# Apoptotic Effect of Co-Treatment with Valproic Acid and 17AAG on Human Osteosarcoma Cells

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Valproic acid (VPA) is a well-known anticonvulsive agent and has been used in the treatment of epilepsy for almost 30 years. VPA emerged in 1997 as an antineoplastic agent. And it is known that antitmor activity of VPA is associated with its targeted at histone deacetylases. 17AAG, Inhibition of HSP90 leads to the proteasome degradation of the HSP90 client proteins, such as Akt, Raf/Ras, Erk, VEGF, cyclin D and p53, and causes potent antitumor activity. It is reported that 17AAG-induced HSP90 inhibition results in prevention of cell proliferation and induction of apoptosis in several types of cancer. This study was undertaken to investigate the synergistic apoptotic effect of co-treatment with the histone deacetylases inhibitor, VPA and the HSP90 inhibitor, 17AAG on human osteosarcoma (HOS) cells.

Cell viability was evaluated by trypan-blue exclusion. Induction and augmentation of apoptosis were confirmed by Hoechst staining, flow cytometry (DNA hypoploidy and MMP change), Westen blot analysis and immunofluorescent staining.

In this study, HOS cells co-treated with VPA and 17AAG showed several lines of apoptotic manifestation such as nuclear condensations, the reduction of MMP, the decrease of DNA content, the release of cytochrome c into cytosol, the translocation of AIF onto nuclei, and activation of caspase-3, caspase-7 and PARP whereas each single treated HOS cells did not. Although the single treatment of 1 mM VPA or 0.5 μM 17AAG for 48 h did not induce apoptosis, the co-treatment with them induced prominently apoptosis. Therefore our data in this study provide the possibility that combination therapy with VPA and 17AAG could be considered as a novel therapeutic strategy for human osteosarcoma.

Key words : Apoptosis, Valproic acid, 17AAG, Human osteosarcoma

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## Ⅰ. INTRODUCTION

The acetylation state of histone is reversibly regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). An inappropriate acetylation state of histones causes abnormal outgrowth and the altered pattern of cell death, which leads to neoplasmic transformation.[1)] HDACs are overexpressed under specific environmental conditions, such as hypoxia, hypoglycemia, and serum deprivation.[2)] Among these conditions,

hypoxia is one of the key factors to trigger angiogenesis via the induction of angiogenic factors. Regulation of such gene expression through the acetylation of histone is highly involved in the control of angiogenesis.[2-4] HDAC inhibitors were known to cause the arrest of growth, differentiation, or apoptosis of a variety of transformed cells in culture, including human bladder, breast, prostate, lung, ovary and colon cancer cells.[5] Several classes of HDACIs are identified, which include organic hydroxamic acids (e.g., TSA and suberoyl anilide bisydroxamine [SAHA]), short-chain fatty acides (e.g., butyrates and valproic acid [VPA]), cyclic tetrapeptides (e.g., MS-275).[6]

Valproic acid (VPA) is a well-known anti-convulsive agent and used in the treatment of epilepsy for almost 30 years. VPA emerged in 1997 as an antineoplastic agent as well, when findings indicated that the substance inhibited proliferation and induced differentiation of primitive neuroectocdermal tumor cells in vivo.[7] Antitmor activity of VPA is associated with its targeted at histone deacetylases. VPA in particular, was able to down-regulate class II HDAC protein levels significantly in several cells in contrast to TSA, which implies that VPA might be a more selective HDAC inhibitor than TSA.[8-11]

The heat shock protein 90 (HSP90) is a molecular chaperone required of protein refolding, conformational maturation, activation of several proteins involved in signal transduction pathways, and assembly of many proteins associated with the initiation and development of cancer. Inhibition of HSP90 leads to the proteasome degradation of the HSP90 client proteins, such as Akt, Raf/Ras, Erk, VEGF, cyclin D and p53, and causes potent antitumor activity. 17AAG (17-allylamino-17-demethoxygeldanamycin) is one of the most studied inhibitors of the HSP90. It is reported that 17AAG-induced HSP90 inhibition results in prevention of cell proliferation and induction of apoptosis in several types of cancer. [12-14]

Cells undergoing apoptosis usually develop characteristic changes, including nuclear condensation and degradation of DNA into oligonucleosomal fragments.[15] Apoptotic cell death is thought to result ultimately from the proteolytic actions of caspase.[16] and alterations in mitochondrial function play a key part in the regulation of apoptosis.[17] Moreover, the proteasome system is shown to be implicated as a negative or positive mediator of apoptosis. The proteasome pathway is mostly known to work upstream of mitochondrial alterations and caspase activation.[18]

Osteosarcoma is one of the most common primay malignant tumors of bone. Treatment of this tumor with systemic chemotherapy improves dramatically the prognosis. Numerous studies depicted that the therapeutic effect of various chemotherapeutic agents on osteosarcoma depending on the induction of apoptosis.[19-21]

To date, there is no report about the synergistic apoptotic effects of co-treatment with VPA and 17AAG on human osteosarcoma cells. Therefore, this study was undertaken to investigate the synergistic apoptotic effect of co-treatment with VPA and 17AAG on human osteosarcoma (HOS) cells.

## II. MATERIALS AND METHODS

### 1. Reagents

The following reagents were obtained commercially: Mouse monoclonal anti-human caspase-3, caspase-7, poly(ADP-ribose) polymerase (PARP), cytochrome c, apoptosis-inducing factor (AIF) antibodies, and FITC-conjugated goat anti-mouse and anti-rabbit IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); HRP-conjugated sheep anti-mouse and anti-rabbit IgGs were from Amersham GE Healthcare (Little Chalfront, UK). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR, USA). Suc-LLVY-AMC was from Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and FBS were from Gibco

(Gaithersburg, MD, USA). Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, aprotinin, leupeptin, PMSF, thiazolyl blue tetrazolium bromide and propidium iodide (PI) were from Sigma (St. Louis, MO, USA); SuperSignal West Pico enhanced chemiluminescence Western blotting detection reagent was from Pierce (Rockford, IL, USA).

### 2. Cell culture

The HOS human osteosarcoma cell line was purchased from ATCC (Rockville, USA). Cells were maintained at 37℃ with 5% $CO_2$ in air atmosphere in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate supplemented with 10% FBS.

### 3. Assessment of co-treatment of valproic acid (VPA) and 17AAG

The stock solutions of VPA (2 M) made by dissolving the drug in PBS and 17AAG (10 mM) which made by dissolving the drug in ethanol were kept frozen at -20℃ until use. In twenty four hours after HOS cells were subcultured. the original medium was removed. The cells were washed with phosphate-buffered saline (PBS) and then incubated in the same fresh medium. Since 1 mM VPA and 0.5 μM 17AAG are approximately the concentration not to induce HOS cell death, it is determined to utilize this single concentration of each chemical for the combination treatment study. HOS cells were co-treated with 1 mM VPA and 0.5 μM 17AAG for 48 h. Cells were harvested, stained with trypan blue and then counted by using a hemcytometer.

### 4. Hoechst staining

Cells were harvested and cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/mL Hoechst 33342 for 30 min at 37℃ and fixed for 10 min in 4% paraformaldehyde.

### 5. Quantification of DNA hypoploidy by flow cytometry

After the treatment for 48 h, cells were harvested by trypsinization and ice cold 95% ethanol with 0.5% Tween 20 was added to the cell suspensions up to a final concentration of 70% ethanol. Fixed cells were pelleted, and washed in 1% BSA-PBS solution. Cells were resuspended in 1 mL PBS containing 20 μg/mL RNase A, incubated at 4℃ for 30 min, washed once with BSA-PBS, and resuspended in PI solution (10 μg/mL). After cells were incubated at 4℃ for 5 min in the dark, DNA content were measured on a CYTOMICS FC500 flow cytometry system (Beckman Coulter, Brea, CA, USA) and data was analyzed by using the Multicycle software which allowed a simultaneous estimation of apoptosis.

### 6. Assay of mitochondrial membrane potential (MMP)

JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS. Flow cytometry to measure MMP was performed on a CYTOMICS FC500 flow cytometry (Beckman Coulter, FL, CA, USA). Data were acquired and analyzed by using CXP software version 2.2.

### 7. Immunofluorescent staining

Cells were cytocentrifuged and fixed for 10 min in 4% paraformaldehyde, incubated with each primary antibody for 1 h, washed 3 each for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS. Fluorescent images were observed and analyzed under Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany).

8. Western blot analysis

Cells (2 x $10^6$) treated with VPA and/or 17AAG were washed twice with ice-cold PBS, which is resuspended in 200 μL ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 μL/mL aprotinin and 2 μL/mL leupeptin] and incubated at 4℃ for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4℃. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 50 μg of proteins were loaded onto 7.5-15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham GE Healthcare, Little Chalfont, UK) and reacted with each antibody. Immunostaining with antibodies was performed in using SuperSignal West Pico enhancing chemiluminescence substrate and detecting with Alpha Imager HP (Alpha Innotech, Santa Clara, USA).

## Ⅲ. RESULTS

1. Co-treatment with VPA and 17AAG augmented the reduction in viability of HOS cells.

Single treatment of 1 mM VPA or 0.5 μM 17AAG for 48 h reduced slight viability of HOS cells (VPA, 90.03% ; 17AAG, 84.00%). Co-treatment of VPA and 17AAG significantly reduced cell viability compared to the effect of each single treatment (co-treatment, 44.70%) (Fig. 1).

2. Co-treatment with VPA and 17AAG augmented the nuclear condensation and fragmentation in HOS cells.

To explore whether nuclear condensation and fragmentation were induced, Hoechst staining, a hallmark of apoptosis, was conducted. The co-treatment with VPA and 17AAG showed a variety of condensed and fragmented nuclei compared to the single treatment (Fig. 2).

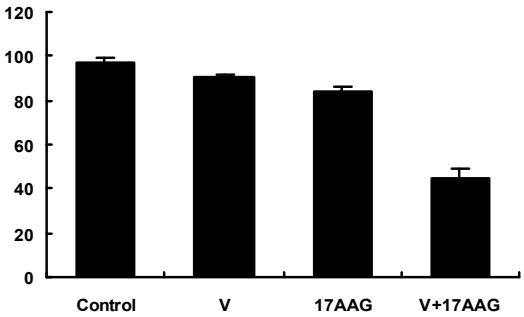


Fig. 1. Co-treatment with VPA and 17AAG significantly reduced cell viability in HOS cells. Cell viability was determined by hematocytometer. Three independent assays were performed. Values are means ± SD of triplicates of each experiment (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

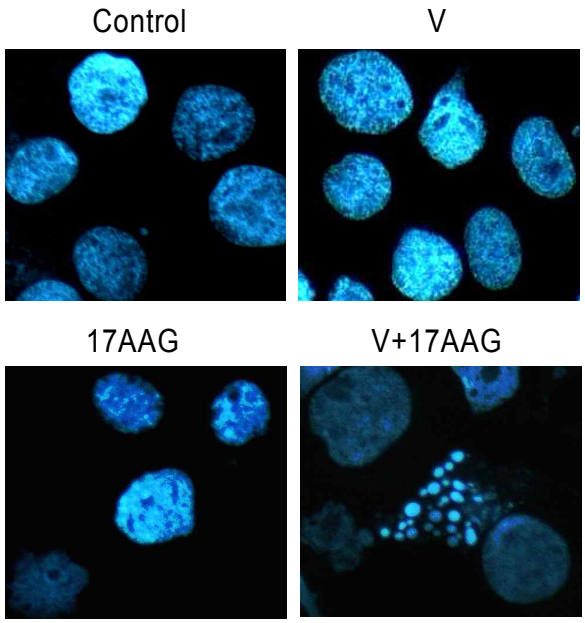


Fig. 2. Immunofluorescent micrographs showing nuclear morphology after Hoechst staining. Co-treatment with VPA and 17AAG showed numerous condensed and fragmented nuclei in HOS cells compared to the single treatment (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

3. Augmentation of apoptosis by co-treatment with VPA and 17AAG was demonstrated by the decrease of DNA content in HOS cells.

The flow cytometry showed that co-treatment with VPA and 17AAG remarkably increased apoptotic cells with DNA hypoploidy compared to the single treatment (Fig. 3).

4. Augmentation of apoptosis by co-treatment with VPA and 17AAG was demonstrated by reduction of mitochondrial membrane potential (MMP) in HOS cells.

The single treatment with VPA and 17AAG did not show the loss of MMP compared to control group. But the co-treatment with VPA and 17AAG reduced significantly MMP compared to the single treatment (Fig. 4).

5. Efficient apoptotic effect of co-treatment with VPA and 17AAG was demonstrated by Western blot assay.

The co-treatment of VPA and 17AAG induced the cleavage of PARP, and the degradation of caspase-3 and caspase-7 whereas the single treatment did not (Fig. 5).

6. Co-treatment with VPA and 17AAG showed to lead to the translocation of AIF from mitochondria onto the nuclei.

The confocal microscopy showed that AIF was located at mitochondria in the single treatment of VPA or 17AAG whereas AIF was evidently translocated onto nuclei in the co-treatment (Fig. 6).

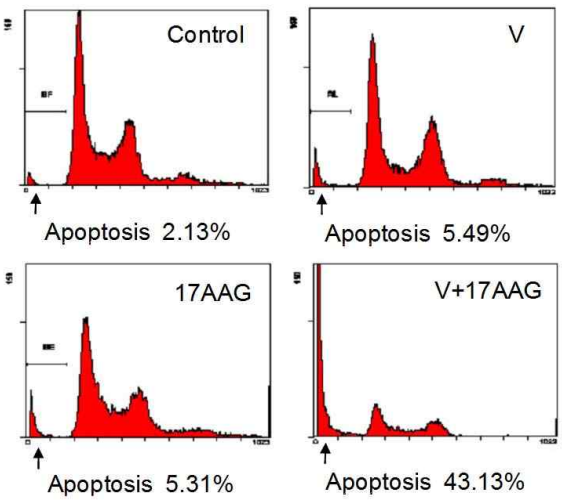


Fig. 3. The kinetic analysis of the effect of co-treatment on HOS cell cycle progression and induction of apoptosis by flow cytometry. Co-treatment showed remarkably the increase of apoptotic cells with DNA hypoplpoidy compared to the single treatment (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

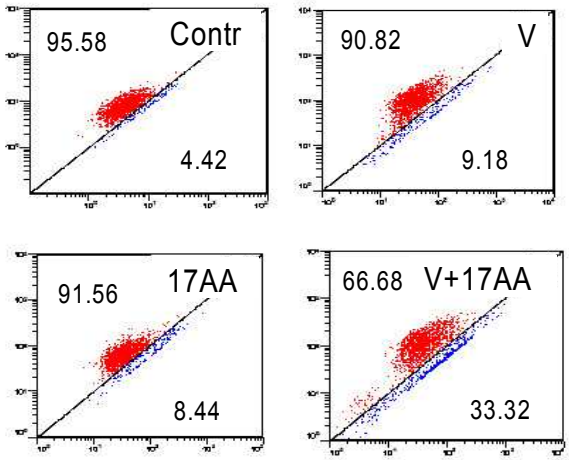


Fig. 4. Co-treatment with VPA and 17AAG showed significantly the loss of MMP (△Ψm) compared to the single treatmemt. MMP was measured by JC-1 with flow cytometry (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

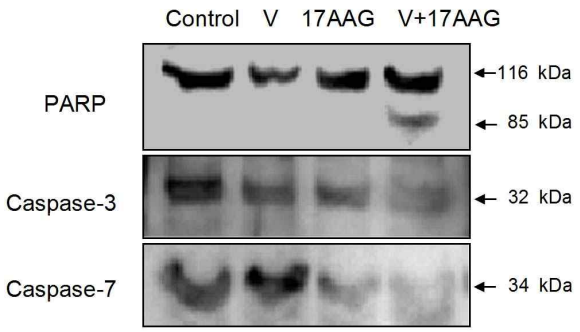


Fig. 5. Western blot analysis showing that the co-treatment with VPA and 17AAG in HOS cells remarkably induced cleavage of PARP, and caspase-3 and caspase-7 degradations (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

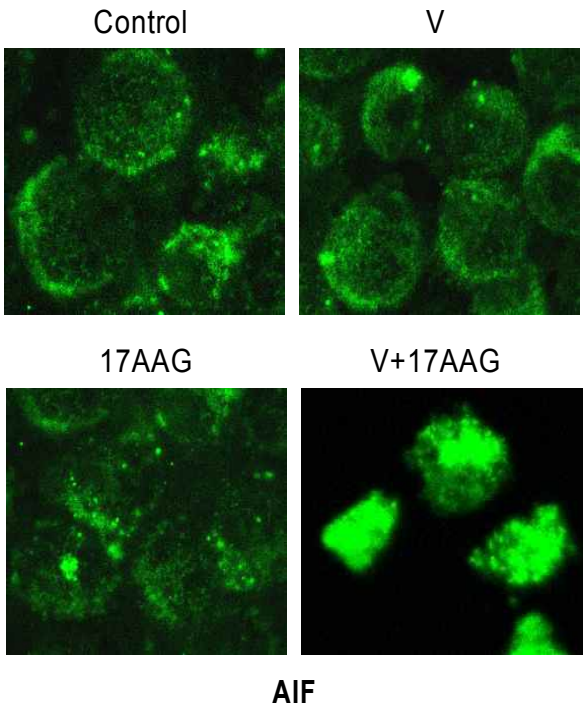


Fig. 6. The confocal microscopy showed that AIF was evidently translocated onto nuclei in HOS cells when co-treated with VPA and 17AAG (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

7. Co-treatment with VPA and 17AAG showed to lead to the release of cytochrome c from mitochondria into the cytosol.

The confocal microscopy showed that cytochrome c was located at mitochondria in the single treatment of VPA whereas cytochrome c was evidently released into the cytosol in the co-treatment or the single treatment of 17AAG (Fig. 7).

## Ⅳ. DISCUSSION

VPA was employed as monotherapy or comination therapy for various types of malignancy. [22-26] Those

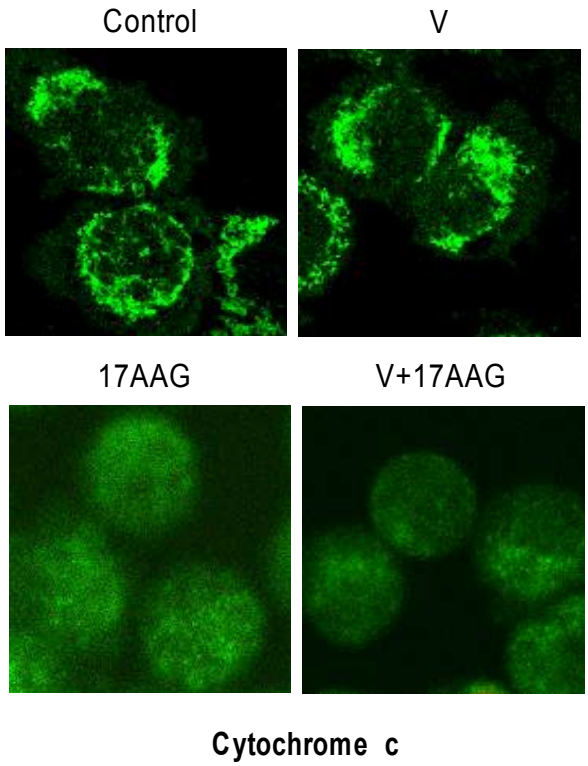


Fig. 7. The confocal microscopy showed that cytochrome c was evidently released to the cytosol in HOS cells when co-treated with VPA and 17AAG or single treated with 17AAG (V, cells treated with 1 mM VPA for 48 h; 17AAG, cells treated with 0.5 μM 17AAG for 48 h; V+17AAG, cells treated with 1 mM VPA plus 0.5 μM 17AAG for 48 h).

studies indicated that VPA as monotherapy or combination therapy showed antiproliferative activity on cancer cells. It is noticeable that VPA, at concentrations of clinical interest, enhanced significantly the antiprolifearative activity.[24,27] This range of concentrations of VPA can be achieved in an patient's serum when receiving a daily dose of 20-30 mg/kg for epilepsy. Thus, VPA is being considerd as a promising potential therapeutic agent for cancers. Combination of anticancer therapies using VPA and other drugs, especially non-toxic drugs, may offer a substantial advantage over VPA monotherapy in a clinical setting. VPA which was combined with all-trans retinoic acid[28] or interferon alpha (IFN-α)[11] was demonstrated to enhance the efficacy of each antitumor agent. Relatively recently, heat shock protein 90 (HSP90) has emerged as an important target in cancer therapy. HSP90 normally accounts for approximately 1-2% of the total cytosolic protein content, while under stress conditions, its levels increase up to 4-6% of the whole proteomic load of the cell.[29] The HSP90 inhibitor, 17AAG is reported to lead to potent antitumor activity through the proteasome degradation of the HSP90 client proteins and result in prevention of cell proliferation and induction of apoptosis.[12,14] We, in the present study, designed VPA combination anticancer therapy using 17AAG for HOS.

Mitochondria plays an important role in apoptosis and induction of the mitochondrial permeability transition play a key part in the regulation of apoptosis.[30-32] Outer mitochondrial membrane becomes permeable to intermembrane space proteins such as cytochrome c and AIF (apoptosis inducing factor) during apoptosis.[33] Releasing cytochrome c and disruption of mitochondrial membrane potential (MMP) are in fact known features in apoptosis triggered by proteasome inhibition.[34,35] On induction of apoptosis, AIF is translocated to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation.[36] This study showed evidently that co-treatment with VPA and 17AAG in HOS cells results in significant decrease of MMP, the release of cytochrome c into cytosol and the translocation of AIF onto nuclei whereas the single treatment does not.

A common final event of apoptosis is nuclear condensation, which is controlled by caspases, DFF, and PARP. Caspases, the cysteinyl aspartate-specific intracellular proteinases, play an essential role during apoptotic death.[37] Once activated, the effector caspases (caspase-3, caspase-6 or caspase-7) are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, leading ultimately to cell death. This study demonstrated that co-treatment with VPA and 17AAG in HOS cells results in the activation of caspase-3, caspase-7 and PARP whereas the single treatment does not.

In the study, HOS cells co-treated with VPA and 17AAG showed several lines of apoptotic manifestation such as nuclear condensations, the decrease of MMP, the decrease of DNA content, the release of cytochrome c into cytosol, the translocation of AIF, and activation of caspase-7, caspase-3 and PARP whereas each single treated HOS cells did not.

In conclusion, combination therapy with VPA and 17AAG could be considered, in the future, as an alternative therapeutic strategy for human osteosarcoma. Its clinical application awaits further extensive studies.

## V. CONCLUSION

Valproic acid (VPA) is a well-known anticonvulsive agent and has been used in the treatment of epilepsy for almost 30 years. VPA emerged in 1997 as an antineoplastic agent. And it is known that antitmor activity of VPA is associated with its targeted at histone deacetylases. 17AAG, Inhibition of HSP90 leads to the proteasome degradation of the HSP90 client proteins, such as Akt, Raf/Ras, Erk, VEGF, cyclin D and p53, and causes potent antitumor activity. It is reported that 17AAG-induced HSP90 inhibition results in prevention of cell proliferation and induction of apoptosis in several types of cancer. This study was

undertaken to investigate the synergistic apoptotic effect of co-treatment with the histone deacetylases inhibitor, VPA and the HSP90 inhibitor, 17AAG on human osteosarcoma (HOS) cells.

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국문초록

# Valproic acid와 17AAG의 병용처리가 사람골육종세포에 미치는 세포자멸사 효과에 대한 연구

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Valproic acid(VPA)는 아주 잘 알려진 항경련제로서, 30년 동안 간질치료제로서 사용되어져 왔다. VPA는 1997년에 최초로 항암제의 효능이 밝혀졌으며, VPA의 항암효과는 히스톤탈아세틸화효소 억제제의 기전에 기인한다고 규명되었다. 17AAG(17-Allyamnio-17-demethoxygeldanamycin)는 HSP90의 억제제이며, HSP90은 세포증식과 세포생존에 관여하며, 최근 17AAG가 세포자멸사를 유도한다는 연구들이 보고되어지고 있다. 본 연구는 히스톤탈아세틸화효소억제제인 VPA와 HSP90 억제제인 17AAG의 병용처리가 사람골육종세포에 상승 세포자멸사 효과가 있는지를 알기 위해서 수행되었다.

VPA과 17AAG의 병용처리가 단독처리에 비해서 효과적인 세포생존율 감소가 있는지 확인하기 위해서 trypan-blue법을 시행하였고, 세포자멸사의 유도와 증가를 확인하기 위해서 Hoechst 염색법, flow cytometry(DNA hypoploidy와 MMP 측정), Western bot 분석법 그리고 면역형광염색법을 수행하였다.

병용처리 된 사람골육종세포는 단독처리 된 사람골육종세포에서 거의 관찰할 수 없었던 핵 응축과 조각남, 사립체막 전위와 DNA 양의 감소, cytochrome c의 세포질로의 유리, AIF의 핵으로의 이동, caspase-3과 caspase-7의 파괴 및 PARP의 분절화와 같은 세포자멸사 증거를 보였다.

48시간 동안 1 mM의 VPA와 0.5 μM 17AAG을 각기 단독처리 한 결과에서는 세포자멸사를 유도 못했으나, 병용처리한 결과에는 아주 탁월한 세포자멸사의 유도를 보였다. 이러한 병용처리 결과는 사람골육종의 새로운 치료적 전략으로 응용될 수 있다고 생각한다.

주요어: 세포자멸사, valproic acid, 17AAG, 사람골육종