Clobenpropit Enhances Anti-Tumour Effect of Gemcitabine
In Pancreatic Cancer

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Background: Histamine is associated with carcinogenesis through activation of its 4 membrane-specific receptors. We evaluated the anti-tumour effect of clobenpropit, which is the specific H3 antagonist and H4 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 H3 and H4 receptor in pancreatic cancer cell was identified with Western blotting. Effects of clobenpropit on cell proliferation, migration and apoptosis were evaluated. 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: H4 receptors were present as 2 subunits in human pancreatic cancer cells, while there was no expression of H3 receptor. Clobenpropit inhibited cell migration and increased apoptosis of pancreatic cancer cells in combination with gemcitabine. Clobenpropit up-regulated E-cadherin, whereas down-regulated vimentin and matrix metalloproteinase 9 in real-time PCR. Also, clobenpropit inhibited tumour growth and enhanced apoptosis in combination with gemcitabine by up-regulation of E-cadherin and down-regulation of Zeb1 in Panc-1 xenograft mouse.

Conclusion: Clobenpropit enhanced anti-tumour effect of gemcitabine in pancreatic cancer cells through inhibition of epithelial-mesenchymal transition process.

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

INTRODUCTION

Pancreatic cancer is a very aggressive human cancer and has dismal prognosis with only 6% of patients survive 5 years after diagnosis (Siegel et al, 2012). In spite of the progresses of treatments, the attempts of survival prolongation, especially in the advanced stage, have failed and resulted in no significant improvement (Hidalgo, 2010). Surgical resection is the only potentially curative treatment in pancreatic cancer, but only 15% of patients could be candidate for resection (Conlon et al, 1996; Mancuso et al, 2006). Some chemotherapeutic agents are used in pancreatic cancer, and gemcitabine became the standard chemotherapeutic agent in pancreatic cancer since the randomized trial in 1997 (Burris et al, 1997). 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 (Huang et al, 1991). However, pancreatic cancer is highly resistant to chemotherapy including gemcitabine (Ying et al, 2012), and the disappointing circumstance of pancreatic cancer is mainly due to late diagnosis at which chemoresistance in patients is a critical issue (Hung et al, 2012). Resistance to gemcitabine has been increasing and the effectiveness of gemcitabine has been reduced to less than 20% (Bafna et al, 2009). Hence, new therapeutic targets and chemotherapeutic agents of pancreatic cancer are desperately required.

High concentration of histamine has been shown in melan-
Histamine participates in tumor proliferation or apoptosis through activation of its four membrane-specific receptors, H₁, H₂, H₃ and H₄ (Cianchi et al., 2008). As in other human cancers, expressions of histidine decarboxylase and histamine contents have been reported in pancreatic cancer (Rivera et al., 2000; Tanimoto et al., 2004). Histamine inhibits the cell proliferation through H₁ and H₂ receptor, which is associated with a partial differentiation in pancreatic cancer (Cricco et al., 2000). Through H₂ receptor, histamine induces G₀/G₁ phase arrest and modulation of mitogen activated protein kinase (MAPK) and Bcl-2 family proteins (Cricco et al., 2004; Cricco et al., 2006; Martin et al., 2002). Furthermore, the previous study suggests that H₃ and H₄ receptors are involved in pancreatic cancer cell growth, with proliferation increased through H₃ receptor and diminished via H₄ receptor (Cricco et al., 2008). However, the mechanism of anti-cancer effect through histamine receptor still remains unclear.

Clobenpropit, which is a specific H₃ antagonist and H₄ agonist, inhibits the spread of mammary adenocarcinoma by decreasing invasion potential (Medina et al., 2008). The recent study suggested that the modulation of H₄ receptor by clobenpropit disrupts epithelial-mesenchymal transition (EMT) processes, extracellular matrix (ECM) breakdown, and invasion potential and decreases tumour growth in cholangiocarcinoma (Meng et al., 2011). Similarly, EMT plays a crucial role in tumour progression and metastasis of pancreatic cancer (Nakajima et al., 2004). Furthermore, EMT regulators including Zeb1 are known to induce chemoresistance of human pancreatic cancer cells (Anumugam et al., 2009). Thus, therapeutic agents targeting EMT process could restore the chemoresistance of pancreatic cancer. Therefore, we aimed to investigate the anticancer 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 containing 10% fetal bovine serum. They were incubated at 37°C and 5% CO₂.

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 MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 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 H₃ and H₄ receptor antibodies (Millipore, Billerica, MA). Immunoreactivity was developed using a peroxidase conjugate antiseraum (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.

Wound healing assay. Three kinds of pancreatic cancer cells (5 × 10⁵) 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.
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 described in Supplementary Table S1 (Chang et al, 2012; Kapral et al, 2012; Nakamura et al, 2010; Yu et al, 2010). 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.

MTS proliferation assay. Cells were plated into 96-well plates at a density of 4 × 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)-2H-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.

Apoptosis determination. Pancreatic cancer cells were cultured and divided into 4 groups according to the treatment: (i) control, (ii) gemcitabine (5 μM) alone, (iii) clobenpropit (50 μM) alone and (iv) gemcitabine (5 μM) and clobenpropit (50 μM) 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 tumours, Panc-1 was subcutaneously inoculated with 1 × 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 40 days), (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 40 days) and clobenpropit (every other day intraperitoneal injection at 20 μmol/kg for 40 days) (Lee et al, 2008; Meng et al, 2011). The body weight of mouse was measured weekly with electronic scale. Tumour size was measured every week with electronic caliper and the volume was calculated by the following formula: tumour volume = (length × width^2) × π/6 (Lee et al, 2011). At 1 week later after finishing the treatment schedule, mice were anesthetized with isoflurane and tissue, organs and tumours were harvested for analysis.

The expression of EMT markers was investigated by real-time PCR in whole tumour mRNA. Also, tumour 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. Western blots were
conducted for the assessment of E-cadherin, vimentin, MMP-9 and Zeb1 expressions. 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 all cell lines (Data not shown). When clobenpropit (50 \( \mu \)M) and/or gemcitabine (5 \( \mu \)M) were treated, \( H_4 \) receptor expression was also showed but not significantly changed in all cell lines (Fig. 1B).

Clobenpropit inhibited cell migration by inhibition of EMT process. The inhibition of cell migration was assessed by wound healing assay. Clobenpropit inhibited the migration of pancreatic cancer cells in wound healing assay (Fig. 2). Wound healing ranges were more decreased depending on the higher clobenpropit concentration. In addition, the migration rate was also inhibited after treatment of gemcitabine and clobenpropit combination compared with control and gemcitabine or clobenpropit alone (Supplementary Fig. S1).

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. MMP-9 was reduced in MiaPaCa-2, and vimentin and MMP-9 were reduced to about half after treatment of clobenpropit in AsPC-1 (Supplementary Fig. S2). Therefore, clobenpropit down-regulated epithelial marker, while up-regulated mesenchymal markers and it means that clobenpropit might disrupt EMT process of pancreatic cancer cells.

Clobenpropit enhanced gemcitabine-induced apoptosis. Clobenpropit alone did not affect the proliferation of pancreatic cancer cells (Supplementary Fig. S3). However, the exposure to gemcitabine (5 \( \mu \)M) and/or clobenpropit (50 \( \mu \)M) induced apoptosis of pancreatic cancer cells. Gemcitabine and clobenpropit combination therapy significantly increased apoptosis of Panc-1, MiaPaCa-2 and AsPC-1 compared with control (Fig. 3). However, gemcitabine alone did not increase apoptosis of pancreatic cancer cells significantly compared with control.

Xenograft mouse model. In the xenograft mouse model of Panc-1, the body weight of mice was checked every week (Fig. 4A). There was no significant difference in mean body weight between the groups. There was no mortality of mouse until the end of the treatment. The combination treatment of gemcitabine with clobenpropit showed significant tumour growth inhibition while up-regulated mesenchymal markers and it means that clobenpropit might disrupt EMT process of pancreatic cancer cells.
Figure 2. Effect of clobenpropit on the cell migration. Clobenpropit inhibits the migration of Panc-1 (A), MiaPaCa-2 (B) and AsPC-1 (C) in wound healing assay. Wound healing range was inversely correlated with clobenpropit concentration in MiaPaCa-2 and AsPC-1.

Figure 3. Enhanced apoptosis of pancreatic cancer cells by gemcitabine and clobenpropit combination treatment. Clobenpropit enhanced apoptotic cell death with combination of gemcitabine in human pancreatic cancer 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), MiaPaCa-2 (B) and AsPC-1 (C) were illustrated as asterisk (*).

G, Gemcitabine; C, Clobenpropit.

E-cadherin was up-regulated after clobenpropit administration in real-time PCR (Fig. 5A). Also, immunohistochemical staining showed up-regulation of E-cadherin in clobenpropit alone and combination group (Fig. 5B). To quantify apoptosis of tumours, TUNEL staining was performed. The percentage of TUNEL-stained cells was more increased in gemcitabine and clobenpropit compared with other treatment groups (Fig. 4B and C).
Figure 4. Inhibition of tumour growth in Panc-1 xenograft mouse by gemcitabine and clobenpropit combination treatment. Body weight (A) and tumour 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. Tumour volume of gemcitabine and clobenpropit combination therapy group was significant lower than control and other treatment groups. Tumour bearing mice and excised tumour of each treatment group (C).

G, Gemcitabine; C, clobenpropit.

*p < 0.05; **p < 0.01; ***p < 0.001.

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 tumour 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 H4 receptor.

Three kinds of pancreatic cancer cells (Panc-1, MiaPaCa-2 and AsPC-1) were used in this study, and all the cells had H4 receptors. The H4 specific antibody showed immunoreactivity mainly as a band at 73,000 and 40,000 Da, which is in consistent with previous report (van Rijn et al, 2006).

Increasing evidence indicates that cancer cells are subjected to the EMT, a process by which epithelial cells undergo remark-
Figure 5. Effect of gemcitabine and clobenpropit combination treatment in Panc-1 xenograft mouse. Real-time PCR shows increased E-cadherin expression after clobenpropit treatment compared with gemcitabine alone (A). Pathological evaluation of tumour tissue determined by H&E staining, TUNEL staining and immunohistochemistry of E-cadherin and vimentin (B). Immunohistochemical staining shows up-regulation 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.

E-cadherin is associated with invasion and metastasis of tumours (Vlemingckx et al, 1991). Furthermore, the loss of E-cadherin expression has been associated with a poor clinical outcome in several cancers (Arumugam et al, 2009; Dohadwala et al, 2006; Pena et al, 2005). H4 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 up-regulation of E-cadherin.

Transcriptional repressors of E-cadherin such as Zeb1, Zeb2, Twist, Snail and Slug are associated with EMT (Comijn et al,
Besides, diverse signal pathways such as Wnt cascade, TGF-β and PI3K/Akt pathway are connected with these transcriptional repressors of E-cadherin (Iwatsuki et al., 2010; Larue and Bellacosa, 2005). Zeb1 expression was decreased in clobenpropit treated mice group compared 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 H4 receptor remains unsolved.

Vimentin is a mesenchymal marker which is up-regulated with EMT (Kong et al., 2010). Down-regulation of vimentin after clobenpropit administration also suggests that H4 receptor agonist disrupts EMT process. For the invasion and metastasis of tumour, breakdown of the ECM should be present (Nakanuma et al., 2010). Clobenpropit may protect the ECM from breakdown by down-regulation 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 (Arumugam et al., 2009). 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 (Arumugam et al., 2009). In order to clarify the effect of clobenpropit, we used the most chemoresistant cells (Panc-1) in xenograft model. It is known that pancreatic cancer cells undergoing EMT with increased expression of Snail and Twist become invasive and develop chemoresistance (Shah et al., 2007). In addition, EMT reversion by silencing Zeb1 increases cellular sensitivity to gemcitabine (Arumugam et al., 2009). The apoptosis of pancreatic cancer cells was significantly increased after gemcitabine and clobenpropit combination treatment in present study. Moreover, tumour volume of xenograft mouse was significantly decreased in combination group compared with control and clobenpropit or gemcitabine 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 H4 receptor.

In conclusion, clobenpropit enhanced gemcitabine-induced apoptosis in human pancreatic cancer cells by inhibition of EMT process. The novel role of H4 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 H4 receptor.

ACKNOWLEDGEMENTS

This study was supported in part by the Korean Society of Internal Medicine Research Fund (2012) and the Seoul National University College of Medicine Research Fund (2011). Grateful thanks to Hye Jo Ryu for the assistance of experiments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

dual faces of the coin. Cancer Biol Ther 7: 36-37


Supplementary

Table S1. The sequences of primers used for real-time PCR

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<th>Gene</th>
<th>Sequences</th>
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<td>H4 receptor</td>
<td>Forward: 5’-GTGGTTAGCATAGGTATATC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-ATGCCACTGCCTCCTGC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-ACGGATTTTGCTATTTGGAAG-3’</td>
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<td>Reverse: 5’-TGATTTGAGGGATGATCCTGC-3’</td>
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<td>E-cadherin</td>
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<td></td>
<td>Reverse: 5’-TCGCACTGCTCGTCTCAAATCCG-3’</td>
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**Figure S1.** 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 S2. 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 S3. 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.