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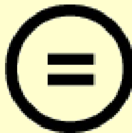
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2024년 2월
박사학위 논문

Effects of 2-D08 (2′,3′,4′-trihydroxy-flavone) on Proliferation and Apoptosis in Uterine Leiomyosarcoma Cells

- Effect of 2-D08 on Uterine Leiomyosarcoma Cells -

조 선 대 학 교 대 학 원
의 학 과
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2-D08 (2′,3′,4′-trihydroxy-flavone)이 자궁 평활근 육종 세포의 증식과 세포사멸에 미치는 영향에 대한 연구

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Effects of 2-D08 (2′,3′,4′-trihydroxy-flavone) on Proliferation and Apoptosis in Uterine Leiomyosarcoma Cells

- Effect of 2-D08 on Uterine Leiomyosarcoma Cells -

지도교수 최 상 준

이 논문을 의학 박사학위신청 논문으로 제출함

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Table of Contents

I. Korean abstract---ii

II. Abstract--iv

III. Introduction---1

IV. Materials and Methods-------------------------------4

V. Results---9

A. Effects of 2-D08 on cell viability of Ut-LMS cells

B. 2-D08 inhibits colony formation of Ut-LMS cells

C. 2-D08 suppresses the proliferation of Ut-LMS cells *in vitro*

D. Effect of 2-D08 on the protein expression related to cell proliferation and apoptosis in Ut-LMS cells

E. 2-D08 treatment inhibits the degradation of p21 by Ub-proteasome pathway in Ut-LMS cells

F. The impact of 2-D08 on the expression of differentiation marker proteins in Ut-LMS cells

VI. Discussion---13

VII. Figures--17

VIII. Reference---29

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2-D08 (2′,3′,4′-trihydroxy-flavone)이 자궁 평활근 육종 세포의 증식과 세포사멸에 미치는 영향에 대한 연구

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(국문초록)

목 적: SUMO E2 억제제인 2-D08은 항암 효과를 비롯한 여러 생물학적 기능을 가지고 있다. 그러나 자궁 평활근 육종에서 2-D08의 효과는 알려져 있지 않았다. 본 연구는 인간 유래 자궁 평활근 육종 세포에서 2-D08의 항암 활성을 조사했다.

방 법: SK-LMS-1 (인간 유래 자궁 평활근 육종 세포주) 세포를 본 연구에 사용했다. 세포 생존성 (MTT 분석), 콜로니 형성, Ki67 염색 및 BrdU 증식 분석을 통해 세포 증식에 대한 2-D08의 효과를 평가했다. 카스파제-3 활성도 분석을 통해 2-D08에 의한 세포사멸을 측정하였다. 세포 신호전달에 대한 2-D08의 영향을 평가하기 위해 웨스턴 블롯 분석 및 면역침강법을 사용하였다.

결 과: 2-D08은 SK-LMS-1 세포에서 세포 생존성을 유의하게 억제하였다. 2-D08을 처리하면 SK-LMS-1 세포의 콜로니 형성 능력이 유의하게 억제되었다.

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Ki67 염색 및 BrdU 분석에서 2-D08이 처리가 된 SK-LMS-1 세포는 항증식 효과가 있음을 발견했다. 또한, 2-D08은 SK-LMS-1 세포에서 p21 단백질을 증가하는 것으로 나타났지만, 카스파제-3 활성화 분석에서 세포자멸을 유도하지 않았다. 그리고 프로테아좀 억제는 SK-LMS-1 세포에서 p21 단백질의 안정 수준을 증가시켰고, 2-D08은 이러한 신호 전달 경로를 조절한다. 게다가 SK-LMS-1 세포에서 α-SM-액틴, TAGLN 및 칼포닌 1의 발현 변화를 확인한 결과 2-D08은 평활근 표현형 전환을 직접 유도하지 않았다.

결 론: 이러한 결과는 SK-LMS-1 세포에서 2-D08이 p21의 안정화를 조절하여 세포 증식을 억제한다는 것을 나타낸다. 따라서 2-D08은 자궁 평활근 육종의 후보 치료 물질이 될 수 있다. 그러나 추가적인 연구를 통해 이 화합물의 적절한 용량을 신중하게 선택해야 한다.

핵심어:

2-D08; 자궁; 평활근 육종; SK-LMS-1; 증식

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ABSTRACT

Effects of 2-D08 (2′,3′,4′-trihydroxy-flavone) on Proliferation and Apoptosis in Uterine Leiomyosarcoma Cells

- Effect of 2-D08 on Uterine Leiomyosarcoma Cells -

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Objective: 2-D08, a SUMO E2 inhibitor, has several biological functions, including anti-cancer effects. However, the effects of 2-D08 in uterine leiomyosarcoma (Ut-LMS) are unknown. This study explored the anti-cancer activity of 2-D08 against human Ut-LMS cells.

Methods: We used SK-LMS-1 (human Ut-LMS cells) as an *in vitro* model. Cell viability (MTT assay), colony formation, Ki67 staining, and BrdU proliferation assays were used to evaluate the effect of 2-D08 on cellular proliferation. Caspase-3 activity was assessed by measuring 2-D08-induced apoptosis. The impact of 2-D08 on cellular signaling was evaluated through western blotting and immunoprecipitation techniques.

Results: 2-D08 significantly inhibited cell viability in the SK-LMS-1 cells. 2-D08 treatment markedly suppressed the potential of SK-LMS-1 cells to form colonies. Using

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Ki67 staining and BrdU assay, we found that 2-D08 treatment had anti-inhibitory effects on the proliferation of SK-LMS-1 cells. Moreover, 2-D08 was also shown to upregulate p21 proteins in SK-LMS-1 cells, though 2-D08 did not further promote Caspase-3 activity and apoptosis. In SK-LMS-1 cells, the inhibition of proteasomes led to elevated steady-state levels of p21, and 2-D08 was observed to modulate these signaling pathways. Moreover, the evaluation of α-SM-actin, TAGLN, and calponin 1 expression indicated that 2-D08 did not directly initiate smooth muscle phenotypic switching in SK-LMS-1 cells.

Conclusion: These results indicate that in SK-LMS-1 cells, 2-D08 inhibits cell proliferation by regulating the stabilization of p21. Therefore, 2-D08 show potential as a promising candidate for human Ut-LMS. However, it is crucial to meticulously choose the proper dosage for this compound.

Keywords:

2-D08; Uterine; Leiomyosarcoma; SK-LMS-1; Proliferation

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Introduction

Uterine leiomyosarcoma (Ut-LMS) is a malignant tumor that occurs in the smooth muscles of the uterus (myometrium). It is an uncommon condition, representing less than 1% of malignant cases in the uterus and 25–36% of uterine sarcomas [1, 2], but can spread to the surrounding tissues and organs [3]. In particular, in the case of patients with stages 3 or 4 Ut-LMS, the 5-year disease-specific survival (DSS) rates were 29–45% [4]. Patients with Ut-LMS show symptoms such as abdominal pain and vaginal bleeding. As the tissue appearances of Ut-leiomyoma and Ut-LMS are similar, these are differentiated through a surgical biopsy [5]. Although it is extremely rare for Ut-leiomyoma to change to Ut-LMS, it is possible that an Ut-leiomyoma is a precursor form of Ut-LMS. Furthermore, if the tumor grows quickly or Ut-LMS is suspected after an ultrasound scan, it is necessary to periodically monitor whether it progresses to malignant tumors, and additional examinations, such as MRI, are required.

Thus, Ut-LMS is difficult to diagnose early, and there are few methods that can be used for the diagnosis before curative surgery. However, there have been a few studies on new biomarkers and molecular mechanisms of Ut-LMS to overcome these difficulties. For example, the modulation of the Akt-mTOR pathway is pivotal in the development of Ut-LMS [6], and compounds that inhibit the PI3K and mTOR pathways demonstrate the ability to hinder the growth of Ut-LMS [7]. In addition, PTK787, a VEGFR/PDGFR inhibitor, can influence the survival of Ut-LMS [8]. LMP2 deficiency in mice leads to spontaneous development of Ut-LMS, and an inadequate expression of LMP2 could pose

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a substantial risk factor for Ut-LMS development [9]. A report published in 2017 showed that the data of The Cancer Genome Atlas (TCGA) on Ut-LMS revealed the presence of mutations and deletions corresponding to cancer genes such as *RB1, TP53 (tumor protein p53),* and *PTEN* [10]. In addition, a similar study has indicated that whole-exome sequencing of Ut-LMS showed frequent alterations in the *TP53, RB1,* ATRX, and MED12 genes [11, 12].

Cellular proliferation stands as a key hallmark of malignant tumors, and leiomyosarcomas, in particular, demonstrate notable levels of proliferative activity [13]. Numerous published studies have explored the relationship between leiomyosarcoma and Ki67. Ki67, functioning as an indicator of cellular proliferation, is acknowledged as a predictive and prognostic factor in multiple subtypes of cancers, such as breast cancer [14, 15], prostate cancer [16, 17] and adrenocortical carcinoma [18]. Some studies propose that Ki67 holds prognostic significance in leiomyosarcoma as well [19, 20].

2-D08 (2’,3’,4’-trihydroxyflavone) is a synthetic flavone that mechanistically is a specific inhibitor of protein SUMOylation [21]. Recent research has showcased diverse biological functions of 2-D08, including its anti-cancer effects. For example, the migration of pancreatic cancer cells was hindered by the presence of 2-D08 through the induction of K-Ras deSUMOylation [22]. In addition, 2-D08 induces apoptosis by fostering the accumulation of reactive oxygen species (ROS), likely through NOX2 deSUMOylation in AML cells [23]. Lastly, 2-D08 treatment has an inhibitory effect on both the proliferation and differentiation of C2C12 myoblast cells [24].

However, the effect of 2-D08 on the growth of Ut-LMS is not yet clearly understood.

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Hence, in this investigation, we examined the suppressive effects of 2-D08 on the growth of human LMS cell lines (SK-LMS-1) and explored the potential molecular mechanisms that may underlie its effects.

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Materials and Methods

Cell line and reagents

SK-LMS-1 cells were purchased from ATCC. Cells were grown in a humidified incubator maintained in DMEM (11995-065, Gibco) containing 10% FBS (Hyclone) and 1% streptomycin-penicillin (Gibco) at 37 °C with 5% CO_2. 2-D08 was obtained from Merck Millipore (SML1052-5MG). The proteasome inhibitor MG132 was sourced from Selleckchem.

MTT assays

After seeding cells (5×10^3 cells per well) in 96-well plates, various concentrations of 2-D08 were administered, and the cell were incubated for 24 and 48 h. Each well received the addition of MTT stock solution (M2128, Sigma-Aldrich), and was then incubated for 3 h, and after discarding the medium, DMSO was added to all wells. Thereafter, cell viability was assessed at 570 nm employing the INNO microplate spectrophotometer (LTek) and normalized to the control group.

Colony formation assay

Each well of 6-well plate (1000 cells per well) was seeded with SK-LMS-1 cells and permitted to incubate for 24 h, the medium was removed, and 2-D08 was introduced into a fresh medium with varying concentrations. Cell cultivation continued for 7 days, allowing the colonies to become visible. To observe the growth of colonies formed, we

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used a crystal violet assay kit (ab232855, Abcam). In brief, the cells were fixed and then subjected to staining with 2% crystal violet solution for 20 min, and the colonies were enumerated. In addition, images capturing the colonies formed were acquired through microscope under bright-field.

Ki67 immunostaining

SK-LMS-1 cells were fixed with IC Fixation Buffer (00-8222-49, Invitrogen) for 20 min and permeabilized for 5 min. Cells were then blocked with 2% normal goat serum (#5425, Cell Signaling Technology) in PBS and incubated with the anti-Ki67 (Invitrogen, MA5-14520) antibody at 4 °C overnight. Detection involved the utilization of the Ki67 antibody (MA5-14520, Invitrogen) with subsequent incubation of the cells using an anti-rabbit IgG antibody (Fab2 Alexa Fluor 594 Conjugate, 8889S, Cell Signaling Technology). The proportion of Ki67-positive cells was determined by dividing the count of Ki67-positive cells by the count of DAPI-positive cells.

BrdU Assay

BrdU Assay Kit (6831S, Cell Signaling Technology) was used to study the proliferating cells. In brief, cells (5×10^3 cells per well) were seeded into 96-well plates and subsequent treatments with 2-D08 were administered at the specified concentrations for either 24 or 48 h. The cells were subjected to the BrdU label for 24 h, fixed and incubated with mouse anti-BrdU antibody, and the treatment was administered following the protocol provided by the manufacturer. Finally, the reading were taken at a wavelength of 450 nm.

Protein preparation and western blot analysis

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Cells lysis was carried out using RIPA buffer (Biosesang) supplemented with a protease inhibitor. Equal amounts of protein, denatured by heat, were loaded onto a 12% SDS-PAGE gel. After the electrophoretic separation, the proteins were transferred from the gel to PVDF membranes (Merck Millipore). Blocking of membranes was achieved using 5% non-fat milk for 1 h, and then probed with the indicated antibodies with gentle shaking. In this study, we utilized the following primary antibodies: p21 (2947S), p27 (3686T), p53 (2527S), Caspase-3 (9665S), cleaved Caspase-3 (9664S), β-Actin (4967S) (Cell Signaling Technology), α-smooth muscle (SM) actin (ab5694), TAGLN (ab14106), calponin 1 (ab46794) (Abcam), and Ub (BML-PW0930-0100, Enzo Life Sciences). Following a wash with the TBST solution, the membranes were subjected to incubation with HRP-linked antibody (7074S or 7076S, Cell Signaling Technology). The signals quantified using the Azure c280 chemiluminescent imaging system.

qPCR

Total RNA was isolated from SK-LMS-1 cells utilizing NucleoZOL (740404.200, MACHEREY-NAGEL). The RNA samples underwent transcription to generate cDNA, utilizing the ReverTra Ace qPCR RT Kit (TOYOBO). QuantiTect SYBR green RT-PCR Kit (QIAGEN) was utilized for the qPCR. The RNA level was expressed as a relative result via $2^{-\Delta\Delta Ct}$ method against the endogenous standard control β-actin. The sequences of the primers were as follows: α-SM-actin, F: 5'-acccagcaccatgaagatca-3', R: 5'-tttgcggtggacaatggaag-3'; TAGLN, F: 5'-ggctgaagaatggcgtgatt-3', R: 5'-ctgccatgtctttgccttca-3'; Calponin 1, F: 5'-gtgaagccccacgacatttt-3', R: 5'-tgatgttccgcccttctctt-3'; β-actin, F: 5'-catccgcaaagacctgtacg-3', R: 5'-cctgcttgctgatccacatc-

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3'.

Caspase-3 activity assay

We evaluated Caspase-3 activity utilizing the Caspase-3 Assay Kit (ab39401, Abcam). Briefly, the cell lysates underwent centrifugation at 10,000 g for 1 min. An equal amount of supernatant (100 μg of protein) was incubated with the DEVD-pNA substrates in reaction buffer, containing dithiothreitol (DTT). The Caspase-3 activation was quantified using SpectraMax Plus instrument (Molecular Devices) at a wavelength of 405 nm.

Transfection and in vivo ubiquitination assays

Cells were transfected with 4 μg pcDNA3-Ub plasmid using the Lipofectamine Reagent (18324-012, Invitrogen) with the Plus Reagent (Invitrogen) in DMEM, following the manufacturer's protocol, followed by treatment with 2-D08 (50 μM) for 48 h. The cellular pellet was lysed in RIPA buffer supplemented protease inhibitors, and anti-p21 antibody was used for the immunoprecipitation of the lysates at 4 °C overnight. The immune complexes were allowed to incubate with agarose affinity beads (sc-2003, Santa Cruz Biotechnology), and the beads were washed twice with the RIPA buffer. The complexes were analyzed using the western blot analysis for the presence of Ub-conjugated p21 using an anti-Ub antibody.

Statistical analysis

GraphPad Prism software (version 6.0) was employed for the statistical analysis. The results were depicted as the mean ± SEM, and the statistical significance in the distinctions between the 2-D08-treated and control (untreated) groups was evaluated

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through Student's unpaired *t-test*. Significance level were defined at *$p < 0.05$ and **$p < 0.01$.

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Results

Effects of 2-D08 on cell viability of Ut-LMS cells

We first examined the cytotoxic impact of 2-D08 **(Figure 1)** treatment on uterine leiomyosarcoma cells, employing the established SK-LMS-1 cell model. We determined the viability of SK-LMS-1 cells using MTT assay followed by 2-D08 treatment with different concentrations (0–100 μM) for 24 or 48 h. As observed in **Figure 2A**, the viability of SK-LMS-1 cells was significantly decreased to 84% in the 100 μM 2-D08-treated group at 24 h, although different doses did not cause statistically significant changes. Additionally, the viability of the SK-LMS-1 cells was also significantly decreased to 85% and 80% at 2-D08 concentrations of 50 and 100 μM, respectively, after 48 h of treatment with 2-D08 **(Figure 2B)**. These results suggest that the viability of SK-LMS-1 cells is notably impacted by 2-D08 treatment.

2-D08 inhibits colony formation of Ut-LMS cells

We further investigated whether 2-D08 affects the proliferation of SK-LMS-1 cells using a colony formation assay. As demonstrated in the crystal violet-stained (upper panels) and bright field images (lower panels) in **Figure 3A**, the size of a single colony in the 2-D08-treated groups (20, 50, and 100 μM) after 7 d was smaller than that in the control group. In contrast to control cells, exposing SK-LMS-1 cells to varying concentrations of 2-D08 (10, 20, 50, and 100 μM) for a duration of 7 d resulted in a dose-dependent reduction in colony forming efficiency. Treatment with 50 and 100 μM 2-D08

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decreased cell colony numbers to 8.2% and 0.33%, respectively **(Figure 3B)**. These findings indicate that 2-D08 is pivotal in suppressing the formation of cell colonies and is a potential drug for clinical applications in Ut-LMS cells.

2-D08 suppresses the proliferation of Ut-LMS cells *in vitro*

The above studies have demonstrated that a consistent growth inhibitory effect occurs with 50 μM 2-D08 at 48 h and that high doses (100 μM) have the potential to induce nonspecific cytotoxicity. Therefore, further experiments were conducted using 20 and 50 μM doses for 48 h.

In addition, cell proliferation was detected using Ki67 immunostaining and BrdU cell proliferation assay. **Figure 4A** display the expression of the proliferation marker Ki67. The proportion of Ki67-positive cells reduced from 72.9% to 53.6% after treatment with 50 μM 2-D08 at 48 h (n = 10) **(Figure 4B)**. Furthermore, BrdU incorporation in proliferating cells was significantly decreased in 50 μM 2-D08-treated cells at 48 h compared with that in the control (n = 10) **(Figure 5, right graph)**. Conversely, no notable difference was observed in the BrdU incorporation proliferation assay after treatment with 20 and 50 μM 2-D08 at 24 h **(Figure 5, left graph)**. These results indicate that 2-D08 treatment induces anti-proliferative effects in SK-LMS-1 cells.

Effect of 2-D08 on the protein expression related to cell proliferation and apoptosis in Ut-LMS cells

To study the detailed mechanism(s) of 2-D08, protein levels of cell cycle-regulatory proteins, specifically examining p21, p27, and p53, were assessed through western blot

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analysis. The results showed that treatment of 2-D08 on the SK-LMS-1 cells significantly increased p21 protein levels at 20 and 50 μM but not the p53 levels. In addition, the low dose of 2-D08 (20 μM) increased p27 levels, while the high dose of 2-D08 (50 μM) did not show a significant increase **(Figure 6A and B)**.

We further examined whether treatment with 2-D08 induced cell death in SK-LMS-1 cells. First, we measured the activation of Caspase-3 as an early marker of apoptosis induction in cells treated with 2-D08. The Caspase-3 Assay Kit detected the activation of Caspase-3 in cell lysates. **Figure 7A** shows that there was no significant elevation in Caspase-3 activity in cells subjected to 2-D08 treatment compared to that in the control group. We then conducted the western blot analysis with Caspase-3 and pro-apoptotic protein cleaved Caspase-3 antibody using cell lysates. No Caspase-3 cleavage was observed under these conditions, indicating that 2-D08 was unable to activate Caspase-3 **(Figure 7B)**. Together, these findings suggest that the regulation of cell cycle proteins may be involved in 2-D08-induced anti-proliferation in SK-LMS-1 cells.

2-D08 treatment inhibits the degradation of p21 by Ub-proteasome pathway in Ut-LMS cells

To analyze the regulation of cell proliferation regulatory proteins by the proteasome pathway, treatment of SK-LMS-1 cells involved exposing them to MG132, a proteasome inhibitor, at a concentration of 20 μM. As shown in **Figure 8A and B**, the level of p21 protein was significantly increased after 4 h treatment with MG132. In contrast, the levels of p27 and p53 proteins were not affected. To further test whether 2-D08 inhibits p21 ubiquitination, we transfected the pcDNA3-Ub construct into SK-LMS-1 cells treated

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with 2-D08 (50 μM) for 48 h and conducted an *in vivo* ubiquitination assay. In this assay, western blot analysis first confirmed the high level of Ub expression in SK-LMS-1 cells following transfection **(Figure 9A)**. Next, when endogenous p21 protein was immunoprecipitated with an anti-p21 antibody, Ub was detected in the complex **(Figure 9B)**. Thus, 2-D08 decreased p21 ubiquitination compared to that in the control **(Figure 9B, lane 3)**. This suggests that the inhibition of proteasomes results in increased steady-state levels of p21, and 2-D08 can modulate these pathways in the SK-LMS-1 cells.

The impact of 2-D08 on the expression of differentiation marker proteins in Ut-LMS cells

Smooth muscle cells (SMCs) at the differentiation stage are distinguished by elevated levels of α-SM-actin, calponin 1, TAGLN (SM22α), and SM-MHC, serving as specific markers for the contractile apparatus [25, 26]. Various malignancies of SMCs can modulate the differentiated to dedifferentiated phenotype in response to alterations in the surrounding environment [27-31]. For example, human Ut-LMS cell lines, including SK-LMS-1, demonstrate low expression of SM-specific markers because these cancerous cells have a dedifferentiated SMC phenotype [31]. Therefore, we investigated whether 2-D08 affects the expression of SM-specific markers. As shown in **Figure 10A and B**, the treatment of SK-LMS-1 cells with 2-D08 did not significantly affect the expression of α-SM-actin, TAGLN, and calponin 1. Furthermore, the mRNA levels of SM-specific markers, excluding TAGLN, showed no significant difference compared to the group treated with 2-D08 **(Figure 11)**. Taken together, the above findings demonstrate that 2-D08 does not directly regulate the phenotypic modulation of SK-LMS-1 cells.

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Discussion

In this study, we first identified the novel role of 2-D08 in the proliferation of Ut-LMS cells **(Figure 12)**. Although 2-D08 is currently known as a SUMO inhibitor, several studies have shown the biological functions of 2-D08 in different cells. Recently, 2-D08 was shown to suppress the proliferation and differentiation of C2C12 myoblast cells [24]. In this study, we assumed that 2-D08 can affect the proliferation of Ut-LMS, and conducted *in vitro* studies. Our data revealed that 2-D08 suppressed the proliferation of SK-LMS-1 cells **(Figure 2-5)**. In particular, both low-dose (10–20 μM) and high-dose (50–100 μM) treatment with 2-D08 significantly inhibited long-term colony formation as compared to that in the control **(Figure 3)**. However, our data showed that the growth inhibitory effect of 2-D08 was not accompanied by an induction of apoptosis **(Figure 7)**. According to a previous report, 2-D08 induces apoptosis in AML cells [23]. Previous studies suggest that the biological processes during the treatment with 2-D08 may depend on differences between experimental conditions, such as cell type.

p21, p27, and p53 are direct or indirect suppressors of tumor development and play different roles in human [32]. Uterine leiomyosarcomas exhibit elevated p53 expression in contrast to smooth muscle tumors of indeterminate malignancy [33, 34]. In addition, p21 upregulation contributes to cell cycle arrest via the activation of p53 [35]. Consistent with these reports, we hypothesized that 2-D08 affects the expression of p21, p27, and p53 in SK-LMS-1 cells. As shown in **Figure 6**, there was no anti-proliferative effect of 2-D08 when cells were treated with a low dose (20 μM) for 48 h, although the expressions

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of p21 and p27 proteins were significantly increased. Furthermore, the expression of p21 protein only increased at the high dose (50 μM) of 2-D08 through the induction of anti-proliferative activity. Nevertheless, the protein levels of p53 did not exhibit a significantly association with either of the administered doses and the regulation of p21 and p27 by 2-D08 did not trigger apoptosis **(Figure 7)**. This is because p21 has the potential to protect cells from apoptosis [36]. Therefore, these results indicated that p21 and p27 can act individually under each experimental condition and p21 is required more than p27 for 2-D08-induced anti-proliferation. However, as shown in **Figure 3**, p27 can also be involved in the inhibition of Ut-LMS cell proliferation at low doses of long-term 2-D08 treatment. To further confirm the mechanism involved, additional experiments are required to identify the differences.

Ubiquitination plays key roles in normal development, but when a part of this process is disrupted, uncontrolled proliferation of cells occurs, leading to tumor growth [37]. According to our results, the cellular p21 level was influenced by 2-D08 in SK-LMS-1 cells **(Figure 6)**. Moreover, p21 protein levels are controlled by ubiquitin-proteasome degradation in SK-LMS-1 cells, and p21 ubiquitination was inhibited when cell proliferation was suppressed by 2-D08 **(Figure 8 and 9)**. These results indicate that proteasome-mediated degradation of p21 promotes cell proliferation in SK-LMS-1 cells, and the modulation of cell proliferation by 2-D08 is attributed, in part, to its role in reducing the ubiquitination of p21.

In particular, E3 ubiquitin ligases play a crucial role in facilitating the ubiquitination process, and the deregulated expression of these enzymes is related to malignant

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progression in human cancers. Various E3 ubiquitin ligases, including MDM2, Parkin, FBXO22, and Trim27, are involved in the ubiquitination and subsequent degradation of p21 [38-41]. In addition, USP11 induces deubiquitination of p21 and stabilizes p21 protein from ubiquitin-mediated degradation [42]. Hsp90 also protects p21 from proteasome-mediated degradation [43]. However, the molecular mechanisms of p21 ubiquitination on the growth of Ut-LMS remain unclear. Therefore, to fully understand these mechanisms, additional investigations are necessary to identify the specific E3 ubiquitin ligase involved and candidate genes for the p21 ubiquitination process in Ut-LMS cells. These studies will contribute to improving the treatment of Ut-LMS.

Unlike cardiac or skeletal muscle cells, SMCs are unique in their capacity to switch their phenotypes [30, 44, 45]. SMC phenotypic conversion has been extensively studied in vascular diseases, such as atherosclerosis [46]. Although vascular SMC conversion has been intensively studied, several studies have shown that phenotypic modulation is strongly correlated with the development of Ut-LMS. In a study published in 2010, human Ut-LMS cell lines were found to have a less-differentiated SMC phenotype, whereas the exogenous expression of myocardin in Ut-LMS cells induced the increased expression of SM-specific markers and the stabilization of actin fibers [31]. We thus assumed that 2-D08 is associated with an altered phenotype of SK-LMS-1 cells. However, our results showed that the expression of SMC marker genes did not significantly alteration in the treatment of SK-LMS-1 cells with 2-D08 **(Figure 10)**. These results suggest that 2-D08 should be referred to as an anti-proliferative effector rather than a phenotypic modulator for the SK-LMS-1 cells. Although the mechanisms responsible for the phenotypic conversion induced by 2-D08 remain unclear, other candidate genes or

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different drugs can play pivotal roles in the phenotypic conversion of Ut-LMS.

There are several limitations to this study: i) further examination with different Ut-LMS cell lines is recommended to demonstrate its significance; ii) extensive studies such as animal models is essential to discern the biological effects of 2-D08 and the mechanisms underlying the roles; iii) most of the experiments were conducted with two short term (48 h) doses (20 and 50 μM) of 2-D08 for *in vitro* molecular studies. Determining the optimal dose of 2-D08 is crucial for its potential therapeutic application in human Ut-LMS.

In summary, this study demonstrated that 2-D08 partially inhibited cell proliferation in SK-LMS-1 cells by targeting p21 for ubiquitination and proteasomal degradation **(Figure 12)**. Although further research is necessary to gain a comprehensive understanding of the underlying biological mechanisms, these finding indicated that 2-D08 treatment could potentially suppress proliferation and influence the progression of tumor development in Ut-LMS.

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Figures

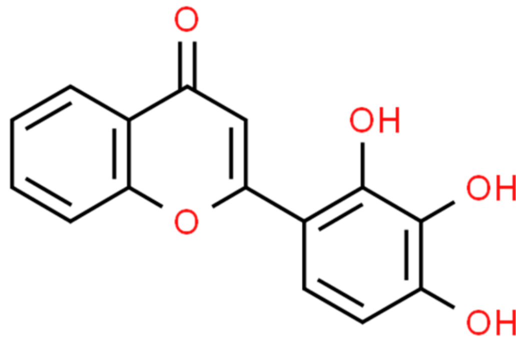

Figure 1. Chemical structure of 2-D08.

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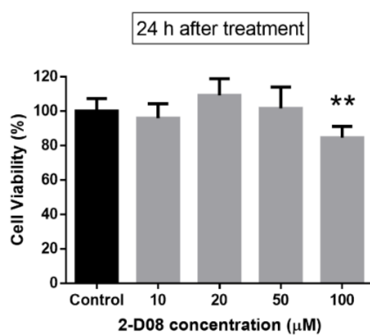

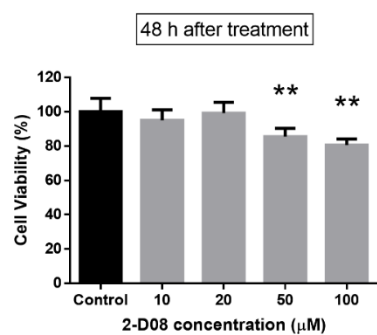

Figure 2. 2-D08 decreased cell viability in the SK-LMS-1 cells. **(A, B)** The cells were treated with 2-D08 in various concentrations (0–100 μM) for 24 or 48 h. After the end of incubation, MTT assay was employed to evaluate the viability of SK-LMS-1 cells. *p < 0.05; **p < 0.01 vs. control.

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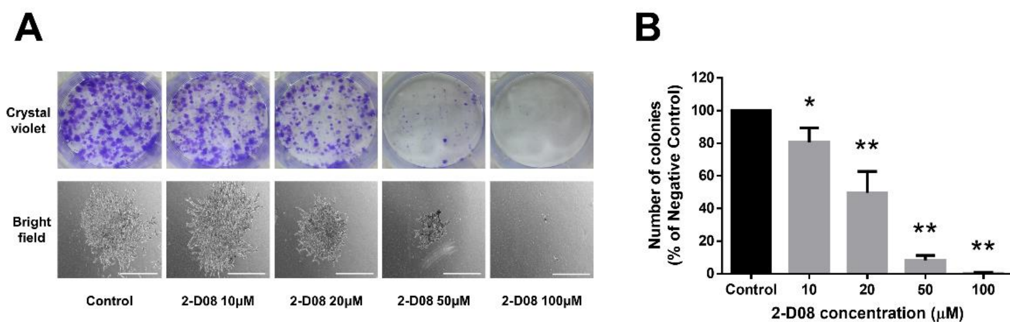

Figure 3. 2-D08 decreased colony formation ability in the SK-LMS-1 cells. **(A)** Cells underwent a 7 d treatment with 2-D08 at concentrations ranging from 0 to 100 μM). Microscopic images illustrating representative colonies were stained with crystal violet (scale bar = 1000 μm). **(B)** After 7 d, statistical analysis was conducted on colony formation in both the control and 2-D08-treated groups. Data are reported as means ± SEM. The experiments were replicated three times for validation. *$p < 0.05$; **$p < 0.01$ vs. control.

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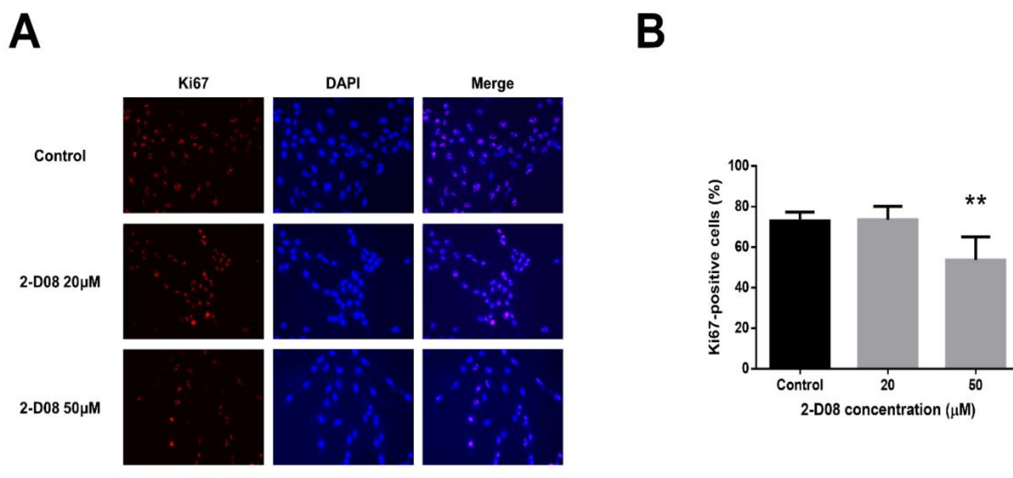

Figure 4. Inhibition of SK-LMS-1 cell proliferation by 2-D08. **(A)** During a 48 h period, SK-LMS-1 cells underwent treatment with 20 and 50 μM concentrations of 2-D08, and the immunofluorescence assay was used to analyze cell proliferation marker Ki67 and its expression in 2-D08-treated SK-LMS-1 cells. Red, Ki67; Blue, nuclear DNA (DAPI). Bar, 200 μm. **(B)** The ratio of Ki67-positive cells as a percentage of the total cell number at the indicated concentrations at 48 h. The data is expressed as mean ± SEM based on three independent tests. *$p < 0.05$; **$p < 0.01$ vs. control.

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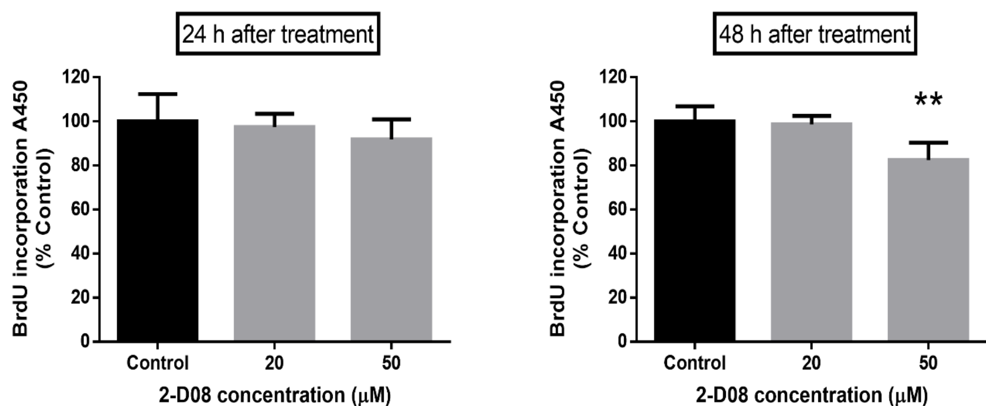

Figure 5. The cell proliferation assay using BrdU labeling. BrdU assay was conducted following 24 and 48 h treatment of SK-LMS-1 with 2-D08 (20 or 50 μM). Cells were exposed to 20 or 50 μM of 2-D08 for 24 h for the BrdU incorporation assay. 2-D08 did not lead to any clear changes in the result of BrdU incorporation assay in SK-LMS-1 cells after exposure for 24 h. Absorbance was measured at 450 nm. The results are presented as mean ± SEM from three distinct tests. $*p < 0.05$; $**p < 0.01$ vs. control.

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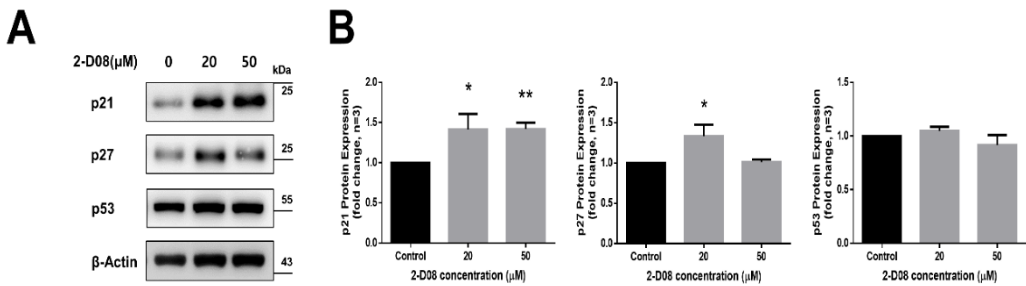

Figure 6. The effects of 2-D08 on the expression of cell proliferation genes in SK-LMS-1 cells. **(A)** Expression of p21, p27, and p53 protein in SK-LMS-1 cells exposed to 2-D08. **(B)** The bar graph shows the density ratios of p21, p27, and p53 protein bands relative to β-actin bands. Data are presented as mean ± SEM of three independent tests. *$p < 0.05$; **$p < 0.01$ vs. control

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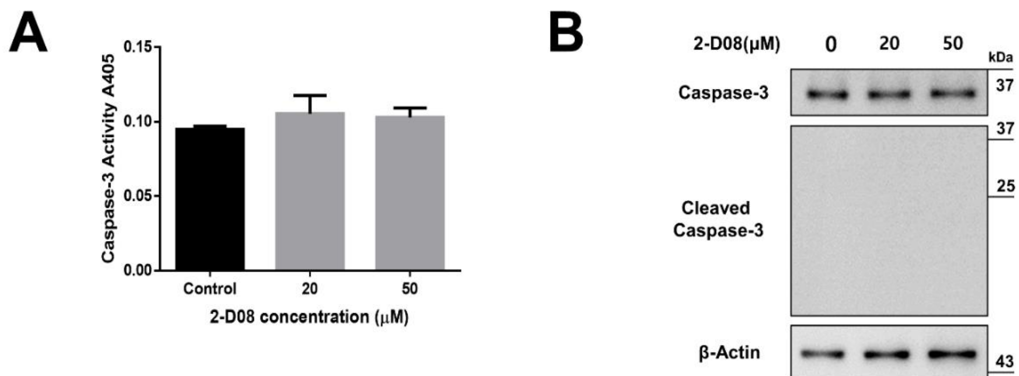

Figure 7. The effects of 2-D08 on the expression of apoptosis-related genes in SK-LMS-1 cells. **(A)** Effect of 2-D08 on the Caspase-3 activity in the SK-LMS-1 cells. **(B)** Western blot analysis of the effect of 2-D08 on the activation and proteolytic cleavage of Caspase-3 in the SK-LMS-1 cells.

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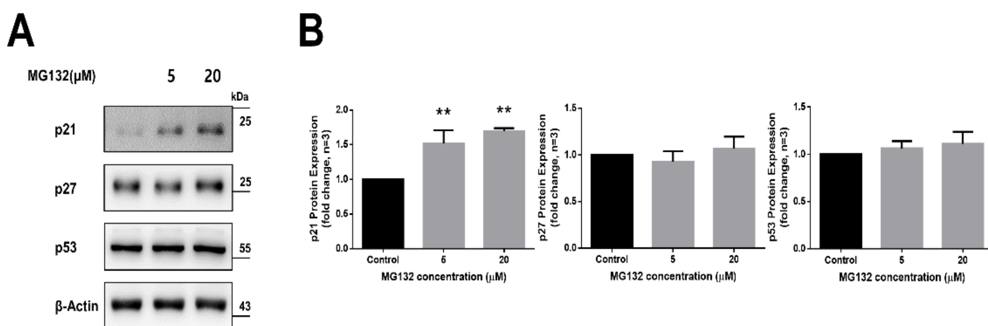

Figure 8. Proteasome inhibition increased the steady-state levels of p21 in SK-LMS-1 cells. **(A)** Cells were exposed to the indicated concentrations of MG132 for 4 h, and p21, p27, and western blot analysis was employed to assess the level of p53. **(B)** Bar graphs depict the quantification of the p21, p27, and p53 protein levels normalized to β-actin in each condition.

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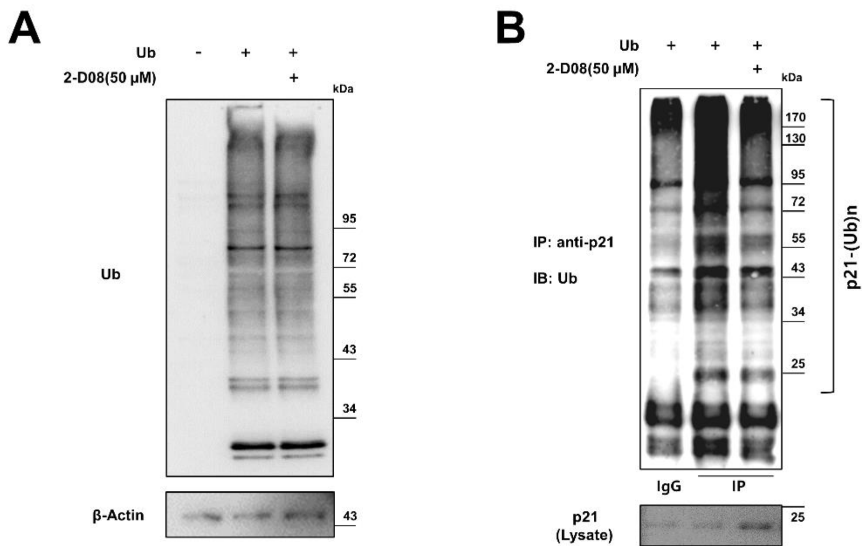

Figure 9. 2-D08 inhibits p21 ubiquitination in SK-LMS cells. **(A)** Cells were transfected with Ub expression vector and were treated with 50 µM of 2-D08 for 48 h. Cell lysates were immunoprecipitaed by anti-p21 antibody. Ubiquitinated p21 proteins were analyzed using western blot analysis with the anti-Ub antibody. **(B)** Transfection of cells was performed both with and without the Ub expression vector. Western blot analysis of Ub proteins was performed

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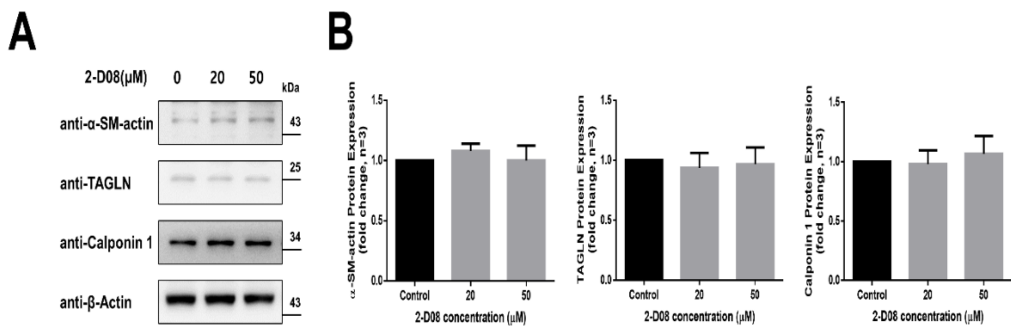

Figure 10. The analysis of contractile SMC-specific proteins expression was conducted using western blot analysis. **(A)** The expressions of α-SM-actin, TAGLN, and Calponin 1 were assessed using the western blot analysis. **(B)** Quantification of α-SM-actin, TAGLN, and Calponin 1. The bands in the left panel were quantified by the image analyzer. The results are presented as means ± SEM (n = 3).

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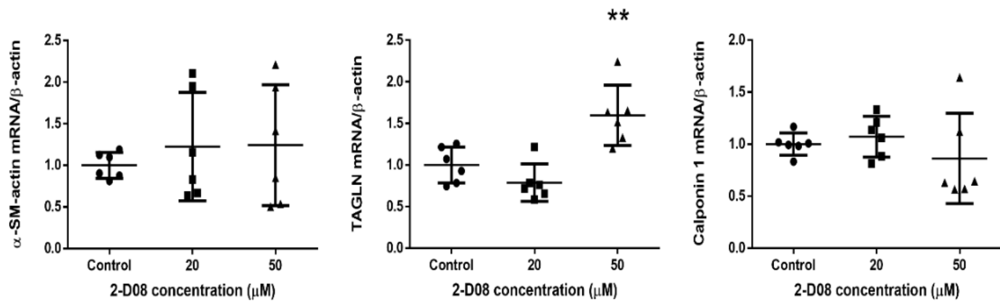

Figure 11. Expression of contractile SMC-specific proteins analyzed using quantitative real-time PCR analysis. The mRNA levels of α-SM-actin, TAGLN, and Calponin 1 were assessed using quantitative real-time PCR analysis. The results are presented as means ± SEM (n = 6).

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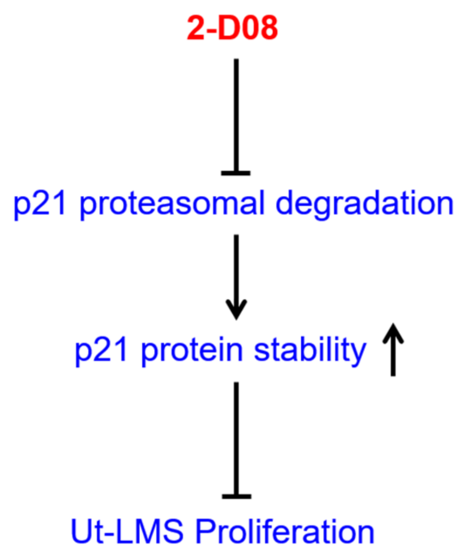

Figure 12. Diagram. 2-D08 inhibits cell proliferation by regulating the stabilization of p21.

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References

1. Echt G, Jepson J, Steel J, Langholz B, Luxton G, Hernandez W, Astrahan M, Petrovich Z. Treatment of uterine sarcomas. Cancer 1990; 66(1): 35-9.
2. Kim WY, Chang SJ, Chang KH, Yoon JH, Kim JH, Kim BG, Bae DS, Ryu HS. Uterine leiomyosarcoma: 14-year two-center experience of 31 cases. Cancer Res Treat 2009; 41(1): 24-8.
3. Tirumani SH, Deaver P, Shinagare AB, Tirumani H, Hornick JL, George S, Ramaiya NH. Metastatic pattern of uterine leiomyosarcoma: retrospective analysis of the predictors and outcome in 113 patients. J Gynecol Oncol 2014; 25(4): 306-12.
4. Chern JY, Boyd LR, Blank SV. Uterine Sarcomas: The Latest Approaches for These Rare but Potentially Deadly Tumors. Oncology (Williston Park) 2017; 31(3): 229-36.
5. Murakami M, Ichimura T, Kasai M, Matsuda M, Kawamura N, Fukuda T, Sumi T. Examination of the use of needle biopsy to perform laparoscopic surgery safely on uterine smooth muscle tumors. Oncol Lett 2018; 15(6): 8647-51.
6. Hernando E, Charytonowicz E, Dudas ME, Menendez S, Matushansky I, Mills J, Socci ND, Behrendt N, Ma L, Maki RG, Pandolfi PP, Cordon-Cardo C. The AKT-mTOR pathway plays a critical role in the development of leiomyosarcomas. Nat Med 2007; 13(6): 748-53.
7. Babichev Y, Kabaroff L, Datti A, Uehling D, Isaac M, Al-Awar R, Prakesch M, Sun RX, Boutros PC, Venier R, Dickson BC, Gladdy RA. PI3K/AKT/mTOR

CHOSUN UNIVERSITY 1946
조선대학교
CHOSUN UNIVERSITY

inhibition in combination with doxorubicin is an effective therapy for leiomyosarcoma. J Transl Med 2016; 14: 67.

8. Gaumann AK, Drexler HC, Lang SA, Stoeltzing O, Diermeier-Daucher S, Buchdunger E, Wood J, Bold G, Breier G. The inhibition of tyrosine kinase receptor signalling in leiomyosarcoma cells using the small molecule kinase inhibitor PTK787/ZK222584 (Vatalanib(R)). Int J Oncol 2014; 45(6): 2267-77.

9. Hayashi T, Horiuchi A, Sano K, Hiraoka N, Kanai Y, Shiozawa T, Tonegawa S, Konishi I. Molecular Approach to Uterine Leiomyosarcoma: LMP2-Deficient Mice as an Animal Model of Spontaneous Uterine Leiomyosarcoma. Sarcoma 2011; 2011: 476498.

10. Cancer Genome Atlas Research Network. Electronic address edsc, Cancer Genome Atlas Research N. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. Cell 2017; 171(4): 950-65 e28.

11. Makinen N, Aavikko M, Heikkinen T, Taipale M, Taipale J, Koivisto-Korander R, Butzow R, Vahteristo P. Exome Sequencing of Uterine Leiomyosarcomas Identifies Frequent Mutations in TP53, ATRX, and MED12. PLoS Genet 2016; 12(2): e1005850.

12. Tsuyoshi H, Yoshida Y. Molecular biomarkers for uterine leiomyosarcoma and endometrial stromal sarcoma. Cancer Sci 2018; 109(6): 1743-52.

13. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144(5): 646-74.

14. DeCensi A, Guerrieri-Gonzaga A, Gandini S, Serrano D, Cazzaniga M, Mora S, Johansson H, Lien EA, Pruneri G, Viale G, Bonanni B. Prognostic significance

조선대학교
CHOSUN UNIVERSITY

of Ki-67 labeling index after short-term presurgical tamoxifen in women with ER-positive breast cancer. Ann Oncol 2011; 22(3): 582-87.

15. Davey MG, Hynes SO, Kerin MJ, Miller N, Lowery AJ. Ki-67 as a Prognostic Biomarker in Invasive Breast Cancer. Cancers (Basel) 2021; 13(17).

16. Tollefson MK, Karnes RJ, Kwon ED, Lohse CM, Rangel LJ, Mynderse LA, Cheville JC, Sebo TJ. Prostate cancer Ki-67 (MIB-1) expression, perineural invasion, and gleason score as biopsy-based predictors of prostate cancer mortality: the Mayo model. Mayo Clin Proc 2014; 89(3): 308-18.

17. Maia R, Santos GAD, Reis S, Viana NI, Pimenta R, Guimaraes VR, Recuero S, Romao P, Leite KRM, Srougi M, Passerotti CC. Can we use Ki67 expression to predict prostate cancer aggressiveness? Rev Col Bras Cir 2022; 49: e20223200.

18. Zhang F, Zhang F, Liu Z, Wu K, Zhu Y, Lu Y. Prognostic Role of Ki-67 in Adrenocortical Carcinoma After Primary Resection: A Retrospective Mono-Institutional Study. Adv Ther 2019; 36(10): 2756-68.

19. Akhan SE, Yavuz E, Tecer A, Iyibozkurt CA, Topuz S, Tuzlali S, Bengisu E, Berkman S. The expression of Ki-67, p53, estrogen and progesterone receptors affecting survival in uterine leiomyosarcomas. A clinicopathologic study. Gynecol Oncol 2005; 99(1): 36-42.

20. Travaglino A, Raffone A, Catena U, De Luca M, Toscano P, Del Prete E, Vecchione ML, Lionetti R, Zullo F, Insabato L. Ki67 as a prognostic marker in uterine leiomyosarcoma: A quantitative systematic review. Eur J Obstet Gynecol Reprod Biol 2021; 266: 119-24.

21. Kim YS, Keyser SG, Schneekloth JS, Jr. Synthesis of 2',3',4'-trihydroxyflavone

조선대학교
CHOSUN UNIVERSITY

(2-D08), an inhibitor of protein sumoylation. Bioorg Med Chem Lett 2014; 24(4): 1094-7.

22. Choi BH, Philips MR, Chen Y, Lu L, Dai W. K-Ras Lys-42 is crucial for its signaling, cell migration, and invasion. J Biol Chem 2018; 293(45): 17574-81.
23. Zhou P, Chen X, Li M, Tan J, Zhang Y, Yuan W, Zhou J, Wang G. 2-D08 as a SUMOylation inhibitor induced ROS accumulation mediates apoptosis of acute myeloid leukemia cells possibly through the deSUMOylation of NOX2. Biochem Biophys Res Commun 2019; 513(4): 1063-69.
24. Liu H, Lee SM, Joung H. 2-D08 treatment regulates C2C12 myoblast proliferation and differentiation via the Erk1/2 and proteasome signaling pathways. J Muscle Res Cell Motil 2021; 42(2): 193-202.
25. Beamish JA, He P, Kottke-Marchant K, Marchant RE. Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering. Tissue Eng Part B Rev 2010; 16(5): 467-91.
26. Xie C, Ritchie RP, Huang H, Zhang J, Chen YE. Smooth muscle cell differentiation in vitro: models and underlying molecular mechanisms. Arterioscler Thromb Vasc Biol 2011; 31(7): 1485-94.
27. Amada S, Nakano H, Tsuneyoshi M. Leiomyosarcoma versus bizarre and cellular leiomyomas of the uterus: a comparative study based on the MIB-1 and proliferating cell nuclear antigen indices, p53 expression, DNA flow cytometry, and muscle specific actins. Int J Gynecol Pathol 1995; 14(2): 134-42.
28. Sprogoe-Jakobsen S, Holund B. Immunohistochemistry (Ki-67 and p53) as a tool in determining malignancy in smooth muscle neoplasms (exemplified by a

조선대학교
CHOSUN UNIVERSITY

myxoid leiomyosarcoma of the uterus). APMIS 1996; 104(10): 705-8.

29. Horiuchi A, Nikaido T, Ito K, Zhai Y, Orii A, Taniguchi S, Toki T, Fujii S. Reduced expression of calponin h1 in leiomyosarcoma of the uterus. Lab Invest 1998; 78(7): 839-46.

30. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 2004; 84(3): 767-801.

31. Kimura Y, Morita T, Hayashi K, Miki T, Sobue K. Myocardin functions as an effective inducer of growth arrest and differentiation in human uterine leiomyosarcoma cells. Cancer Res 2010; 70(2): 501-11.

32. Philipp-Staheli J, Kim KH, Liggitt D, Gurley KE, Longton G, Kemp CJ. Distinct roles for p53, p27Kip1, and p21Cip1 during tumor development. Oncogene 2004; 23(4): 905-13.

33. O'Neill CJ, McBride HA, Connolly LE, McCluggage WG. Uterine leiomyosarcomas are characterized by high p16, p53 and MIB1 expression in comparison with usual leiomyomas, leiomyoma variants and smooth muscle tumours of uncertain malignant potential. Histopathology 2007; 50(7): 851-8.

34. Schaefer IM, Hornick JL, Sholl LM, Quade BJ, Nucci MR, Parra-Herran C. Abnormal p53 and p16 staining patterns distinguish uterine leiomyosarcoma from inflammatory myofibroblastic tumour. Histopathology 2017; 70(7): 1138-46.

35. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B. Requirement for p53 and p21 to sustain G2 arrest

조선대학교
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after DNA damage. Science 1998; 282(5393): 1497-501.

36. Gorospe M, Cirielli C, Wang X, Seth P, Capogrossi MC, Holbrook NJ. p21(Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells. Oncogene 1997; 14(8): 929-35.

37. Sun T, Liu Z, Yang Q. The role of ubiquitination and deubiquitination in cancer metabolism. Mol Cancer 2020; 19(1): 146.

38. Xu H, Zhang Z, Li M, Zhang R. MDM2 promotes proteasomal degradation of p21Waf1 via a conformation change. J Biol Chem 2010; 285(24): 18407-14.

39. Park MH, Lee HJ, Lee HL, Son DJ, Ju JH, Hyun BK, Jung SH, Song JK, Lee DH, Hwang CJ, Han SB, Kim S, Hong JT. Parkin Knockout Inhibits Neuronal Development via Regulation of Proteasomal Degradation of p21. Theranostics 2017; 7(7): 2033-45.

40. Zhang L, Chen J, Ning D, Liu Q, Wang C, Zhang Z, Chu L, Yu C, Liang HF, Zhang B, Chen X. FBXO22 promotes the development of hepatocellular carcinoma by regulating the ubiquitination and degradation of p21. J Exp Clin Cancer Res 2019; 38(1): 101.

41. Xing L, Tang X, Wu K, Huang X, Yi Y, Huan J. TRIM27 Functions as a Novel Oncogene in Non-Triple-Negative Breast Cancer by Blocking Cellular Senescence through p21 Ubiquitination. Mol Ther Nucleic Acids 2020; 22: 910-23.

42. Deng T, Yan G, Song X, Xie L, Zhou Y, Li J, Hu X, Li Z, Hu J, Zhang Y, Zhang H, Sun Y, Feng P, Wei D, Hu B, Liu J, Tan W, Ye M. Deubiquitylation and stabilization of p21 by USP11 is critical for cell-cycle progression and DNA

조선대학교
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damage responses. Proc Natl Acad Sci U S A 2018; 115(18): 4678-83.

43. Warfel NA, El-Deiry WS. p21WAF1 and tumourigenesis: 20 years after. Curr Opin Oncol 2013; 25(1): 52-8.

44. Le Guen L, Marchal S, Faure S, de Santa Barbara P. Mesenchymal-epithelial interactions during digestive tract development and epithelial stem cell regeneration. Cell Mol Life Sci 2015; 72(20): 3883-96.

45. Scirocco A, Matarrese P, Carabotti M, Ascione B, Malorni W, Severi C. Cellular and Molecular Mechanisms of Phenotypic Switch in Gastrointestinal Smooth Muscle. J Cell Physiol 2016; 231(2): 295-302.

46. Chakraborty R, Chatterjee P, Dave JM, Ostriker AC, Greif DM, Rzucidlo EM, Martin KA. Targeting smooth muscle cell phenotypic switching in vascular disease. JVS Vasc Sci 2021; 2: 79-94.