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Clobenpropit Enhances Antitumor Effect of Gemcitabine in Pancreatic Cancer

췌장암에서 Clobenpropit에 의한 Gemcitabine의 항암효과 강화

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논문 제목: Clobenpropit Enhances Antitumor Effect of Gemcitabine in Pancreatic Cancer

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ABSTRACT

Introduction: Pancreatic cancer shows dismal prognosis due to early metastasis, frequent recurrence and chemoresistance. However, there is no effective treatment to overcome these problems. Histamine is associated with carcinogenesis through activation of its 4 membrane-specific receptors, thus the agents targeting histamine receptor could be the new therapeutic drug in pancreatic cancer. The aim of this study was to evaluate the anti-tumor effect of clobenpropit, which is the specific H_3 antagonist and H_4 agonist, with gemcitabine combination in pancreatic cancer cell line.

Methods: Three kinds of human pancreatic cancer cell lines (Panc-1, MiaPaCa-2, and AsPC-1) were used in this study. Expression of H_3 and H_4 receptor in pancreatic cancer cell was identified with Western blotting. Effects of clobenpropit on cell proliferation, migration, invasion, and apoptosis were evaluated by MTS proliferation assay, wound healing assay, invasion assay and annexin V binding assay. Alteration of epithelial and mesenchymal markers after administration of clobenpropit was analyzed. *In vivo* study with Panc-1 xenograft mouse model was also performed.

Results: H_4 receptors were present as 2 subunits in human pancreatic cancer cells, while there was no expression of H_3 receptor. Clobenpropit inhibited migration and increased apoptosis of pancreatic cancer cells in combination with gemcitabine, while did not affect cell invasion property. Clobenpropit upregulated E-cadherin, whereas downregulated vimentin and matrix

metalloproteinase 9 in real-time PCR. Also, clobenpropit enhanced apoptosis in combination with gemcitabine by upregulation of E-cadherin and downregulation of Zeb1 in Panc-1 xenograft mouse.

Conclusions: Clobenpropit enhanced antitumor effect of gemcitabine in pancreatic cancer cells through inhibition of EMT process.

Keywords: Pancreatic neoplasm, Clobenpropit, Histamine, Histamine receptors, Epithelial mesenchymal transition

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G, gemcitabine; C, clobenpropit.

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G, Gemcitabine; C, Clobenpropit.

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G, Gemcitabine; C, Clobenpropit.

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G, Gemcitabine; C, clobenpropit.

**, $P < 0.01$; *** $P < 0.001$

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E-cadherin was also increased in clobenpropit alone and combination group by Western blotting, whereas Zeb1, the repressor of E-cadherin, was decreased in combination group (D).

G, Gemcitabine; C, clobenpropit.

* $P < 0.05$; ** $P < 0.01$.

LIST OF ABBREVIATIONS

EMT: epithelial mesenchymal transition

ECM: extracellular matrix

MMP-9: matrix metalloproteinase 9

PMSF: phenylmethylsulfonyl fluoride

DMEM: Dulbecco modified Eagle medium

FBS: fetal bovine serum

TUNEL: terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling

Introduction

Pancreatic cancer is a very aggressive human cancer and has dismal prognosis with only 6% of patients survive 5 years after diagnosis.[1] In spite of the progresses of treatments, the attempts at improving survival of pancreatic cancer in the past 15 years, especially in the advanced disease setting, have failed and resulted in no significant improvement.[2] Surgical resection is the only potentially curative treatment in pancreatic cancer, however only 15% of patients could be candidate for resection.[3, 4] Some chemotherapeutic agents are used in pancreatic cancer, and gemcitabine became the standard chemotherapeutic agent in pancreatic cancer after randomized trial in 1997.[5] Gemcitabine is a nucleoside pyrimidine analogue which exerts its cytotoxic actions primarily by the incorporation of gemcitabine triphosphate into DNA, leading to masked chain termination.[6] However, pancreatic cancer is highly resistant to chemotherapy including gemcitabine,[7] and the disappointing circumstance of pancreatic cancer is mainly due to late diagnosis at which chemoresistance in patients is a critical issue.[8] Resistance to gemcitabine has been increasing and the effectiveness of gemcitabine has been reduced to less than 20%.[9] Hence, new therapeutic targets and chemotherapeutic agents of pancreatic cancer are desperately required.

High concentration of histamine has been shown in melanoma,[10] small cell lung carcinoma,[11] breast carcinoma[12] and colorectal carcinoma.[13] Histamine participates in tumor proliferation or apoptosis through activation of its four membrane-specific receptors, H_1, H_2, H_3 and H_4.[14] As in other human cancers,

expressions of histidine decarboxylase and histamine contents have been reported in pancreatic cancer.[15, 16] Histamine inhibits proliferation through the H_1 receptor and H_2 receptor, which is associated with a partial differentiation in pancreatic cancer.[17] Through H_2 receptor, histamine induces G0/G1 phase arrest and modulation of MAPK and Bcl-2 family proteins.[18-20] Furthermore, previous study suggests that H_3 and H_4 receptors are involved in pancreatic cancer cell growth, with proliferation increased through H_3 receptor and diminished via H_4 receptor.[21] However, the mechanism of anti-cancer effect through histamine receptor still remains unclear.

Clobenpropit, which is a specific H_3 antagonist and H_4 agonist, inhibits the spread of mammary adenocarcinoma by decreasing invasion potential.[22] Recent study defines the modulation of H_4 receptor by clobenpropit disrupts epithelial mesenchymal transition (EMT) processes, extracellular matrix (ECM) breakdown, and invasion potential and decreases tumor growth in cholangiocarcinoma.[23] Similarly, EMT plays a crucial role which is associated with tumor progression and metastasis of pancreatic cancer.[24] Furthermore, EMT regulators including Zeb1 are known to induce chemoresistance of human pancreatic cancer cells.[25] Thus, therapeutic agents targeting EMT process could restore the chemoresistance of pancreatic cancer. Therefore, we aimed to investigate the anti-cancer efficacy of clobenpropit with gemcitabine combination in human pancreatic cancer cells. Additionally, we evaluated the alteration of EMT markers after administration of clobenpropit with *in vitro* and *in vivo* studies.

Materials and methods

Pancreatic cancer cell lines

Human pancreatic cancer cell lines, Panc-1, MiaPaCa-2 and AsPC-1 were obtained from Korea Cell Line Bank and maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). They were incubated at 37°C and 5% CO_2.

Western blotting

After washing with PBS, three kinds of pancreatic cancer cells (Panc-1, MiaPaCa-2 and AsPC-1) were processed and lysed in NP-40 buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mg/mL protease inhibitor mixture). Then, proteins were quantified with a BCA protein assay kit (Pierce, Rockford, IL). Proteins were separated by 10% to 15% SDS-polyacrylamide denaturing gels, transblotted onto nitrocellulose membranes and probed with rabbit antihuman H_3 and H_4 receptor antibodies (Millipore, Billerica, MA). Immunoreactivity was developed using a peroxidase conjugate antiserum (Sigma-Aldrich, St Louis, MO) and detected by enhanced chemiluminescence reagents (Amersham Biosciences, Baied'Urfe, Quebec, Canada). Western blotting of Panc-1, MiaPaCa-2 and AsPC-1 treated with 50 or 100 μM of clobenpropit (Sigma-Aldrich) alone or with 5 μM of gemcitabine (Yuhan, Seoul, Korea) combination was also performed.

MTS proliferation assay

Cells were plated into 96-well plates at a density of 4 x 10^3 cells/well and stimulated with clobenpropit (1 to 100 μM) for up to 48 hours to determine optimal dose and stimulation time. 3-(4,5-Demethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS; Promega, Madison, WI) was added to the cells, and the numbers of live cells were counted after allowing development for 1 hour. The plates were read on a Wallac-1420 plate reader (Perkin-Elmer, Boston, MA) at an absorbance of 490 nm. Data are expressed as fold change of treated cells as compared with basal treated controls.

Wound healing assay and cell invasion assay

Three kinds of pancreatic cancer cells (5 x 10^5) were seeded in 6-well plates and cultured until reaching 70%-80% confluence as a monolayer. A straight scratch was made on cell plates, and then cell plates were gently rinsed to remove the detached cells. After adding 0, 10 and 50 μM of clobenpropit to each plate, cells were grown for additional 48 hours. After washing the cells with PBS twice, photos were taken on a confocal microscope (Leica, Wetzlar, Germany). Wound healing ranges were measured by Aperio ImageScope V11.1.2.752 (Aperio Technologies, Vista, CA). Additional wound healing assays after administration of gemcitabine (5 μM) and/or clobenpropit (50 μM) in Panc-1 and gemcitabine (15 μM) and/or clobenpropit (50 μM) were performed to evaluate the change of cell migration after gemcitabine and clobenpropit combination treatment.

Invasion assay was performed using BD BioCoat tumor invasion system (BD Biosciences). Confluent pancreatic cancer cells were labeled with 10 μg/mL of $DiLC_{12}$ in DMEM containing 10% FBS for 1 hour at 37℃. Cell suspensions were prepared by trypsinizing the cell monolayers and resuspended in serum-free DMEM at 1 x 10^5 cells/mL. After adding 500 μL of the labeled cell suspension to the apical chambers and 750 μL of chemoattractant to basal chamber, cells were incubated at 24-well plate for 24 hours at 37°C, 5% CO_2. Fluorescence of invaded cells was read at wavelengths of 549/565 nm. Only those labeled cells that had invaded the Matrigel (BD Biosciences) matrix and passed through the pores of the membrane were detected and measured.

Real-time PCR

Gene expression was evaluated in mRNA from all pancreatic cancer cell lines. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA samples were diluted to a final concentration of 0.5 mg/mL in RNase-free water and stored at -80°C until use. Synthesis of the cDNA was performed with 1 mg of total RNA with M-MLV reverse transcription reagents (Invitrogen), and real-time PCR reaction was carried out on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) in 20 μL TaqMan Gene Expression Master Mix (Applied Biosystems) using 200 ng cDNA.

Human primers sets were ordered and used according to their protocols. The specific primers were in Table 1.[26-29] The human β–actin gene was used as an

endogenous reference to control for the independent expression of sample-to-sample variability. The relative expression of target genes was normalized by dividing the target Ct value by the endogenous Ct values. All equipment was purchased from Applied Biosystems and used according to manufacturer's protocols.

Apoptosis determination

Pancreatic cancer cells were cultured and divided into 4 groups according to the treatment: (i) control, (ii) gemcitabine (5 μL) alone, (iii) clobenpropit (50 μL) alone and (iv) gemcitabine (5 μL) and clobenpropit (50 μL) combination. After trypsinization, cells were incubated with annexin V-fluorescein isothiocyanate and propidium iodide (BD Biosciences, Franklin Lakes, NJ) for 15 minutes at room temperature in the dark. Degree of apoptosis was analyzed by fluorescence activated cell sorting. The proportion of stained cells in each quadrant was quantified with CellQuest software (BD Biosciences).

Animal experiments

Five-week-old male BALB/c nude mice were purchased from Orient (Gyeonggi-do, Korea). Mice were housed under specific pathogen-free conditions, and a γ-ray-irradiated laboratory rodent diet (Purina Korea, Gyeonggi-do, Korea) and autoclaved water were provided ad libitum. All the protocols for the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Seoul National University

Hospital (IACUC No. 12-0213). All animal procedures were in consistent with the "Guide for the Care and Use of Laboratory Animals" issued by the Institute of Laboratory Animal Resources Commission on Life Science, US National Research Council.

To generate tumors, human pancreatic cancer cells (Panc-1 in the right flank and MiaPaCa-2 in the left flank of each mouse) were subcutaneously inoculated with 1 x 10^6 cells suspended in 0.15 mL of Matrigel. All mice were divided into 4 groups randomly, and each group consisted of 5 mice: (i) control (vehicle alone), (ii) gemcitabine (twice-a-week intraperitoneal injection at 125 mg/kg for 3 weeks), (iii) clobenpropit (every other day intraperitoneal injection at 20 μmol/kg for 40 days), (iv) gemcitabine (twice-a-week intraperitoneal injection of at 125 mg/kg for 3 weeks) and clobenpropit (every other day intraperitoneal injection at 20 μmol/kg for 40 days).[23, 30] The body weight of mouse was measured weekly with electronic scale. Tumor size was measured every week with electronic caliper and the volume was calculated by the following formula: tumor volume = (length x width^2) x $\pi/6$.[31] At 1 week later after finishing the treatment schedule, mice were anesthetized with isoflurane and tissue, organs and tumors were harvested for analysis.

The expression of EMT markers was investigated by real-time PCR in whole tumor mRNA. Also, tumor samples were fixed in 10% buffered formalin, embedded in low-temperature fusion paraffin, and sectioned for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining and immunohistochemical staining. TUNEL staining was

performed using Tumor TACS in situ Apoptosis kit (R&D Systems, Minneapolis, MN). Apoptotic cells were counted as DAB-positive cells (brown stained) at five arbitrarily selected microscopic fields at 200x magnification, together with total number of cells. Apoptotic index was calculated as number of apoptotic cells x 100/total number of cells. The paraffin-embedded tumor sections were incubated with specific primary antibodies, including E-cadherin and vimentin (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 37 °C followed by overnight incubation at 4°C in humidity chamber. The sections were then incubated with appropriate secondary antibody (1:200 dilutions) for 60 minutes at room temperature. Finally, sections were incubated with conjugated horseradish peroxidase streptavidin for 45 minutes, followed with 3,3'-diaminobenzidine working solution, and counterstained with hematoxylin.

Four independent Western blots were conducted for the assessment of E-cadherin, vimentin, MMP-9 and Zeb1 expressions. Proteins were extracted from the harvested tumors which were stored at -70°C. Tissue were minced and incubated on ice for 30 minutes in 0.5 mL of ice-cold buffer composed of 100 mM HEPES (pH 7.9), 1.5 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1% IGEPAL CA-630, and 0.5 mM PMSF. The minced tissue was homogenized using a Dounce homogenizer followed by centrifuging 5000*g* at 4°C for 15 minutes. The crude nuclear pellet was suspended in 200 µL of buffer B (20 mM HEPES (pH 7.9), 20% glycerol, 15 mM MgCl2, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, and 4 µM leupeptidin) and incubated on ice for 30 minutes. The suspension was centrifuged at 14,000 rpm at 4°C for

15 minutes. The protein concentration was determined with a BCA assay kit. The following procedures with extracted proteins were same as previously described. Antibodies of E-cadherin, vimentin, MMP-9 and Zeb1 were purchased from Santa Cruz.

Statistical analysis

All experimental results represent at least 3 independent experiments using cells from a minimum of three separate isolations. Results for continuous variables are expressed as means ± standard error of mean and compared with the Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test or repeated measures ANOVA. $P < 0.05$ was considered statistically significant. Analysis was performed with GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA).

Results

H_4 receptor was present in pancreatic cancer cells

The specific antibody to H_4 receptor showed immunoreactivity mainly as a band at 70,000 and 43,000 Da in Panc-1, MiaPaCa-2 and AsPC-1 (Fig. 1A). However, there was no H_3 receptor expression in these human pancreatic cancer cells (Data not shown). When different concentrations of clobenpropit (50 and 100 μM) were added to the pancreatic cancer cells, expression of H_4 receptor was increased in MiaPaCa-2 (Fig. 1A). When clobenpropit (50 μM) and/or gemcitabine (5 μM) were treated, H_4 receptor expression was also showed in human pancreatic cancer cells (Fig. 1B).

Anti-proliferative effect of clobenpropit in pancreatic cancer cells

Clobenpropit alone did not affect the proliferation of pancreatic cancer cells (Fig. 2). Also it showed little anti-proliferative effect in pancreatic cancer cells even if administrated in combination with gemcitabine in Panc-1, MiaPaCa-2 and AsPC-1 (Fig. 3).

Clobenpropit inhibited cell migration by inhibition of EMT process

The inhibition of cell migration and invasion was assessed by wound healing assay and invasion assay. Clobenpropit inhibited the migration of pancreatic cancer cells in wound healing assay (Fig. 4). Wound healing ranges were more decreased depending on the higher clobenpropit concentration. In addition, the migration rate of Panc-1 and MiaPaCa-2 was also inhibited after

treatment of gemcitabine and clobenpropit combination compared with control and gemcitabine or clobenpropit alone (Fig. 5). However, the invasiveness of pancreatic cancer cells showed no significant difference after clobenpropit alone or combination with gemcitabine administration (Fig. 6).

To investigate the mechanism of clobenpropit to the cell migration, real-time PCR about epithelial and mesenchymal markers was performed. E-cadherin was about 4-fold increased after treatment of clobenpropit in Panc-1 (Fig. 7A). MMP-9 was reduced in MiaPaCa-2 (Fig. 7B), and vimentin and MMP-9 were reduced to about half after treatment of clobenpropit in AsPC-1 (Fig. 7C). Therefore, clobenpropit downregulated epithelial marker, while upregulated mesenchymal markers, and it means that clobenpropit might disrupt EMT process of pancreatic cancer cells.

Clobenpropit enhanced gemcitabine-induced apoptosis

The exposure to gemcitabine (5 μM) and/or clobenpropit (50 μM) induced apoptosis of pancreatic cancer cells. Gemcitabine and clobenpropit combination therapy significantly increased apoptosis of Panc-1 and MiaPCa-2 compared with control (Fig. 8A, B). Besides, gemcitabine and clobenpropit combination therapy tended to increase apoptosis of AsPC-1 compared with other treatment (Fig. 8C).

Xenograft mouse model

In the xenograft mouse model of Panc-1 and MiaPaCa-2, the body weight of mice was checked every week (Fig. 9A). There was no significant difference

in mean body weight between the groups. There was no mortality of mouse until the end of the treatment. Tumor from Panc-1 was evaluated only because tumor of MiaPaCa-2 was palpable in only 2 mice during the experiment. Mean tumor volume of Panc-1 in combination group was significantly lower than in control ($P < 0.001$) or in clobenpropit alone group ($P < 0.01$) (Fig. 9B). However, tumor volume of gemcitabine alone group was significantly lower than in control group only ($P < 0.05$). There was no significant difference of tumor volume between gemcitabine alone and clobenpropit alone group. Besides, the tumor volume showed no significant difference between gemcitabine alone and combination treatment group.

Real-time PCR, immunohistochemical and TUNEL staining, and Western blot were performed to investigate the efficacy of gemcitabine and clobenpropit combination treatment and the alteration of EMT process in Panc-1 xenograft mouse model. E-cadherin was upregulated after clobenpropit administration in real-time PCR (Fig. 10A). Also, immunohistochemical staining showed upregulation of E-cadherin in clobenpropit alone and combination group (Fig. 10B). To quantify apoptosis of tumors, TUNEL staining was performed. The percentage of TUNEL-stained cells was more increased in gemcitabine and clobenpropit combination group compared with other groups (Fig. 10C). The expression of E-cadherin was also increased in clobenpropit alone and combination group by Western blotting, whereas Zeb1 expression, the repressor of E-cadherin, was decreased in combination group (Fig. 10D).

Discussion

The role of histamine and its receptors in carcinogenesis is complex and somehow confusing. We evaluated the effect of specific histamine receptor and its agonist against human pancreatic cancer cells in this study. The significant findings in the present study are that clobenpropit emphasized gemcitabine-induced apoptosis of human pancreatic cancer cells *in vitro* in cell culture as well as *in vivo* in tumor xenograft mice. There was no adverse health effect due to clobenpropit in mice as monitored by body weight. The enhanced cytotoxicity of gemcitabine and clobenpropit combination might result from disruption of EMT through H_4 receptor.

Three kinds of pancreatic cancer cells (Panc-1, MiaPaCa-2 and AsPC-1) were used in this study, and all the cells had H_4 receptors. The H_4 specific antibody showed immunoreactivity mainly as a band at 73,000 and 40,000 Da, which is in consistent with previous report.[32]

Increasing evidence indicates that cancer cells are subjected to the EMT, a process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from an epithelial to a mesenchymal phenotype leading to increased motility and invasion.[33] The process of EMT consists of multiple steps.[34, 35] First, cell-cell adhesion disintegrates with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as vimentin. Next, there is a loss of basoapical polarization and the acquisition of front-rear polarization. Then, the cytoskeleton undergoes remodeling, with changes in cortical actin and actin stress fiber. Finally, cell-

matrix adhesion is altered, with activation of proteolytic enzymes such as MMPs. EMT and its inverse process occur normally during embryonic development to from the body plan and to differentiate multiple tissue and organ.[36] On the other hand, EMT is a developmental process which plays an important role in tumor progression and metastasis in diverse solid tumors, including pancreatic cancer.[25] The process of EMT during cancer progression and metastasis closely resembles that observed in embryologic development.[35] E-cadherin, a calcium-dependent transmembrane glycoprotein, is one of the most important molecules in cell-cell adhesion in epithelial tissue,[37] which localized on the surface of epithelial cells in regions of adherens junctions.[38] It can play a major role in malignant cell transformation, and especially in tumor development and progression. Loss of E-cadherin is associated with invasion and metastasis of tumors.[39] Furthermore, the loss of E-cadherin expression has been associated with a poor clinical outcome in several cancers.[25, 40, 41] H_4 receptor agonist increased the expression of E-cadherin in this study, both *in vitro* in cell culture and *in vivo* in xenograft mouse. Clobenpropit would play an important role with interfering cell migration and increasing chemosensitivity of gemcitabine in pancreatic cancer cells through inhibition of EMT process and upregulation of E-cadherin.

Transcriptional repressors of E-cadherin such as Zeb1, Zeb2, Twist, Snail and Slug are associated with EMT.[42-45] Besides, diverse signal pathways such as Wnt cascade, TGF-β and PI3K/Akt pathway are connected with these transcriptional repressors of E-cadherin.[35, 46] Zeb1 expression was decreased in clobenpropit treated mice group compare with control and gemcitabine

alone group. Zeb1 would act as the main transcriptional repressor of E-cadherin in this study although the relationship between Zeb1 and H_4 receptor remains unsolved.

Vimentin is a mesenchymal marker which is upregulated with EMT.[47] Downregulation of vimentin after clobenpropit administration also suggests that H_4 receptor agnonist disrupts EMT process. For the invasion and metastasis of tumor, breakdown of the ECM should be present.[48] Clobenpropit may protect the ECM from breakdown by downregulation of MMP-9, preventing invasion or metastasis of pancreatic cancer.

The change of epithelial markers or mesenchymal markers after administration with clobenpropit was different according to the cells. E-cadherin was increased in Panc-1 only, while vimentin was decreased in AsPC-1 only. Besides, MMP-9 was decreased in MiaPaCa-2 and AsPC-1. It can be explained by the different expressions of EMT markers according to the cancer cells.[25] However, it was coherent that the change of EMT markers indicated the disruption EMT process by clobenpropit in this study.

The chemosensitivity of pancreatic cancer cells are vary, and Panc-1, MiaPaCa-2 and AsPC-1 are known to be resistant to gemcitabine.[25] In order to clarify the effect of clobenpropit, we used chemo-resistant cells and administered low concentration of gemcitabine. It is known that pancreatic cancer cells undergoing EMT with increased expression of Snail and Twist become invasive and develop chemoresistance.[49] In addition, EMT reversion by silencing Zeb1 increases cellular sensitivity to gemcitabine.[25] The apoptosis of pancreatic cancer cells was significantly increased after

gemcitabine and clobenpropit combination treatment in present study. Moreover, tumor volume of xenograft mouse was significantly decreased in combination group compared with control and clobenpropit alone group, and TUNEL stain also showed increased apoptosis in combination group. These results support the idea that therapeutic targeting to reverse EMT could increase chemosensitivity in pancreatic cancer. However, further studies are needed to clarifying the molecular alterations which reverse EMT through H_4 receptor.

In conclusion, clobenpropit enhanced gemcitabine-induced apoptosis in human pancreatic cancer cells by inhibition of EMT process. The novel role of H_4 receptor in carcinogenesis of pancreatic cancer represents a new therapeutic molecular target and clobenpropit could be the one of the promising drug. Further studies are required to reveal the mechanism of EMT inhibition via H_4 receptor.

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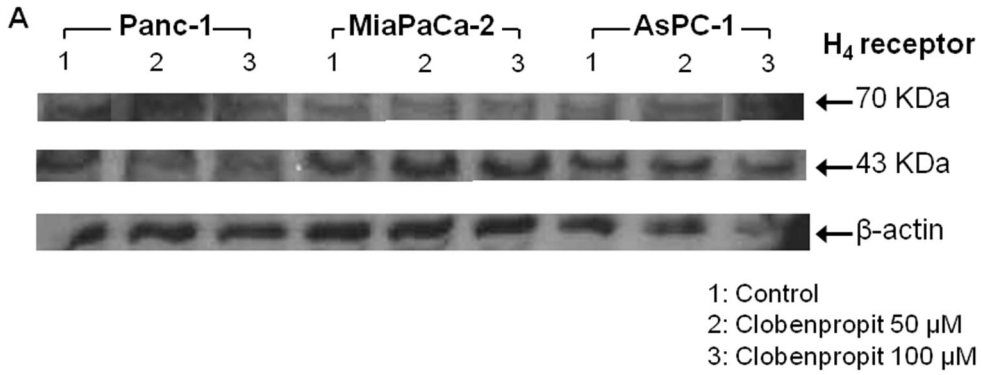
Table 1. The sequences of primers used for real-time PCR.

Gene	Sequences
H_4 receptor	Forward: 5'-GTGGTTAGCATAGGTTATAC-3'
	Reverse: 5'-ATGCCACTGCACTCCTGC-3'
GAPDH	Forward: 5'-ACGGATTTGGTCGTATTGGG-3'
	Reverse: 5'-TGATTTTGGAGGGATCTCGC-3'
E-cadherin	Forward: 5'-GCCTCCTGAAAAGAGAGTGGAAG-3'
	Reverse: 5'-TGGCAGTGTCTCTCCAAATCCG-3'
Vimentin	Forward: 5'-AGGCAAAGCAGGAGTCCACTGA-3'
	Reverse: 5'-ATCTGGCGTTCCAGGGACTCAT-3'
Fibronectin	Forward: 5'-ACAACACCGAGGTGACTGAGAC-3'
	Reverse: 5'-GGACACAACGATGCTTCCTGAG-3'
MMP-9	Forward: 5'-TTCTGCCCCAGCGAGAGA-3'
	Reverse: 5'-GTGCAGGCGGAGTAGGATTG-3'
CK-19	Forward: 5'-GAAGAACCATGAGGAGGAAATCA-3'
	Reverse: 5'-ACCTCATATTGGCTTCGCATGT-3'

Figure 1. Cellular extracts of Panc-1, MiaPaCa-2 and AsPC-1 were separated by SDS-PAGE, transblotted onto nitrocellulose membranes and detected using anti-H_4 receptor antibodies. Pancreatic cancer cell lines were treated with different concentration of clobenpropit and/or gemcitabine, and Western blotting was performed. The specific antibody to H_4 receptor showed immunoreactivity mainly as a band at 70,000 and 43,000 Da. Expression of H_4 receptor was still present after administration of clobenpropit alone or combination with gemcitabine.



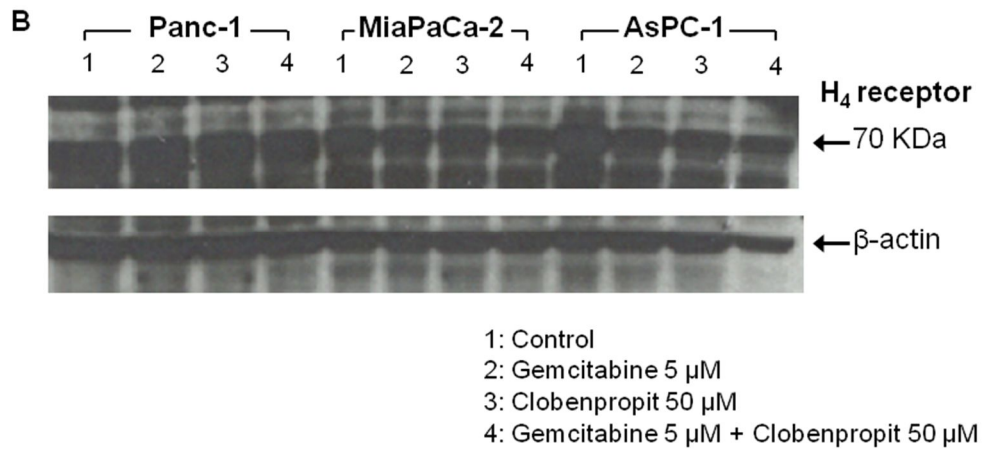


Figure 2. Effect of clobenpropit on pancreatic cancer cell proliferation was evaluated by MTS assay (A: Panc-1, B: AsPC-1). Clobenpropit showed no cytotoxicity on pancreatic cancer cells.

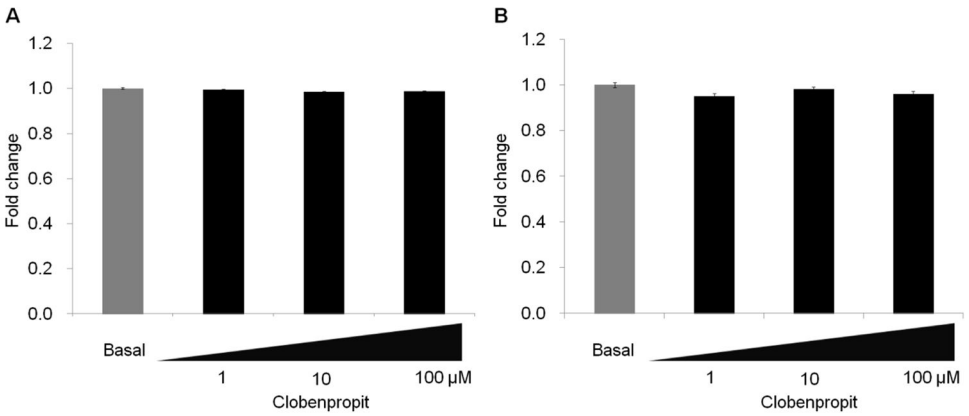


Figure 3. Effect of clobenpropit at 50 μM and gemcitabine combination therapy on pancreatic cancer cell proliferation was evaluated by MTS assay (A: 48 hours, B: 72 hours). There was no significant difference whether clobenpropit added or not.

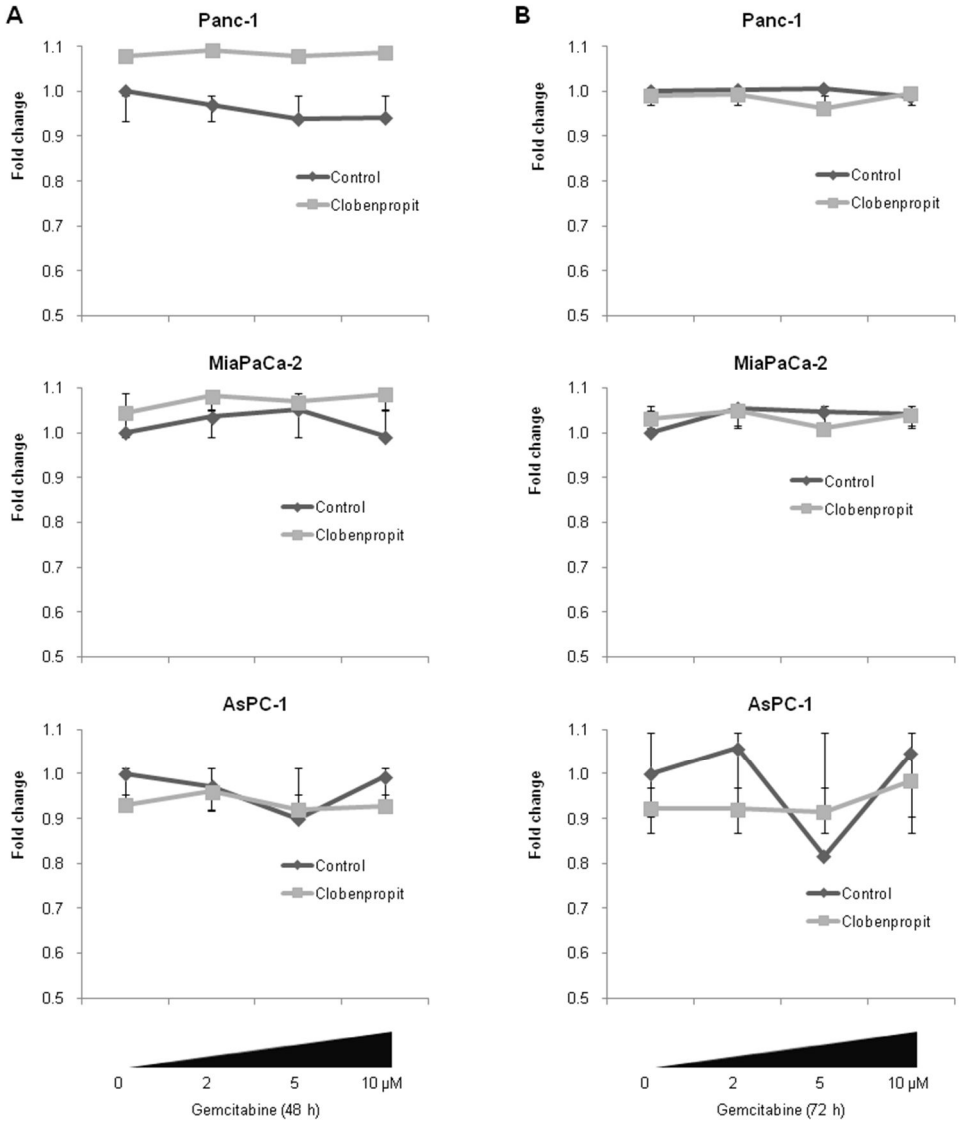
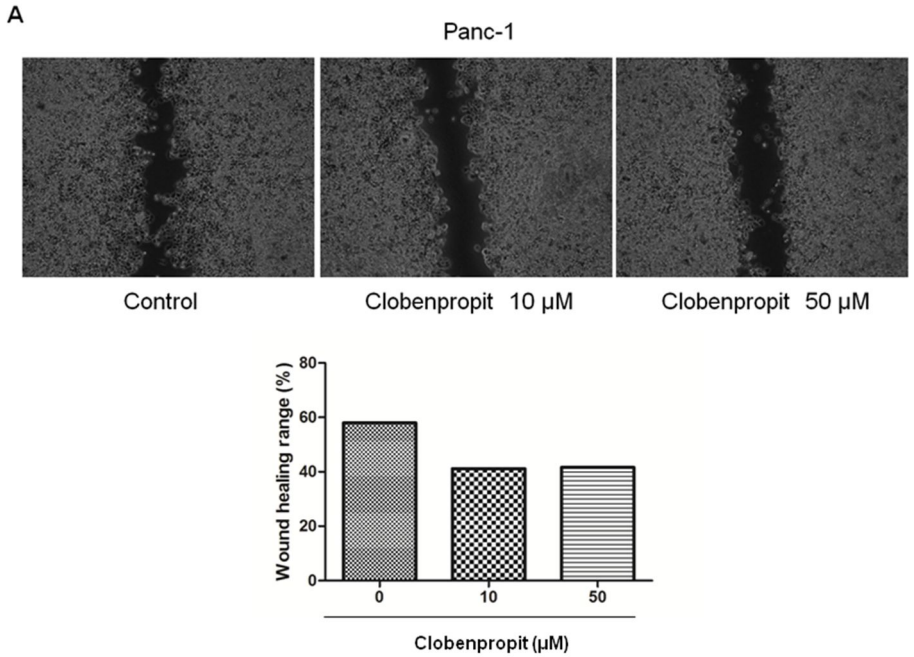
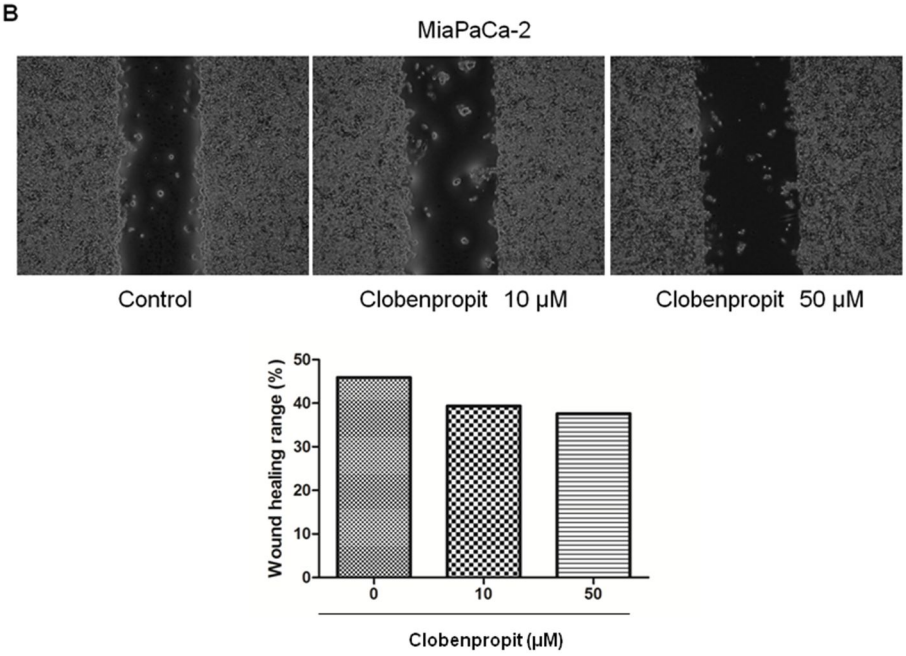


Figure 4. Clobenpropit inhibited the migration of pancreatic cancer cells in wound healing assay. Wound healing range was inversely correlated with clobenpropit concentration in MiaPaCa-2 (B) and AsPC-1 (C).



B


MiaPaCa-2
Control
Clobenpropit 10 μM
Clobenpropit 50 μM
Wound healing range (%)
50
40
30
20
10
0
0
10
50
Clobenpropit (μM)

C

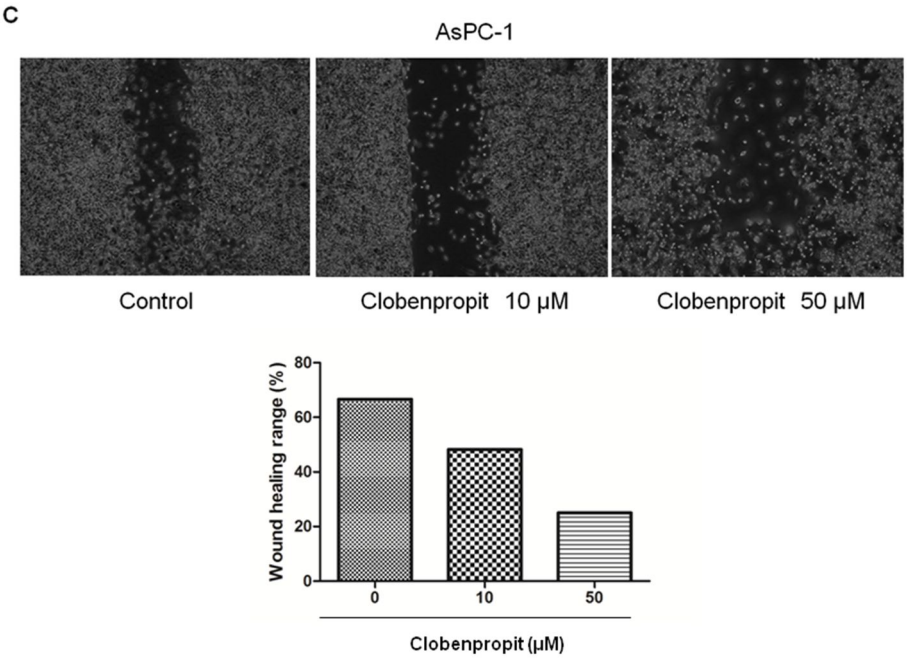

AsPC-1
Control
Clobenpropit 10 μM
Clobenpropit 50 μM
Wound healing range (%)
80
60
40
20
0
0
10
50
Clobenpropit (μM)

Figure 5. The migration of pancreatic cancer cells was inhibited after gemcitabine and clobenpropit combination in wound healing assay. The concentrations of gemcitabine in Panc-1 and MiaPaCa-2 were 5 and 15 μM, respectively. The concentration of clobenpropit was 50 μM.

G, gemcitabine; C, clobenpropit.

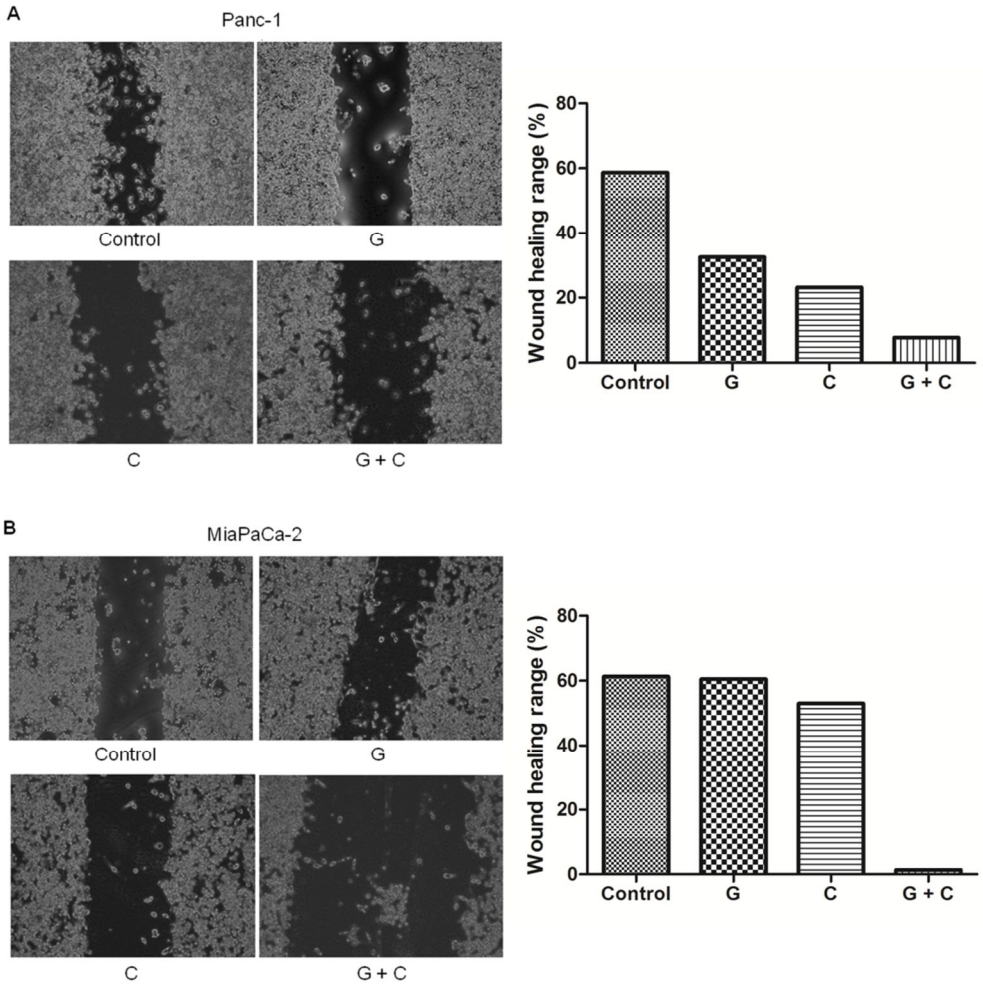


Figure 6. The migratory cells which had invaded through the Matrigel were counted. The invasiveness of pancreatic cancer cells showed no significant difference after clobenpropit alone (A) or gemcitabine (5 μM) and/or clobenpropit (50 μM) (B) administration.

G, Gemcitabine; C, Clobenpropit.

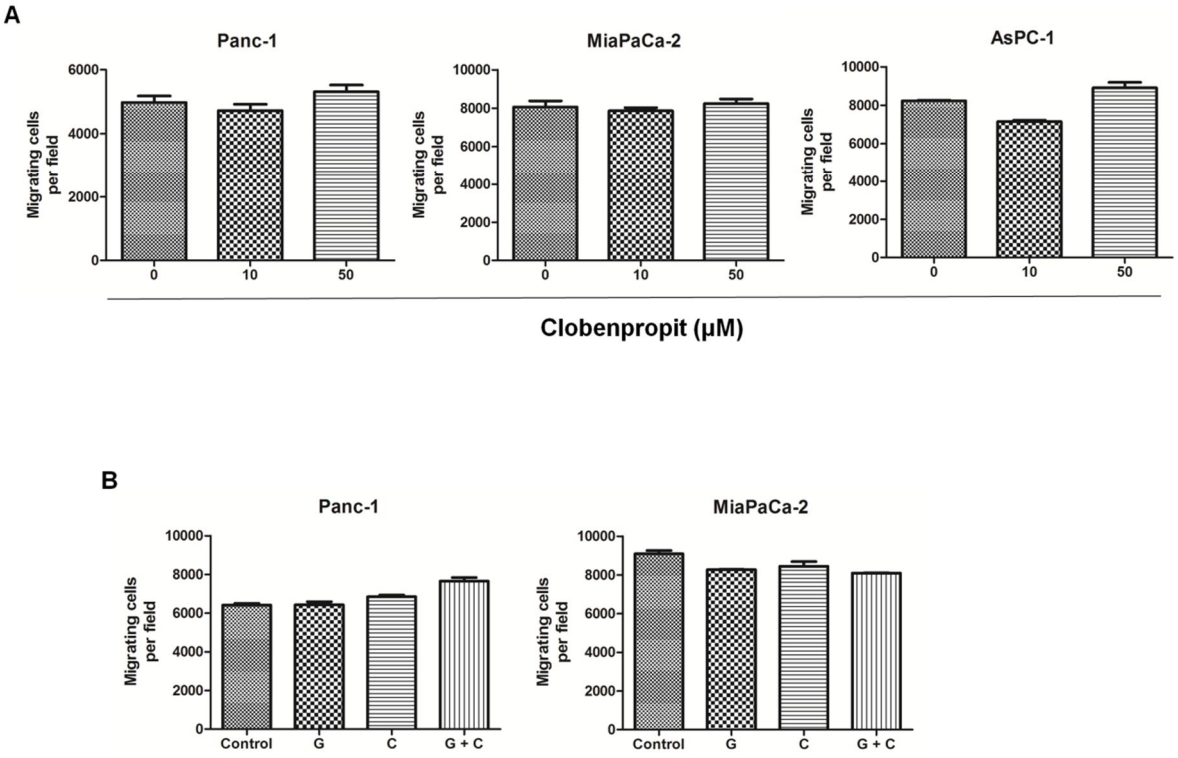


Figure 7. Real-time PCR shows that E-cadherin was upregulated (A), whereas MMP-9 and vimentin were downregulated (B, C) in pancreatic cancer cells after treatment with different concentration of clobenpropit.

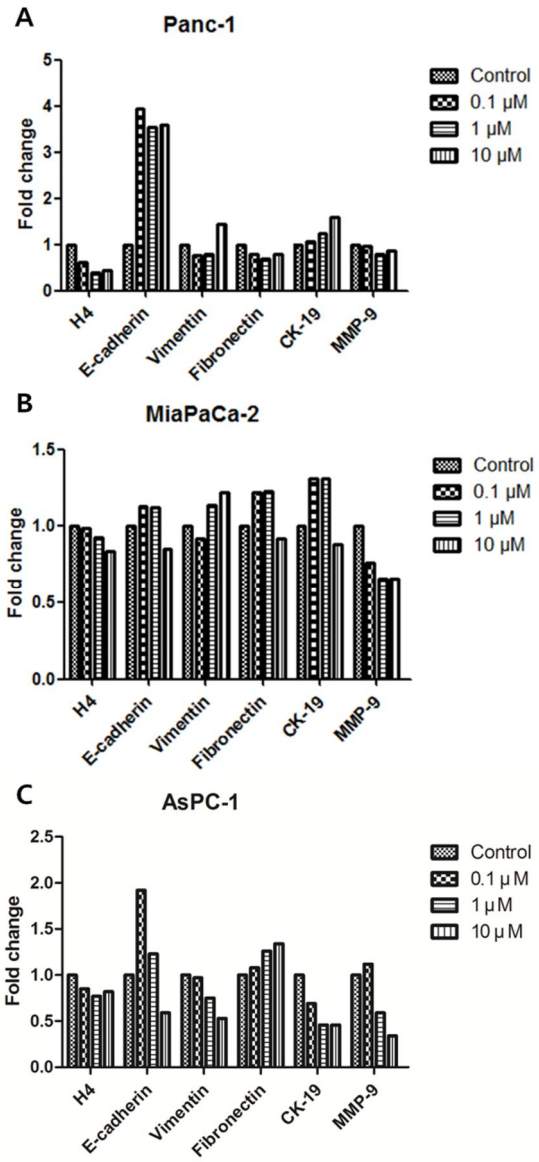
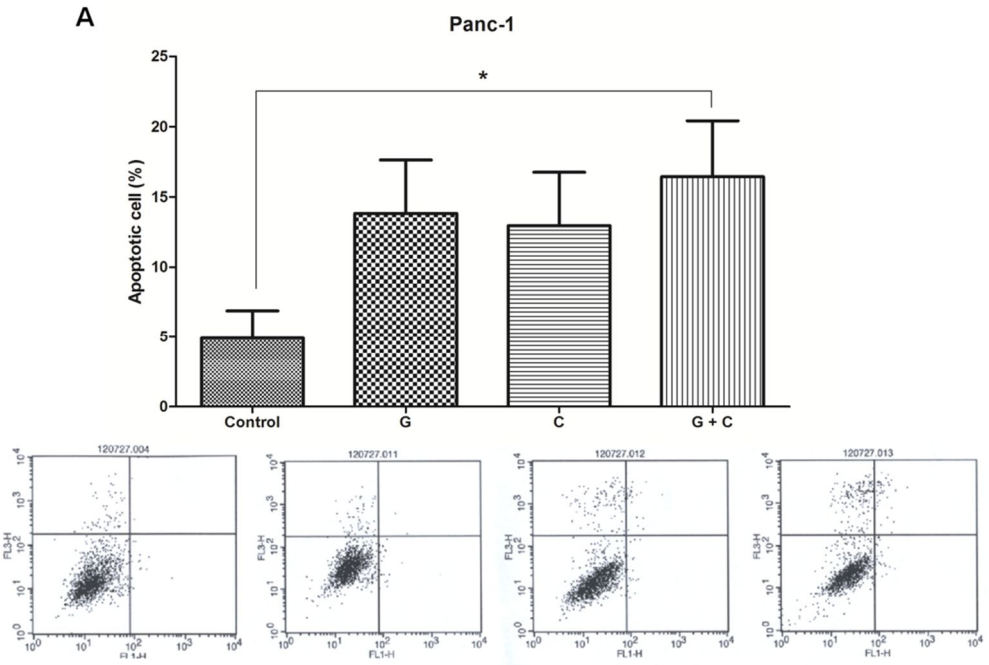
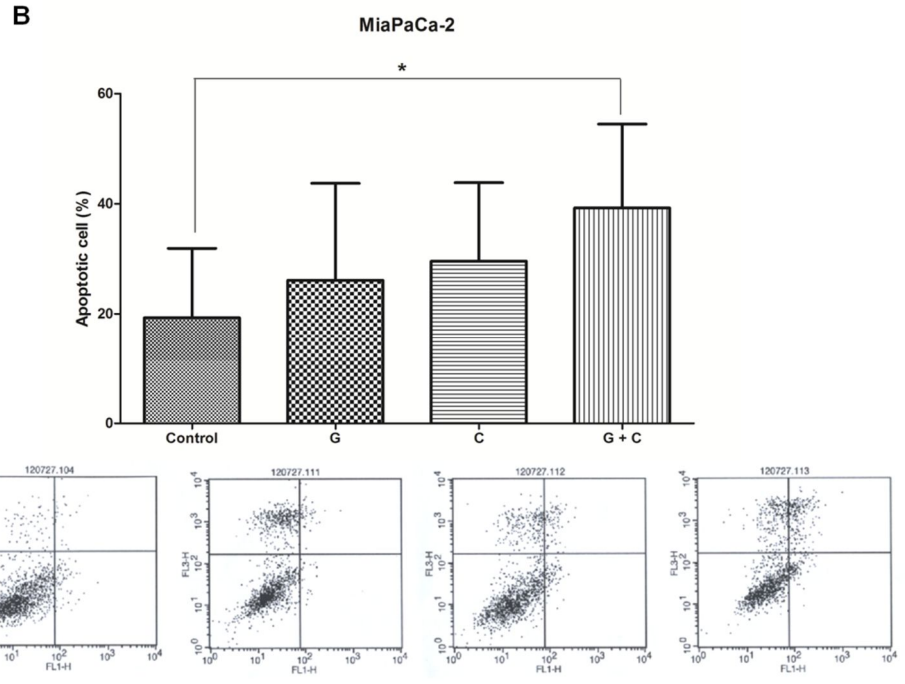


Figure 8. Clobenpropit enhanced apoptotic cell death with combination of gemcitabine in human pancreatic cells. The percentage of apoptotic cell population was determined by fluorescein isothiocyanate-labeled annexin V assay followed by flow cytometry. Statistically significant differences ($P < 0.05$) of the combination treatment of gemcitabine (5 μM) and clobenpropit (50 μM) compared with control in Panc-1 (A) and MiaPaCa-2 (B) were illustrated as asterisk (*).

G, Gemcitabine; C, Clobenpropit.




B
MiaPaCa-2
*
Apoptotic cell (%)
60
40
20
0
Control
G
C
G + C
120727.104
120727.111
120727.112
120727.113
FL3-H
FL1-H

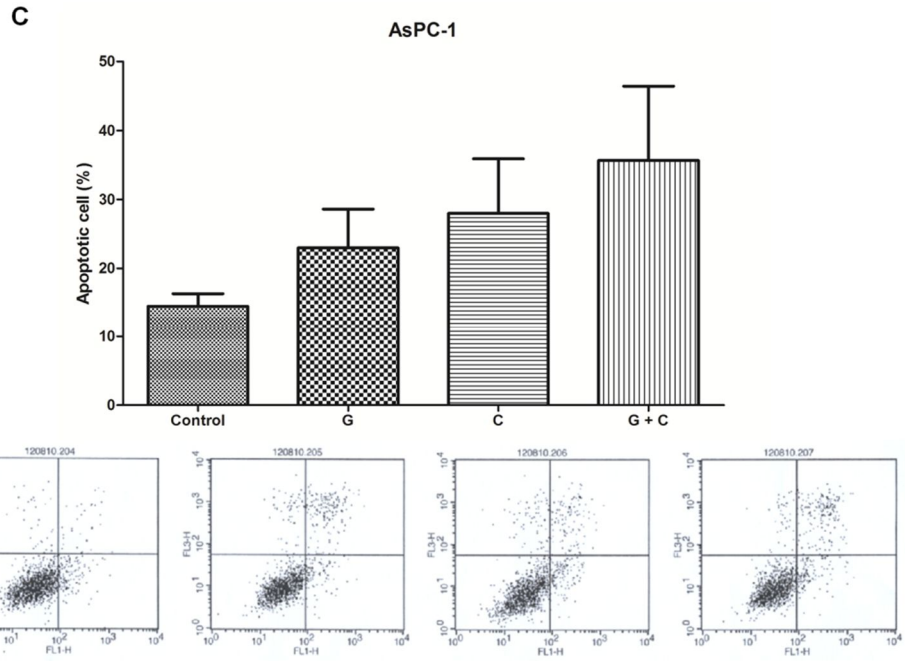
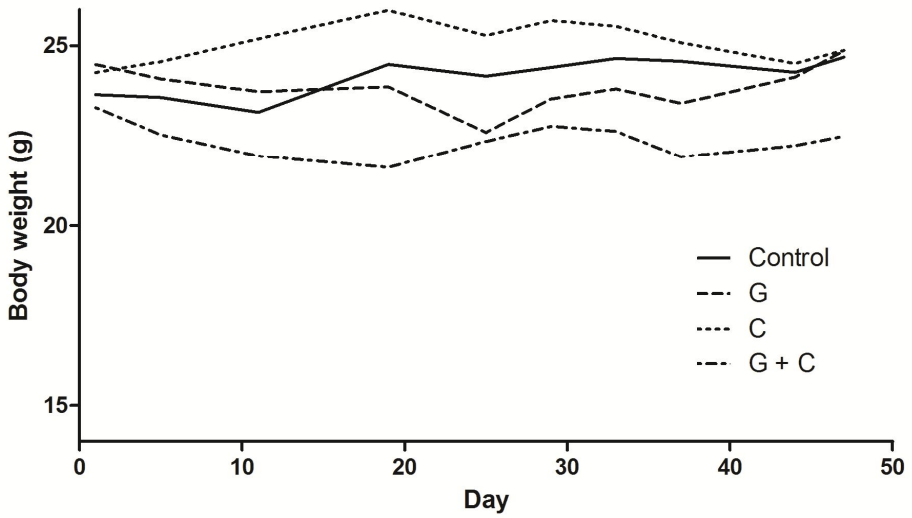

C
AsPC-1
Apoptotic cell (%)
50
40
30
20
10
0
Control
G
C
G + C
120810.204
120810.205
120810.206
120810.207
FL3-H
FL1-H

Figure 9. Body weight (A) and tumor volume (B) curves for Panc-1 xenograft mouse model with administration of vehicle (control), gemcitabine, clobenpropit or their combination. There was no significant difference of body weight between the groups by repeated measures ANOVA. Tumor volume of combination group was significant lower than control or clobenpropit alone group. Tumor bearing mice and excised tumor of each treatment group (C).

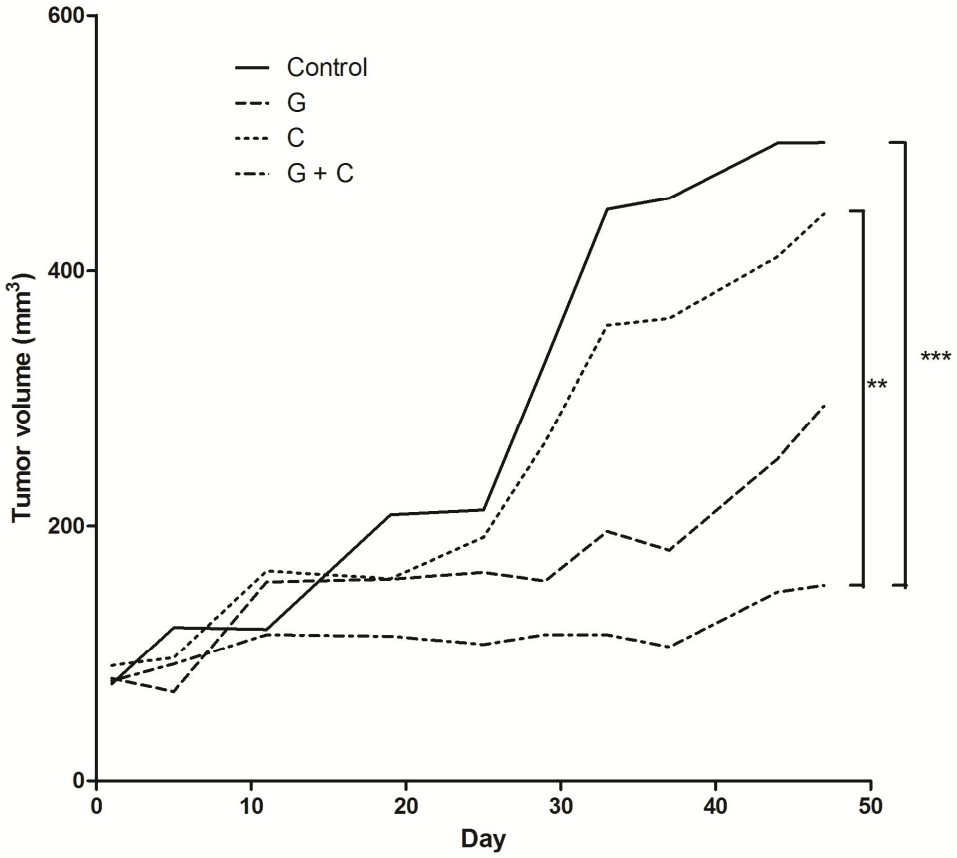
G, Gemcitabine; C, clobenpropit.

**, $P < 0.01$; *** $P < 0.001$

A



B



C

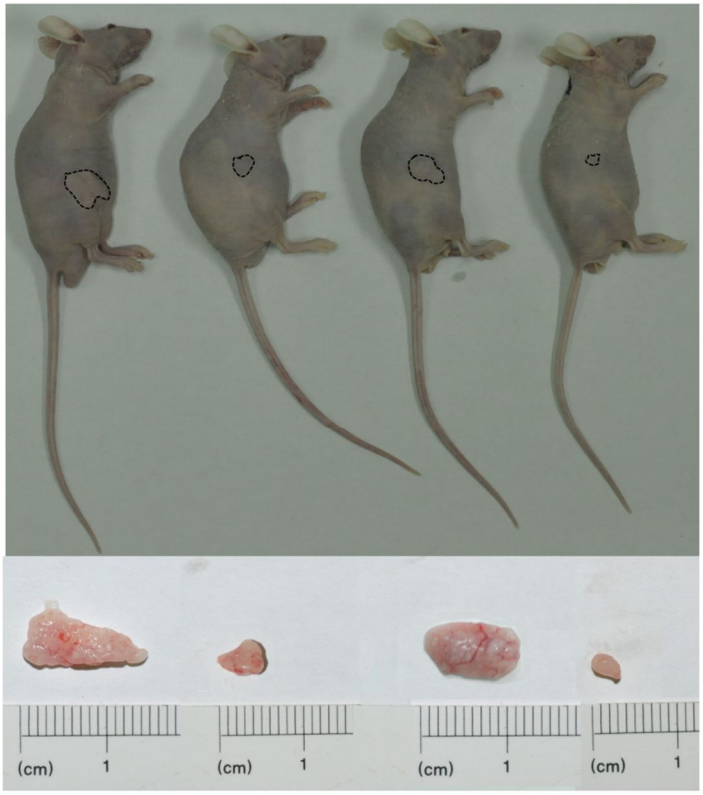


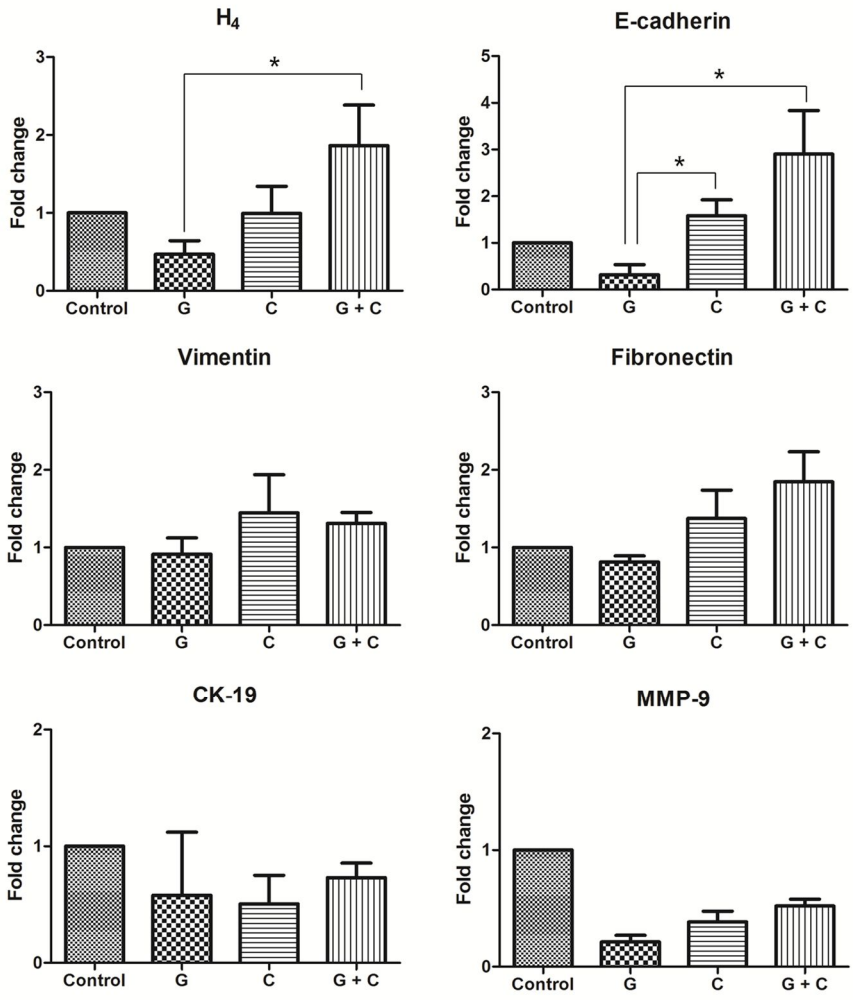
Figure 10. Effect of gemcitabine and clobenpropit combination treatment in Panc-1 xenograft mouse model. Real-time PCR shows increased E-cadherin expression after clobenpropit treatment compared with gemcitabine alone (A). Pathological evaluation of tumor tissue determined by H&E staining, TUNEL staining and immunohistochemistry of E-cadherin and vimentin (B). Immunohistochemical staining shows upregulation of E-cadherin in gemcitabine and clobenpropit combination group. Apoptotic index calculated with TUNEL staining shows increased apoptosis in gemcitabine and clobenpropit combination group (C).

E-cadherin was also increased in clobenpropit alone and combination group by Western blotting, whereas Zeb1, the repressor of E-cadherin, was decreased in combination group (D).

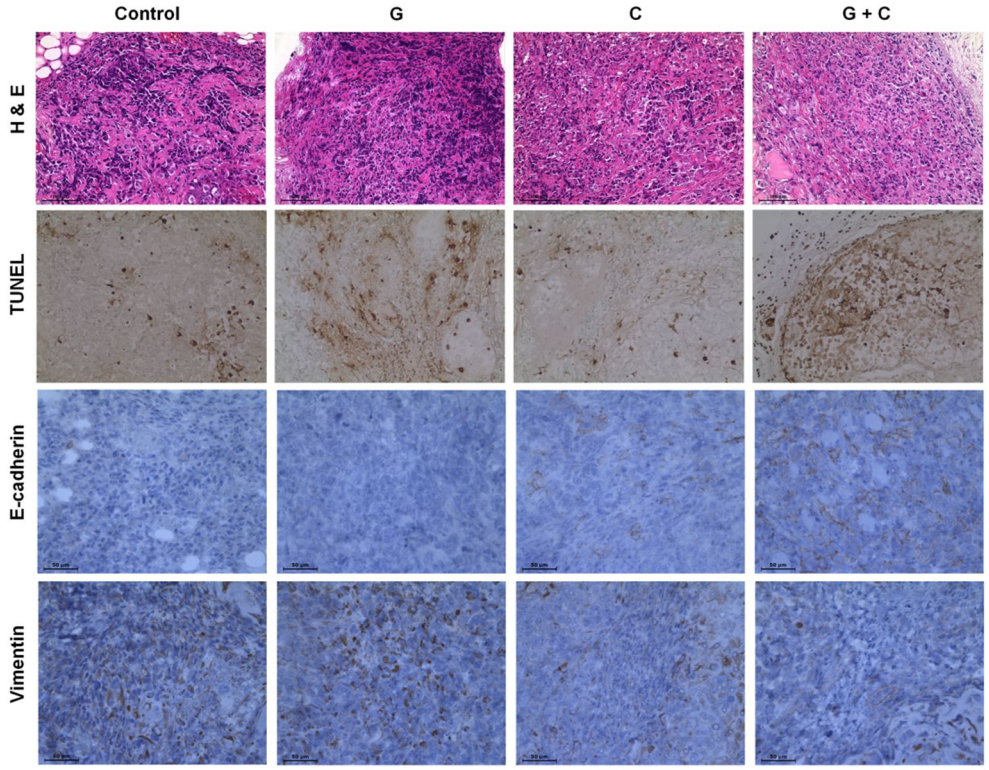
G, Gemcitabine; C, clobenpropit.

* $P < 0.05$; ** $P < 0.01$.

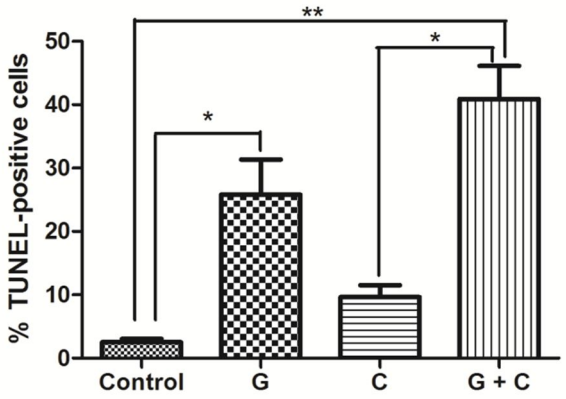
A


H4
E-cadherin
Vimentin
Fibronectin
CK-19
MMP-9
Fold change
Control
G
C
G + C
*

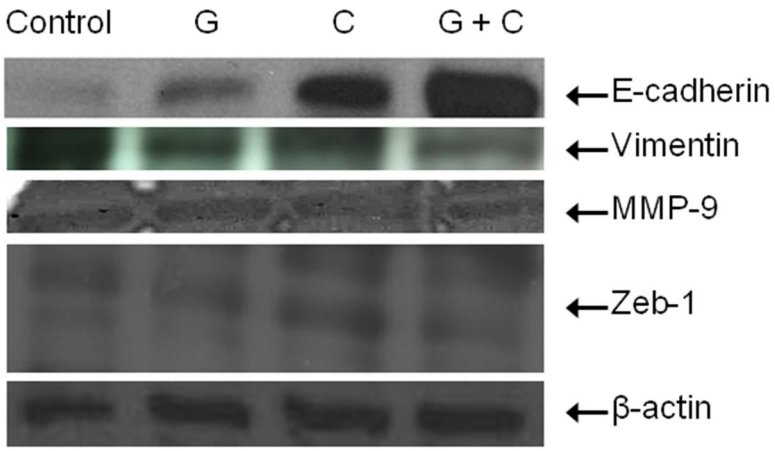
B


Control
G
C
G + C
H & E
TUNEL
E-cadherin
Vimentin

C


% TUNEL-positive cells
50
40
30
20
10
0
**
*
*
Control
G
C
G + C

D



국문초록

목적: 췌장암은 매우 예후가 불량한 암으로, 조기에 전이하며 수술을 시행하더라도 종종 전이하거나 재발이 발생한다. 그러나 현재 이러한 문제를 해결할 효과적인 치료 방법이 없는 상황이다. 히스타민은 4개의 특이 수용체를 통해 암의 발생과 관련이 있는 것으로 알려져 있다. 따라서 히스타민 수용체를 표적으로 하는 약제가 췌장암의 치료에 있어서 도움이 될 것으로 기대된다. 이번 연구에서는 H_3, H_4 수용체에 작용하는 clobenpropit을 이용하여 췌장암에서의 효과를 규명하고자 하였다.

실험 방법: 세 종류의 췌장암 세포(Panc-1, MiaPaCa-2, AsPC-1)가 이번 실험에 사용되었다. 췌장암 세포에서 H_3, H_4 수용체의 발현 정도를 확인하고, 세포 증식, 이동, 침습 및 사멸에 대한 clobenpropit의 효과를 분석하였다. Real-time PCR을 이용하여 clobenpropit 투여에 의한 상피간엽전환 관련 마커의 변화를 측정하였다. 또한 췌장암 세포 이종이식 동물 모델을 통해 gemcitabine과 clobenpropit 병합 치료의 효과를 평가하고, 상피간엽전환 관련 마커의 변화 및 세포 사멸 정도를 확인하기 위해 real-time PCR, 면역화학염색, TUNEL 염색, Western blotting을 시행하였다.

결과: 세 종류의 췌장암 세포 모두에서 H_4 수용체가 발현되었으나 H_3 수용체는 발현되지 않았다. Clobenpropit은 췌장암 세포 증식을

거의 억제하지 못하였으나 췌장암 세포의 이동을 억제하였다. 또한 gemcitabine과 병합 투여 시 췌장암 세포 사멸을 유도하였고, 상피 마커인 E-cadherin의 발현을 증가시킨 반면, 간엽 마커인 vimentin, matrix metalloproteinase 9은 감소시켰다. 동물실험에서도 gemcitabine과 clobenpropit 병합 치료 시 E-cadherin이 증가하는 반면에 E-cadherin의 억제제인 Zeb1은 감소하였고, 이는 세포 사멸을 유도하여 종양의 크기를 감소시켰다.

결론: 췌장암 세포에서 clobenpropit은 H_4 수용체를 통해 상피간엽전환을 억제함으로써 gemcitabine의 항암 효과를 강화시켰다.

Keywords: 췌장암, Clobenpropit, 히스타민, 히스타민 수용체, 상피간엽전환

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