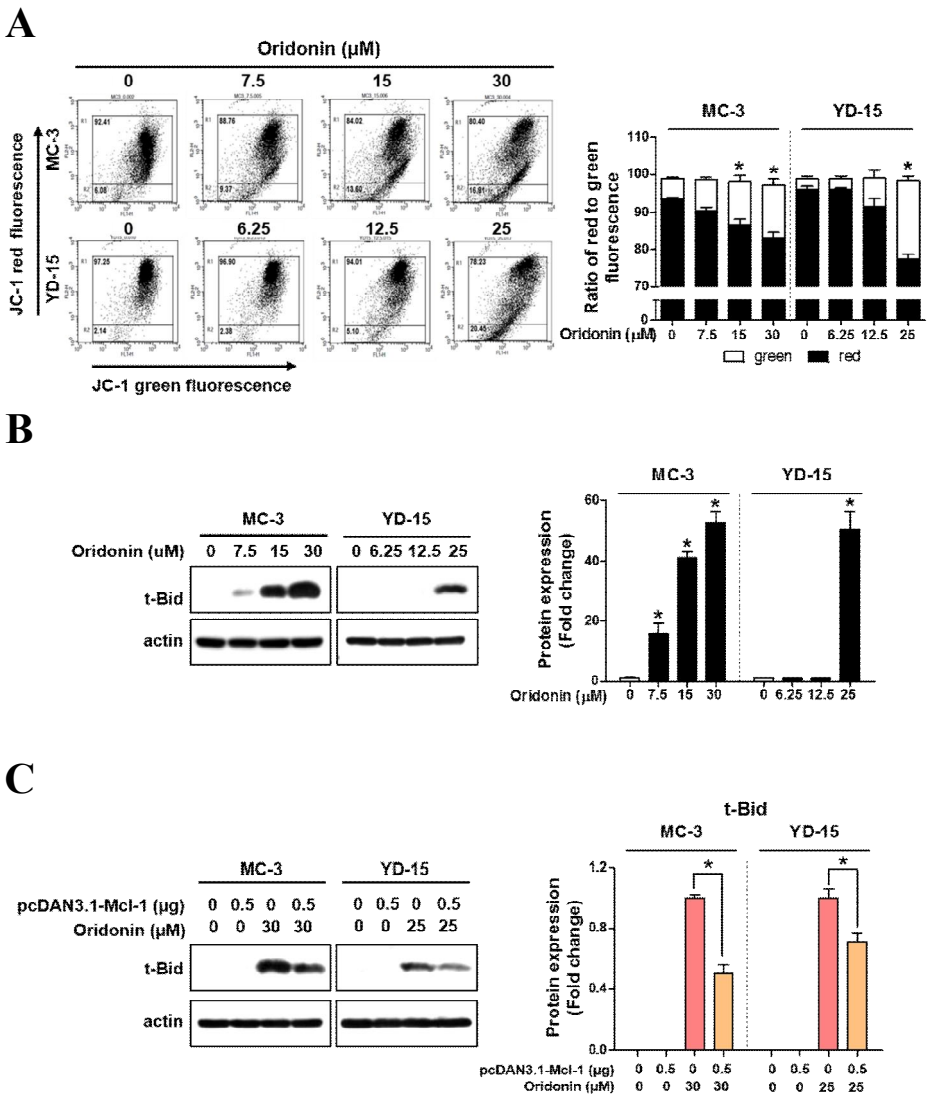

Figure 7. Loss of MOMP and t-Bid expression in oridonin-treated MEC cell lines (A) MOMP was determined using a JC-1 assay. **(B)** The expression level of t-Bid protein was determined by Western

blotting. **(C)** Cells were transiently transfected with 0.5μg of empty pcDNA3.1 or pcDNA3.1-Mcl-1 and treated with oridonin for 48hr. Expression level of t-Bid was determined by Western blotting. Graph shows the means ± SD of three independent experiments. $P<0.05$, significance compared with control group (*).

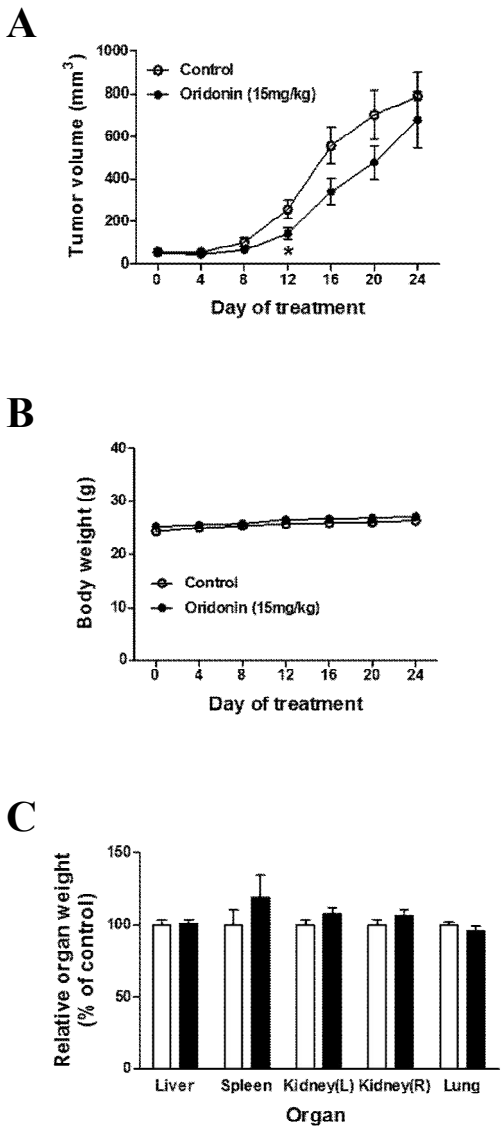

Figure 8. Effects of oridonin on tumor growth in a nude mouse xenograft model bearing MC-3 cell line Athymic nude mice bearing

MC-3 cells were treated with vehicle control or oridonin (15 mg/kg) for 24 days (n = 7/group). Tumor volume **(A),** body weight **(B)**, and organ weights **(C)** were measured. Graph shows the means ± SE of three independent experiments. $P<0.05$, significance compared with control group (*).

4. Discussion

Mcl-1, a pro-survival member of the Bcl-2 family, has been shown to be highly expressed in human malignancies, which has led to increased attention in oral cancer, including other solid tumors [26, 27]. Interestingly, human oral cancer cells showed elevated expression of Mcl-1 protein compared to their normal counterpart, human oral keratinocyte, while Bcl-2 showed negligible expression in most cells [27, 28]. Furthermore, it has been reported that Mcl-1 overexpression is resistant to Bcl-2 family inhibitors such as paclitaxel, vincristine, and gemcitabine, which are well-known anti-cancer therapies [29, 30]. Thus, down-regulation of Mcl-1 may be a key step in promoting apoptosis of cancer cells and the use of natural compounds to modulate the expression or function of Mcl-1 protein has recently been viewed as a promising alternative for elaboration of preventive and therapeutic

regimens [31, 32]. Our previous studies found that natural products such as mithramycin A, fisetin, and fucoidan inhibited the expression of Mcl-1 protein, leading to apoptosis in human oral cancer cells [28, 33, 34]. I also report that oridonin induces apoptosis by increasing the expression of γH2AX on DNA damage [18]. Despite several studies of the apoptotic effects of oridonin, its mode of action in down-regulating Mcl-1 has not yet been fully understood [35, 36]. In this study, it was for the first time that oridonin induces apoptosis through down-regulation of Mcl-1 in human MEC cell lines, independent of transcriptional regulation (Figures 2 and 3). I have also demonstrated that overexpression of Mcl-1 effectively abrogated oridonin-induced apoptosis (Figure 6). In present study, these results support the notion that Mcl-1 is an important survival factor and that its down-regulation is a potential therapeutic strategy for MEC.

In response to apoptotic stimuli, the BH3-only proteins are up-

regulated to allow the activation of BAX and BAK for oligomerization in the outer-mitochondrial membrane known as MOMP, leading to the release of cytochrome *c* and other apoptosis-inducing factors [37]. It means that the impairment of MOMP by the Bcl-2 family has been the cornerstone of intrinsic apoptosis pathways. Here, I found that oridonin treatment significantly induced the loss of MOMP through up-regulation of t-Bid expression in a concentration-dependent manner, accompanied by the marked apoptotic cell death. Liu et al. obtained results with our evidence demonstrating that the gradual increase in MOMP destruction rate by oridonin in HPB-ALL cell line occurred through the up-regulation of t-Bid [38]. Thus, these findings emphasized that oridonin-induced apoptosis is closely related to or dependent on the loss of MOMP through the Mcl-1/t-Bid signaling axis in human MEC cell lines.

Based on the anti-apoptotic effect of oridonin *in vitro*, many studies have shown that oridonin is a potent tumor growth inhibitor *in vivo* [23, 39, 40]. Thus, I conducted a tumor xenograft experiment using nude mice bearing MC-3 cell line. Although the final tumor weight was slightly reduced in oridonin-treated mice (data not shown), tumor volume was almost statistically significantly reduced in oridonin-treated group compared with the control (Figure 8A). I have also shown that the weight of body and organ (liver, spleen, kidney, and lung) has not altered by oridonin administration, indicating that oridonin has negligible toxicity. (Figures 8B and 8C). Regarding the biocompatibility of oridonin, Qiu et al. recently reported that oridonin-conjugated nanoparticles has a high blood solubility and negligible toxicity in blood tests [41]. These findings indicate that oridonin has no *in vivo* side effects.

In conclusion, this study demonstrated for the first time that down-regulation of Mcl-1 by oridonin promotes apoptosis through loss of MOMP and that t-Bid is a critical downstream target of Mcl-1 in oridonin-induced apoptosis in human MEC cell lines. Taken together, Mcl-1 may be a valuable molecular target for oridonin-mediated anti-cancer activity and oridonin is recommended as a naturally derived chemotherapeutic drug candidate for the treatment of MEC.

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국 문 초 록

타액선 점액표피양암종에서 oridonin의 세포사멸 효능 및 분자기전에 관한 연구

한 정 민

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점액표피양암종은 가장 흔히 발생하는 타액선 악성종양으로 고등급인 경우에는 5년 생존율이 최대 40%에 그친다. 현재까지, 타액선 종양의 치료를 위해 많은 화학요법약물이 사용되었으나, 다양한 부작용을 나타낸다고 보고되고 있다. 그로 인해 대체보완 약물 치료방법으로서 천연 식물의 잠재력에 대한 연구가 많이 진행되고 있다. Oridonin은 *Rabdosia*

*rubescen*에서 추출된 디테르페로이드의 일종으로 다양한 암세포에서 항암효능을 나타낸다고 밝혀졌으나, 점액표피양암종에서의 항암효능 및 분자적 기전에 대한 연구는 이뤄진 바 없다. 따라서 본 연구에서는 점액표피양암종에 대한 Oridonin의 항암효능 및 분자기전에 대해 규명하고자 하였다. Oridonin의 항암효능을 평가하기 위해 trypan blue exclusion 분석, live/dead 분석, 4′-6-diamidino-2-phenylindole 염색, western blot 분석, flow cytometric 분석, reverse transcription-polymerase chain reaction (RT-PCR), Quantitative PCR (qPCR), mitochondrial membrane potential (MMP) 분석, Construction of over-expression vector and transient transfection 방법을 수행하였다. 연구결과, Oridonin은 타액선 점액표피양암종 세포주인 MC-3, YD-15의 생존능력을 억제하였고 세포사멸을 유도하였다. Oridonin은 MC-3, YD-15 세포주에서 post-translation modification을 통해 myeloid cell leukemia-1 (Mcl-1) 발현을 감소시켰고 농도 의존적으로 truncated bid (t-

Bid) 발현을 증가시켰으며 MMP를 감소시키는 결과를 확인하였다. 또한 Mcl-1을 과발현 시켰을 때 MC-3, YD-15 세포주에서 Oridonin의 세포사멸 효능이 현저히 줄어들었고 t-Bid 발현 역시 감소하는 것을 확인하였다. 본 연구의 결과를 통해 Oridonin이 점액표피양암종에서 Mcl-1과 t-Bid 조절을 통해 세포사멸을 유도하는 항암효능 및 분자기전을 확인하였다. 따라서 Oridonin이 구강암을 치료할 수 있는 잠재적인 항암물질로 발전 가능성이 있다고 사료된다.

주요어 : 점액표피양암종, Oridonin, 세포사멸, Myeloid cell leukemia-1, truncated-Bid

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