Abstract. A recent study showned that complementary medicine is gradually gaining wide acceptance. In the present study, the herbal mixture extract (H3) composed of 3 oriental herbal plants was investigated for anticancer activity in vitro and in vivo. H3 inhibited PANC1 cell growth by promoting G0/G1 arrest (11% increase) and apoptotic cell death (9% increase). H3 also suppressed stem cell-like side population cells (4% decrease) and migration activity (24% decrease). In contrast, gemcitabine decreased side population cells and migration activity by 3 and 11%, respectively. These effects of H3 and gemcitabine were further studied by examining the expression of apoptosis-associated genes (CXCR4, JAK2 and XIAP) and stem cell-associated genes (ABCG2, POU5F1 and SOX2). We also found that H3 suppressed tumor growth by 46% in a PANC1-xenograft model, while gemcitabine caused a 36% decrease. The antitumor effects of H3 were confirmed by western blot analysis for COX-2 and cytochrome c expression. Furthermore, necrotic cell death and erythrocyte-containing cavities were detected in tumor tissue by hematoxylin and eosin (H&E) staining. Notably, the combinatorial therapy (H3 and gemcitabine) increased tumor growth compared to that in the control. In conclusion, the present study shows that H3 has promise as a therapeutic agent against pancreatic cancer and its cancer stem cells.

Introduction
Pancreatic cancer is ranked fourth among cancer-related deaths worldwide and has a 5-year survival rate ~5% (1). This is likely due to an inadequate response with the currently available treatments along with late detection, to the aggressive pathogenesis course of the disease, and to the development of extreme resistance to radiation, chemotherapies, targeting agents or a combination of these (1,2). Thus, there is a steady movement of ongoing research on various promising therapies for pancreatic cancer (3-5). Gemcitabine is a widely used therapy to treat patients with pancreatic cancer (6,7). Gemcitabine affects the S phase of cell cycle, preventing cell division, and has proven to be effective for symptom management and prolonged survival in advanced pancreatic cancer (8). Recent clinical trials of combination therapy with gemcitabine and another agent had statistical significance, but not significant enough for patients (9-11).

The use of traditional herbal medicines, such as extracts of herbal mixtures, has been revisited since these medicines often present anticancer activities with no or less adverse side-effects (12). Herbal mixture extracts have been studied in pancreatic cancer, as well as other types of cancers (5,13,14). Cocktail therapies of herbal mixture extracts and antitumor drugs may have synergistic effects against tumor growth, allowing the use of lower doses of anticancer drug (15). Thus, the combinatorial use of a herbal mixture and an anticancer drug is of great interest to enhance the beneficial effects of cancer chemotherapeutics.

The present study assesses the antitumor effect of an herbal mixture extract (H3) in the pancreatic adenocarcinoma cell line PANC1 and in a PANC1-induced heterotopic xenograft model. These effects were compared to those of gemcitabine alone and to a H3-gemcitabine combinatorial therapy.

Materials and methods
Preparation of H3. H3 was composed of 40% Meliae fructus (China), 40% Cinnamon bark (Vietnam) and 20% Sparganium rhizome (China) that were obtained from the Oriental Medical Hospital, Dongguk University (Korea). H3
was prepared as follows: the dried and pulverized medicinal herbs were mixed together in 8 kg batches and were soaked in 40% ethanol (80 l). The ethanol extract was concentrated using a rotary evaporator, lyophilized and reconstituted in distilled water.

**In vitro experiment.** The human pancreatic cancer cell line (PANC1; KCLB #21469) was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). PANC1 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained in a humidified incubator at 37°C containing 5% CO$_2$ and 100 µg/ml streptomycin. The cells were seeded in cell culture plates (Sarstedt Inc., Nuermbrecht, Germany) at a density of 5x10^5 cells. The cells were treated with H3 (0.07 mg/ml) or gemcitabine (10 nM; Sigma-Aldrich, St. Louis, MO, USA). For co-treatment, the cells were treated sequentially with 0.05 mg/ml of H3 and 3 nM of gemcitabine, with a 2 h interval between the two treatments. After a 72 h incubation, the cells were harvested using 0.05% trypsin-EDTA.

**Animal model.** Five-week-old female BALB/c-nu/nu mice (Nara Biotech Co., Seoul, Korea) with body weights between 18-20 g were used in the present study. All animals were maintained in standard conditions under 12 h light and 12 h dark cycle, and allowed to acclimate for a week prior to the start of the study. All procedures were approved by the Internal Animal Care and Use Committee, Korea University (KUIACUC-2014-218).

The xenograft models were created by injection of human tumor cells using the methods described by Kim et al (16). The mice were randomly divided into 4 groups, each experimental group contained 5 mice. H3 was administered orally every 6 days and gemcitabine hydrochloride salt (Eli Lilly and Company, Indianapolis, IN, USA) was administered by intra-peritoneal (IP) injection every 3 days. Tumor volumes and body weights were measured 3 times a week. Tumor volumes were calculated by the formula of $a \times b^2 / 2$, where $a$ and $b$ are the long and short dimensions, respectively. Inhibition of tumor growth was expressed as the T/C ratio, the ratio of the median tumor volume in the treated vs. the control group.

**In vitro assays.** Cell viability was determined by trypan blue dye exclusion assay. Cell cycle and apoptosis analysis were performed using the methods described by Jung et al (17). Analysis of the side population (SP) has been previously described by Goodell et al (19). SP cells are a small population of stem cell-like cells that stain faintly or not at all upon treatment with Hoechst 33342 dye. Verapamil was used to confirm the SP as it blocks the ABC transporters leading to a disappearance of the SP phenotype. Briefly, after drug treatment, PANC1 cells were incubated with Hoechst 33342 dye (5 µg/ml) in the presence or absence of verapamil (50 µM/l) (both from Sigma-Aldrich). The cells were analyzed and the SP was sorted using FACSaria (BD Biosciences, San Jose, CA, USA). The CytoSelect 24-Well Wound Healing assay (Cell Biolabs, San Diego, CA, USA) was used to analyze PANC1 migration. The assay was performed according to the manufacturer's protocol.

For the analysis of transcriptional levels, total RNA was isolated from cells using RNAeasy (Qiagen, Valencia, CA, USA), followed by first strand cDNA synthesis using Reverse Transcription System (Promega, Madison, WI, USA) as directed by the manufacturer's instruction. Real-time PCR was performed using TaqMan Gene expression master mix and TaqMan Gene expression assay (both from Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The real-time PCR was performed using CFX-96 (Bio-Rad, Hercules, CA, USA). The PCR cycle conditions were 95°C for 10 min followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Each analysis was performed in triplicate. Gene expression was quantified using the 2$^{-\Delta\Delta CT}$ method and normalized vs. the expression of the housekeeping gene GAPDH.

**In vivo assay.** Tumors were excised and fixed with 4% formaldehyde. Fixed tumors were embedded in paraffin. Paraffin-embedded sample slides were deparaffinized, hydrated and stained with hematoxylin. After rinsing, the slides were stained with eosin, rinsed and sealed with coverslips using Canada balsam. Stained samples were examined and photographed using an Olympus CK40 microscope (Olympus, Tokyo, Japan).

Tumor sample protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 µm; EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk and incubated overnight with primary antibodies at 4°C. Primary antibodies against COX-2, cytochrome $c$ and $\beta$-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). After washing, horse-radish peroxydase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were applied. The blots were visualized using the enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Statistical analysis.** Data are expressed as means ± standard error (SE) of at least 3 independent experiments. Statistical analyses were performed using the SPSS 20.0 software.
Results

**H3 inhibits PANC1 cell growth.** H3 is the ethanol extract of a mixture of *Meliae fructus*, *Cinnamon bark* and *Sparganium rhizome* (Table I). To examine the cytotoxic effects of this herbal mixture extracts (H3) in PANC1 cells, we used the trypan blue exclusion assay. Both H3 and H3-gemcitabine inhibited the growth rate in a dose-dependent manner (Fig. 1). We hypothesized that co-treatment would allow the use of lower concentrations of anticancer drug leading to less harmful side-effects. Thus, the concentration of gemcitabine was varied after fixing the concentration of H3. The half-maximal inhibitions (IC_{50}) of proliferation were 0.07 mg/ml H3, 10 nM gemcitabine or 0.05 mg/ml H3 with 3 nM gemcitabine (co-treatment). The IC_{50} value of gemcitabine was obtained by Park et al (18). These data indicate that H3 inhibits PANC1 cell growth at the relatively low concentration of 0.07 mg/ml.

**H3 affects cell cycle arrest and apoptotic cell death.** After H3 treatment, the PANC1 cells were fixed in 99% cold ethanol for cell cycle progression. Treatment with H3 led PANC1 cells to cell cycle arrest at the G0/G1 phase (59.6±3.9%) with a concomitant reduction in the percentage of cells in the S and G2/M phase, compared with the values for the control (48.8±1.1%; Fig. 2A and B). When the cells were treated with H3 and gemcitabine (co-treatment), the increase in the percentage of cells in the G0/G1 phase (54.9±3.4%)
was slightly lower than with H3 treatment. In contrast, gemcitabine induced S phase arrest in 60.6±0.9% of cells, which is much higher than the percentage seen in the control population (33.2±1.2%; Fig. 2B).

We examined apoptotic cell death in cells treated with H3 and/or gemcitabine. Cell death was evaluated by double-staining with Annexin V-FITC and PI. The percentage of early apoptotic cell death was 14.1±0.7% with H3 treatment, compared to 5.0±0.3% in the control group (Fig. 2C and D). The percentage of early apoptotic cell death was 12.4±1.2% with co-treatment, which is slightly lower than with H3. The percentage of late apoptotic cell death by co-treatment was 12.3±3.3%, which was slightly higher than with H3 only (10.3±0.8%) or in the control (4.1±0.8%). However, with gemcitabine treatment, the percentage of early and late apoptotic cell death was 7.6±0.3 and 4.9±0.3%, respectively, which was lower than with H3 only and co-treatment.

**H3 suppresses SP cells.** To examine the inhibitory effect of H3 and/or gemcitabine in cancer stem cells, we performed an SP assay. The ABC transporter inhibitor verapamil was administered in order to confirm SP cells (19). The SP gate was determined by the disappearance of the cell population in the presence of verapamil. The difference in the percentage of SP cells between the control (9.0±1.4%) and H3 (5.3±1.3%) treatment groups was ~3.7%. With co-treatment (4.4±1.6%), the percentage of SP cells decreased even further. However, with gemcitabine treatment (6.6±0.9%), the percentage of SP cells was greater than with H3 only and co-treatment, indicating that gemcitabine is less effective than the other

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**Figure 3.** Analysis of the PANC1 SP after treatment with H3 and/or gemcitabine. PANC1 cells were treated for 72 h. (A) The SP fraction was analyzed in the presence of 5 µg/ml Hoechst 33342 with or without 50 µM/l verapamil, an ABC transporter inhibitor, using a FACSAria. The data from one representative experiment out of 3 independent experiments is shown. The proportion of SP cells is shown in (B). Data are expressed as the mean ± SE of 3 independent experiments.

**Figure 4.** Effect of H3 and gemcitabine on the migration mobility of PANC1 cells. (A) Wound healing assays were carried out. Wound fields were observed promptly after removal of inserts (0 h) and cell migration was observed after 24 h. (B) Migration activities were measured by the percent closure of PANC1 cells into the wound field. Data are expressed as the mean ± SE of 3 independent experiments; *p<0.05, compared to control.
treatments (Fig. 3). Thus, among the 3 treatments, co-treatment with H3-gemcitabine is most effective at inhibiting SP cells.

**H3 inhibits migration ability.** After treatment with H3 and/or gemcitabine, the migration activity was measured using a wound healing assay. The percent of closure was much lower in the H3-treated cells than in the control (9.9 vs. 33.5%, respectively; Fig. 4). The percent of closure followed the order: control (33.5%) > gemcitabine only (22.2%) > co-treatment (17.3%) > H3 only (9.9%), indicating that H3 treatment is most effective at inhibiting the migration activity of PANC1 cells.

**H3 decreases the mRNA expression of apoptosis-associated genes.** As H3 induced apoptotic cell death, we investigated the effect of H3 on the mRNA expression of apoptosis-associated genes. After treatment with H3 and/or gemcitabine, the mRNA levels of apoptosis-related genes was analyzed by RT-PCR. As shown in Fig. 5A, treatment with H3 and co-treatment with H3-gemcitabine significantly decreased CXCR4 mRNA levels by ~57 and 69%, respectively. JAK2 mRNA levels were reduced by ~56 and 58% with H3 and co-treatment treatment, respectively (Fig. 5B). With co-treatment XIAP mRNA levels were markedly inhibited by ~80%, but the effects were markedly less with H3 treatment only (Fig. 5C).

**H3 decreases the mRNA levels of stem cell-related genes.** As H3 suppressed the percentage of SP cells, we investigated the effect of H3 on the mRNA accumulation of stem cell-related genes. Following H3 and/or gemcitabine treatment, the mRNA levels of stem cell-associated genes were analyzed by RT-PCR. As shown in Fig. 6A, gemcitabine treatment and co-treatment strongly decreased the levels of ABCG2 mRNA by ~56 and 58%, respectively. The level of ABCG2 mRNA with H3 was less significant (15%) than with gemcitabine treatment and co-treatment. The extent of suppression was statistically lower than that of the control. H3 treatment and co-treatment decreased POU5F1 mRNA levels by ~62 and 53%, respectively (Fig. 6B). All the 3 treatments strongly suppressed SOX2 mRNA levels, but the suppression was more significant with gemcitabine treatment and co-treatment (~85% each) than with H3 treatment (~48%; Fig. 6C).

**H3 suppresses PANC1-induced tumor growth in vivo.** The tumor volumes of control, H3, gemcitabine and co-treated mice at day 31 were ~160.5, 113.1, 109.6 and 267.4 mm³, respectively (Fig. 1A and B). The tumor growth was less in
H3 only- and gemcitabine-treated mice than in the control group, and there was not much difference between the H3 group or gemcitabine group. However, the tumor growth was much greater in the co-treated mice than in the other groups (Fig. 7A, B and D). No significant differences in body weight were observed between the groups (Fig. 7C). To quantify the treatment effects in the tumor xenograft experiments, the T/C ratio was used as an antitumor activity rating. It uses an arbitrary cutoff point and typically has no formal statistical inference (20). The tumor inhibition rates of each treatment group were as follows: control (T/C ratio=1.00), H3 (T/C ratio=0.54), gemcitabine (T/C ratio=0.64), co-treatment (T/C ratio=2.00). Consequently, the tumor inhibition rate of the H3 treatment was greater than all the other groups (Fig. 7B).

The antitumor effects of H3 occur through COX-2 and cytochrome c-mediated apoptotic cell death. Induction of
necrosis and erythrocytes in the tumor tissue was examined using H&E staining. Significant necrotic cell death was detected in H3-treated tumor tissue compared to that in the control (Fig. 8A). Erythrocyte-containing cavities lined by tumor cells were also observed (Fig. 8B). These phenomena were also seen in co-treated and gemicitabine-treated tumor tissues but to a lesser degree. These data indicate that H3 treatment leads to significant necrotic cell death and erythrocyte-containing cavities in tumor tissue. Erythrocytes may enter apoptosis-like suicidal death or eryptosis, which is characterized by cell shrinkage and phosphatidylserine scrambling of the cell membrane. Eryptosis may also be triggered by an increase in cytosolic Ca2+ and the changes in Ca2+ mobilization could be linked to altered levels of COX-2 (21). Thus, the levels of apoptosis-associated factor (cytochrome c) and anti-apoptosis-associated factor (COX-2) were investigated. Proteins extracted from tumor tissue samples were analyzed by western blotting. In H3-treated samples, the expression of COX-2 was significantly reduced and the expression of cytochrome c was strongly increased compared to that in the other treatments (Fig. 9).

Discussion

The use of current anticancer drugs can lead to many harmful side-effects such as resistance, metastasis and even death of normal cells (i.e., apoptosis). However, herbal mixture extracts could be a complementary medicine for anticancer drugs (15). Whereas, Western science and medicine focuses mainly on targeting specific malignant molecular mechanisms, complementary medicine using herbal mixture extracts employs a holistic approach that treats the entire human body (22). There is some dispute regarding the use of herbal mixture extracts since complex mixtures of medicinal herbs are employed in contrast to isolated single natural products. It is often suggested that herbal mixture extracts work synergistically to increase the therapeutic effect, while reducing the amount of adverse side-effects to healthy tissues (22). However, scientific data regarding the efficacy and safety of these complex herbal mixture extracts are often insufficient, so further research into this complementary medicine is needed (7,12,13). The present study has shown that the herbal mixture extract H3 is a candidate for novel cancer therapies, and performs better in the selected tests than gemicitabine. There are some reports on each of the individual medicinal herbs, but the present study is the first to report the effect of this herbal mixture extract (23-25).

We investigated the effect of H3 as an alternative to gemicitabine. We also expected that combinatorial treatment (i.e. co-treatment) with H3-gemicitabine could have an enhanced effect against PANC1 cells. Thus, our experiments were performed in the presence of H3 only, gemicitabine only, or H3 and gemicitabine (co-treatment). We found that H3 suppressed cell growth by inducing G0/G1 cell cycle arrest (Figs. 1 and 2), unlike gemicitabine that is an anticancer drug which delays DNA synthesis through inhibition of the S phase of the cell cycle (8). We also observed that H3 induced greater early and late apoptotic cell death than gemicitabine, and that co-treatment resulted in a similar extent of early and late apoptotic cell death as with H3 treatment (Fig. 2C and D). It is known that most chemotherapeutic drugs kill cancer cells by inducing a programmed form of cell death (i.e. apoptosis) (26). Therefore, we hypothesized that H3 may be a possible therapeutic agent for pancreatic cancer or an adjuvant to gemicitabine treatment.

A subset of cancer cells called CSCs are oncogenic, and these cells are more recuperative than normal tumor cells. Thus, in their presence tumors relapse more easily after anticancer drug treatment. CSCs could be one of the best targets for cancer therapy (28). CSCs as therapeutic targets have been studied in various types of cancer such as breast, liver and prostate cancer (29-31). To investigate the effect of H3 on the characteristics of pancreatic CSCs, an SP analysis was conducted (Fig. 3). Most importantly, the average percentage of SP cells was slightly lower with H3 treatment and co-treatment than with gemicitabine treatment, indicating that the presence of H3 helps suppress SP cells. Additionally, the migratory ability of PANC1 cells was suppressed more greatly with H3 treatment and co-treatment than with gemicitabine only. This also indicates that the presence of H3 is critical for suppression of SP cells.

Since H3 induces apoptosis of PANC1 cells, we examined the mRNA levels of several apoptosis-related genes in the 3 different cell treatment groups (Fig. 5). The accumulation of CXCR4 mRNA, a gene related to metastasis and anti-apoptosis, and JAK2, another anti-apoptotic gene, was significantly decreased with all 3 treatments, suggesting that they each induce apoptosis efficiently. The mRNA levels of the anti-apoptotic protein XIAP decreased markedly by co-treatment with H3-gemicitabine, while levels decreased to a lesser degree with H3 or gemicitabine (data not shown). Due to their partial apoptotic resistance, it is known that pancreatic cancer cells often respond poorly to chemotherapy and/or radiotherapy (27). These results indicate that H3 may be involved in the suppression of anti-apoptotic signaling, which could help overcome this resistance. Further studies are required to unveil the details of the relevant mechanistic pathway used by H3 for this purpose.

Since H3 suppresses SP cells, the mRNA accumulation of several stem cell-related genes was investigated (Fig. 6). The expression of ABCG2, which plays a role in multi-drug resistance, significantly decreased with co-treatment and

![Figure 9. Effect of H3 and gemicitabine on the protein expression of COX-2 and cytochrome c in tumor tissue. The expression levels of COX-2 (inflammation factor) and cytochrome c (apoptosis factor) were determined by western blot analysis. β-actin was used as an internal control.](image-url)
gencitabine treatment. The expression of POU5F1, which is related to the self-renewal of undifferentiated embryonic stem cells, was significantly reduced by H3 treatment and co-treatment. The expression of SOX2, which is a transcription factor that is essential for maintaining self-renewal or pluripotency of undifferentiated embryonic stem cells, was significantly decreased by co-treatment and gencitabine treatment. Although there is no specific trend for the suppression of the 3 stem cell-related genes by the 3 different treatments, most of stem cell-related genes were suppressed to some extent by H3 as well as gencitabine. This suggest that H3 is as potent as gencitabine in suppressing pancreatic CSCs.

The in vivo study revealed that H3 and gencitabine effectively suppress tumor growth (Fig. 7). However, contrary to the in vitro results, tumor growth in vivo was enhanced with co-treatment with H3–gencitabine. This suggests that co-treatment with H3 may not be recommended for pancreatic cancer. Significant increases in necrotic cell death and erythrocyte-containing cavities were observed in H3-treated tumor tissue compared to the findings in other treatments (Fig. 8). The effectiveness of H3 as an anticancer agent for pancreatic cancer was also evidenced by western blot analysis which revealed that the expression of apoptosis-associated factor (cytochrome c) was upregulated and the expression of anti-apoptosis-associated factor (COX-2) was downregulated in the tumors of mice having received this treatment (Fig. 9).

In conclusion, our experimental results show the remarkable effect of H3 in pancreatic cancer cells, cancer stem cells, and animal models. H3 is another example of an herbal mixture extracts which requires further studies to be developed into an anticancer therapeutic agent. Further study may be needed for detailed anticancer signaling pathway and, more importantly, application of H3 to other types of cancers.

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References


