Cyclodextrin Conjugated α-Bisabolol Suppresses FAK Phosphorylation and Induces Apoptosis in Pancreatic Cancer

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Abstract. *Background/Aim: α-Bisabolol is an essential oil component extracted from plants, such as chamomile. We have previously reported that α-bisabolol suppressed proliferation, invasion, and motility of pancreas cancer. Cyclodextrin improved the solubility of α-bisabolol, therefore it enabled to administer intravenously. The aim of this study was to clarify the effect of cyclodextrin conjugated α-bisabolol (CD-BSB) and the signals pathways associated with α-bisabolol for pancreatic cancer. Materials and Methods: Human pancreatic cancer cell lines were treated with or without CD-BSB. Cytomorphology and apoptosis were assessed in these treated groups. In addition, several phosphorylated proteins were analyzed to clarify the signal pathway concerning CD-BSB. In subcutaneous xenograft model, tumor volume and Ki-67 expression were evaluated among Control (untreated), CD-BSB, or Gemcitabine (GEM). Results: CD-BSB significantly changed cytomorphology and induced apoptosis in pancreatic cancer cells. CD-BSB suppressed phosphorylation of focal adhesion kinase (FAK). In addition, pFAK 397 was inhibited by CD-BSB in a concentration-dependent manner in cancer cells. In the subcutaneous xenograft models, the tumor volume in the CD-BSB groups was lower than Control groups. Ki67-positive cells in CD-BSB treated group were lower than the GEM-treated groups. Conclusion: We clarified efficiency of CD-BSB in xenograft tumor using intravenous administration. α-Bisabolol suppresses phosphorylation of FAK 397 and impairs cytoskeletal polymerization in a pancreatic cancer.*

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Further investigations are required to reveal the precise mechanisms of the antitumor effects of solubilized α-bisabolol to facilitate its clinical application. Our data indicate that solubilized α-bisabolol has therapeutic potential and could improve the prognosis of cancer patients.

α-Bisabolol is an essential oil component extracted from plants, such as chamomile. Several researchers have reported the effectiveness of α-bisabolol for cancer, such as glioma and non-small cell lung carcinoma (1, 2). We have previously reported that α-bisabolol suppressed proliferation, invasion, and motility of pancreas cancer (3, 4). The latter remains as one of the refractory malignancies (5). Novel treatments are required to improve the prognosis of pancreatic cancer patients. α-Bisabolol is thought to be the candidates of the anticancer drugs for pancreas cancer.

Poorly soluble compounds may cause embolisms during intravenous administration and may be insufficiently absorbed by oral intake (6). α-Bisabolol is a poorly soluble compound, therefore it is difficult to administer it to patients intravenously. Due to this issue, we developed α-bisabolol derivatives which are more potent than the parent compound and may be clinically useful against pancreatic cancer (7). This α-Bisabolol derivative suppressed xenograft tumor growth and disturbed dissemination of pancreatic cancer, even though its solubility did not improve.

Several researchers have reported solubilization methods for insoluble chemicals using micelles, liposomes, and solvents, such as cyclodextrin (8, 9, 10). Cyclodextrin itself is known to be a safe substance with low toxicity and widely used in the food industry and medical products (10). Cyclodextrin improved the solubility of α-bisabolol, therefore it enabled its administration intravenously. In this study, we investigated the effect of cyclodextrin conjugated α-bisabolol (CD-BSB) for pancreatic cancer.

We have also previously reported that α-bisabolol inhibits the activation of Akt and its upstream signals, such as PI3K, PDK1, and mTORC2 (3), and the inhibition of KISS1R

weakened the inhibitory effect of α-bisabolol on the invasiveness of pancreatic cancer cells (4). However, the signaling pathways associated with α-bisabolol are not well understood.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase, which consists of 1030 amino acids (11). It regulates integrin and growth factor signaling and is known to play an important role in cell proliferation, differentiation, and apoptosis (12, 13). In addition, FAK is involved in the cytoskeleton and the formation and alteration of focal adhesions (12). In this study, we focused on FAK and clarified its relevance to α-bisabolol.

Materials and Methods

Materials. α-Bisabolol was purchased from Sigma-Aldrich (St Louis, MO, USA). 2-Hydroxypropyl-β-cyclodextrin was purchased from FUJIFILM (Osaka, Japan). Gemcitabine was purchased from Sawai Seiyaku (Osaka, Japan). Hydroxypropyl-β-cyclodextrin (CD) 2.8 g was dissolved in distilled water 6 ml to prepare 250mM CD solution. Next, α-bisabolol 108.8 μl and 250mM CD solution 4 ml were mixed and incubated at 37°C to prepare a 112.6 mM α-bisabolol solution.

Cell culture. Human pancreatic cancer cell lines (KLM1 and Panc1) were kindly provided from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan). The KP4 human pancreatic cancer cell line was obtained from Riken BioResource Center (Ibaraki, Japan). KLM1 and Panc1 cells were cultured in RPMI 1640 medium (FUJIFILM WAKO, Osaka, Japan) with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Inc. Kerrville, TX, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. KP4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM high glucose; FUJIFILM WAKO) with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Western blot analysis. Whole-cell extracts and tumor tissues were prepared in Laemmli sample buffer. The cell lysate was electrophoresed on Bullet PAGE One Precast Gel (Nacalai tesque, Kyoto, Japan), transferred to PVDF membranes (Immobilon; Millipore, Billerica, MA, USA), and probed with antibodies. Rabbit anti-FAK polyclonal antibody (71433S) (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-phosphorylated FAK Thr[397](8556), Thr[577] (3281) and Thr[925] (3284) polyclonal antibodies (1:1,000; Cell Signaling Technology, Inc.) and mouse anti-β-actin monoclonal antibody (A5316) (1:5,000; Sigma-Aldrich, Inc.) were used as primary antibodies. Anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (#7074S) (1:1,000; Cell Signaling Technology, Inc.) and anti-mouse IgG, HRP-linked antibody (#7076S) (1:1,000; Cell Signaling Technology, Inc.) were applied as secondary antibodies. The signals were detected using Pierce Western blot Substrate (Thermo, Rockford, IL, USA) on a ChemiDoc Touch imaging system (BIO RAD, Tokyo, Japan).

Apoptosis studies. Apoptotic studies were performed using the Muse Annexin V & Dead Cell Kit (Millipore, Inc., Burlington, MA, USA)

according to the manufacturer's instructions. The cells (1×10^5 cells) were cultured in six-well plates. After incubation at 37°C and 5% CO_2 for 24 h, the medium was removed and replaced with 10× diluted fresh medium (1% FBS, 10 U/ml penicillin) containing 75 μM of α-bisabolol diluted in 2-hydroxypropyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, or medium. The apoptosis rate was measured after incubation at 37°C and 5% CO_2 for 72 h. The experiment was performed in triplicate.

Human RKT phosphorylation antibody array. Comprehensive protein phosphorylation was analyzed in untreated and CD-BSB-treated cells using the Human RTK Phosphorylation Antibody Array C1 (RayBiotech, Inc., Peachtree Corners, GA, USA) according to the manufacturer's instructions. The signals were detected using an ECL system. The data were scanned by a transmission-mode scanner. The experiment was performed in triplicate.

Cell morphology analysis. The cells (1×10^5 cells) were cultured in six-well plates and incubated at 37°C and 5% CO_2 for 24 h. The medium was changed to 10x diluted fresh medium (1% FBS, 10 U/ml penicillin) containing 75 μM of α-bisabolol diluted in 2-hydroxypropyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, or medium. After incubation at 37°C and 5% CO_2 for 72 h, the cell morphology was observed under the microscope (BZ-H4XD; Keyence, Osaka, Japan).

Cytoskeleton analysis. Cytoskeleton was investigated using the Cytoskeleton Kit (SiR-Actin and SiR-Tubulin) (Cytoskeleton, Inc., Denver, CO, USA), according to the manufacturer's instructions. KLM1 cells (1×10^5 cells) were cultured in six-well plates at 37°C and 5% CO_2 for 24 h. Next, the cells incubated with the medium (1% FBS, 10 U/ml penicillin) containing 50 μM of α-bisabolol diluted in 2-hydroxypropyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, or PBS alone. The medium was then removed and replaced with staining solution (500 nM SiR-actin and tubulin was diluted to the desired concentration in the cell culture medium). Twenty-four h later, the cells incubated for 10 minutes using Hoechst® 33342 (Invitrogen, Waltham, MA, USA). Thereafter, the cytoskeleton was observed under the microscope (BZ-H4XD; Keyence).

Histology. Tumor samples were fixed immediately in 10% buffered formalin, dehydrated in a graded ethanol series, embedded in paraffin, and then stained with hematoxylin and eosin.

Immunohistochemistry. Ki 67 was stained automatically using rabbit anti-Ki 67 polyclonal antibodies (Abcam, Inc., Cambridge, UK). The Discovery XT automated slide preparation system (Ventana Medical Systems, Inc., Oro Valley, AZ, USA) was used for automated staining, and the procedure was performed according to the manufacturer's instructions.

Animal studies. All animal experiments were conducted in compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine. The experiments were approved by the ethics committee of Nagoya University Graduate School of Medicine (approval number: 20212). Mice were kept in a temperature- and humidity-controlled environment under a 12 h light–dark cycle and had free access to water and food at all times. Male BALB/c nude mice (7 weeks old
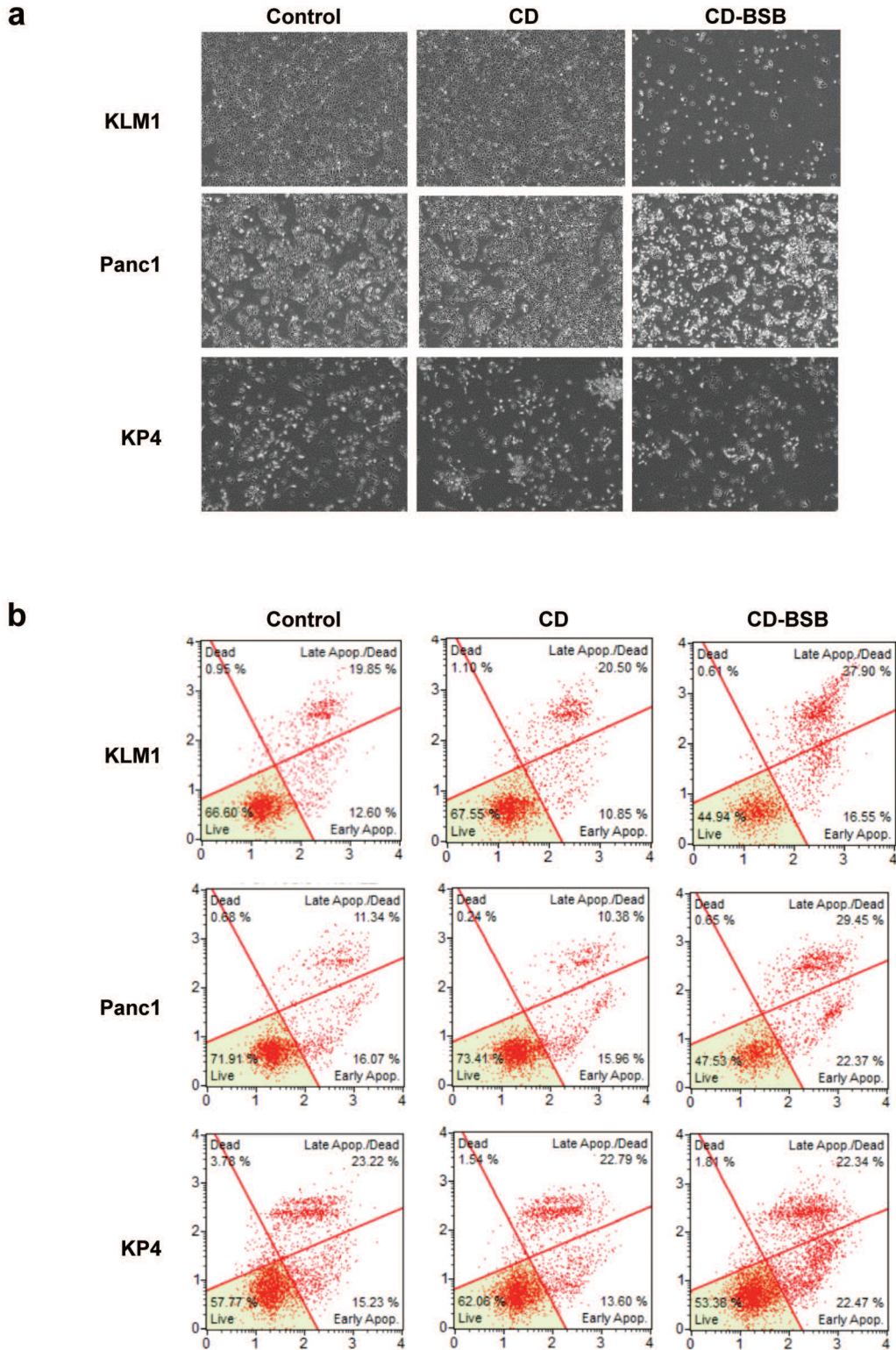
Figure 1. *CD-BSB induced apoptosis in pancreatic cancer cells. (a) Images depicting the morphology of KLM1, Panc1 and KP4 cells that were untreated (Control) or treated with cyclodextrin (CD) or cyclodextrin conjugated α-bisabolol (CD-BSB). (b) Apoptosis was analyzed in KLM1, Panc1 and KP4 cells that were untreated (Control) or treated with cyclodextrin (CD) or cyclodextrin conjugated α-bisabolol (CD-BSB) using the Muse™ Annexin V & Dead Cell Kit. Cells were classified into 4 groups: dead cells, late apoptotic/dead cells, live cells, and early apoptotic cells. A representative of 3 independent experiments is shown.*
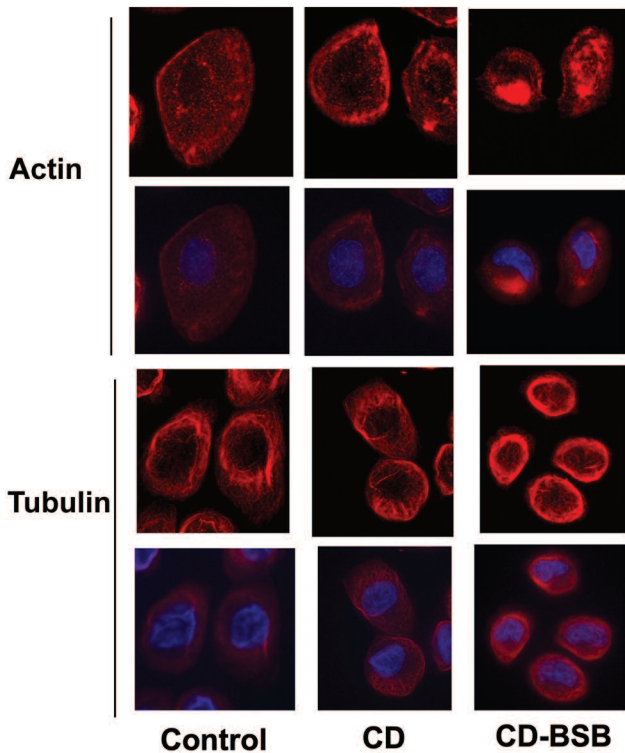
Figure 2. *CD-BSB inhibited the cytoskeleton maintenance. Immunocytochemistry for actin and tubulin in KLM1 cells that were untreated (Control) or treated with cyclodextrin (CD) or cyclodextrin conjugated α-bisabolol (CD-BSB).*

and weighting 20-25g) were purchased from SLC Japan (Nagoya, Japan). Mice were anesthetized using 1% isoflurane in a transparent acrylic chamber. Ten minutes later, they were moved out of the chamber. And then, KLM1 cells (5×10^6 cells/100 μl) were inoculated into the femoral area of each mouse subcutaneously.

After 7 days, the mice were randomly divided into three groups, namely the control group (untreated), the CD-BSB group (the intravenous administration) and the Gemcitabine group (the intraperitoneal administration). In the CD-BSB group, a catheter was inserted into the internal jugular vein and a continuous drug infusion pump was implanted subcutaneously (12). CD-BSB was prepared as α-bisabolol (300 mg/kg), diluted in 2-hydroxypropyl-β-cyclodextrin (total volume 200 μl). CD-BSB was administered to the mice using the infusion pump continuously and intravenously for two weeks. Gemcitabine (40 mg/kg, total volume 100 μl) was injected intraperitoneally twice a week for two weeks. After 22 days, all mice were sacrificed, and tumor size was measured. Tumor growth was assessed by measuring the volume (in mm^3). The volume was calculated using the following equation: $(L \times W^2)/2$, where L is the tumor length (in mm), and W is the tumor width (in mm). After the treatment, the mice were checked for metastatic or disseminated lesions in the thoracic and peritoneal cavities.

Statistical analysis. Differences were tested for significance by Student's *t*-test, ANOVA or Tukey-Kramer test. A difference was considered statistically significant when *p*<0.05.

Results

CD-BSB induced apoptosis in pancreatic cancer cells. Cell viability was analyzed in the pancreatic cancer cell lines KLM1, Panc1, and KP4 with cyclodextrin conjugated α-bisabolol (CD-BSB), cyclodextrin (CD) or no treatment (control). Cell death was assessed in these cell lines 72 hours after the treatments (Figure 1A). Cell death was not present in the CD-treated and control group. In contrast, CD-BSB significantly induced cell death.

Apoptosis was also evaluated in KLM1, Panc1, and KP4 cells treated with CD-BSB or CD and those with no treatment (control). Figure 1B showed the representative results in triple experiments. We defined early plus late apoptotic rate as total apoptotic rate. The total apoptotic rate in the control group, CD-treated group and CD-BSB-treated group was 32.4%, 31.3%, and 54.4% in KLM1, 27.3%, 26.2%, and 51.7% in Panc1, and 38.4%, 36.3%, and 44.8% in KP4 cells, respectively (Figure 1B). CD-BSB significantly increased apoptosis of pancreatic cells, compared with the untreated cells or cells treated with CD in the KLM1, Panc1 and KP4 cell lines. These results indicate that CD-BSB has a strong cytocidal effect on pancreatic cancer cells.

CD-BSB inhibited the cytoskeleton maintenance. Following the effects of CD-BSB on apoptosis, we evaluated the cell morphology and cytoskeleton. As shown Figure 1B, the apoptotic rate was significantly elevated in KLM1 after the treatment. This result suggests that KLM1 is the most sensitive to CD-BSB, therefore we conducted subsequent experiments using this cell line. The cytoskeleton was maintained in CD-treated KLM1 cells and the control group (Figure 2). In contrast, KLM1 cell treated with CD-BSD appear to have shrunk in size. In these cells, actin fibers were ruptured and were accumulated disproportionally. Similarly, tubulin was also found to accumulate on the cell surface. These results can be considered to be an inhibition of polymerization of the cytoskeleton, such as actin and tubulin by CD-BSB.

Comprehensive protein phosphorylation in pancreatic cancer cells after CD-BSB. To further characterize the effect of CD-BSB, the expression of protein phosphorylation was determined in KLM1 cells treated with CD-BSB using the human RTK phosphorylation antibody array. Figure 3A demonstrated that the representative results in triple experiments. Among 71 protein kinases, FAK had significantly lower average protein levels in CD-BSB -treated cells than in control cells (Figure 3A and B). This result indicated that FAK may be associated with the induction of apoptosis of CD-BSB.

Expression of phosphorylated FAK after CD-BSB in pancreatic cancer cells. FAK phosphorylation, such as
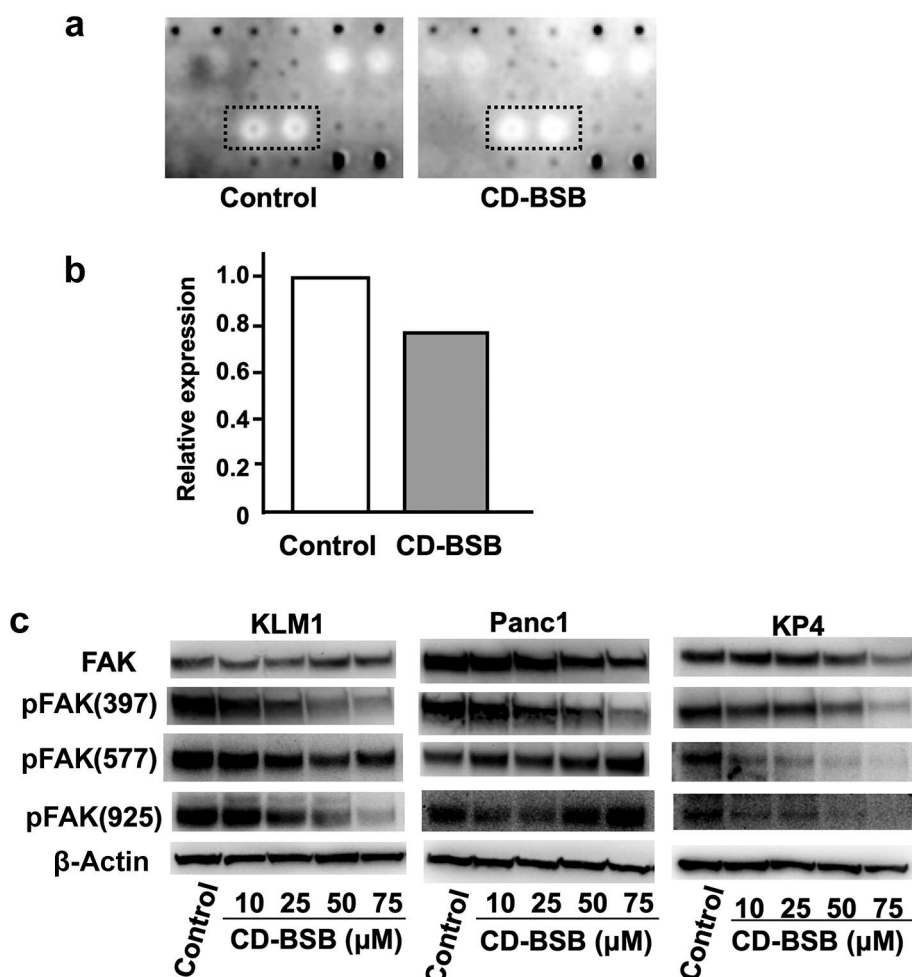
Figure 3. *Comprehensive protein phosphorylation in pancreatic cancer cells after CD-BSB. (a) Comprehensive protein phosphorylation using the Human RTK Phosphorylation Antibody Array in KLM1 cells with Control (Untreated) or cyclodextrin conjugated α-bisabolol (CD-BSB). (b) Level of phosphorylated FAK were analyzed in in KLM1 cells with Control (Untreated) or cyclodextrin conjugated α-bisabolol (CD-BSB). (c) The expression of FAK, pFAK1 (397), pFAK1 (577), and pFAK1 (925) was detected in KLM1, Panc1 and KP4 cells that were untreated (Control) or treated with cyclodextrin conjugated α-bisabolol (CD-BSB) by Western blot analysis. β-actin was used as an internal loading control. THD: Thioridazine.*

pFAK397, pFAK577 and pFAK925, was evaluated in three pancreas cells with CD-BSB or CD and those with no treatment (control). The expression of pFAK397 was decreased in all pancreas cell lines in a concentration-dependent manner. However, CD-BSB inhibited pFAK577 in KP4, but not in KLM1 and Panc1. In pFAK925, it was suppressed in KP4 and KLN1, whereas it was not inhibited in Panc1. The inhibitory effects of pFAK577 and pFAK925 differed by CD-BSB among each cell line (Figure 3C).

Antitumor effects of CD-BSB in xenograft nude mice. To examine the therapeutic potential of CD-BSB in pancreatic cancer, mice with subcutaneous xenograft tumors were treated with CD-BSB intravenously for 2 weeks using the continuous infusion pump. The tumor growth in the CD-BSB or Gemcitabine -treated groups was significantly lower than that in the control group (Figure 4A). The tumor volumes in the control group, CD-BSB-treated group and Gemcitabine-treated group were 974±283, 528±122 and 337±73 mm^3, respectively (Figure 4B). The tumor sizes in the CD-BSB -treated group were similar to those in the Gemcitabine-treated group. This result indicates that CD-BSB had antitumor efficacy as well as Gemcitabine. No apparent side effects, such as vomiting, diarrhea, decreased activity and reduction of food intake, were observed by CD-BSB.

There were significantly more Ki67-positive cells in the tumors of the control group than in the tumors of the CD-BSB group (Figure 4C). Interestingly, there was no
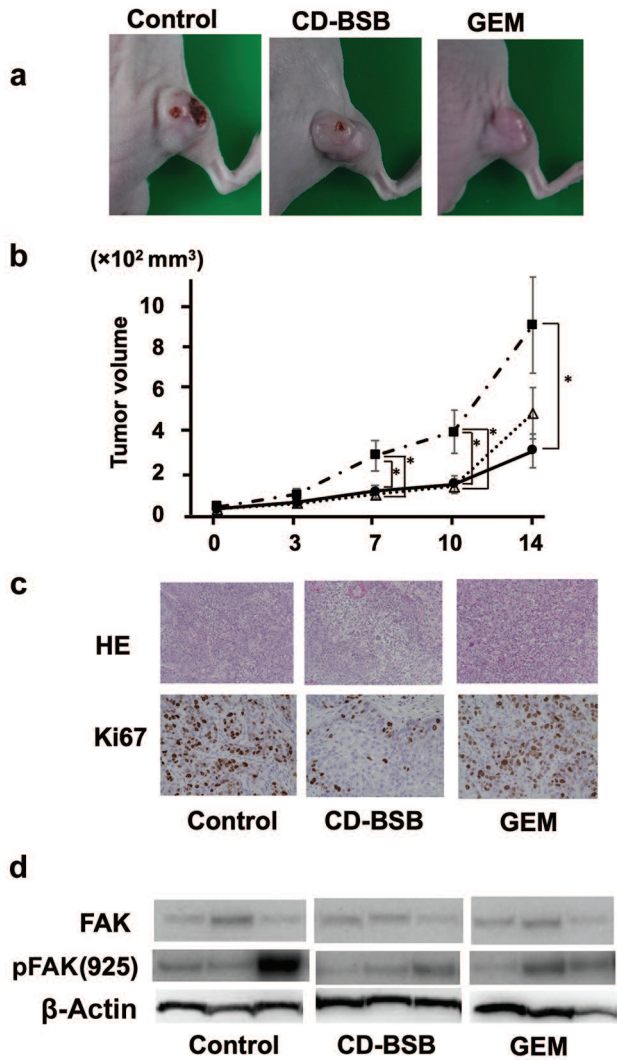
Figure 4. *Antitumor effects of CD-BSB in xenograft nude mice. (a) Representative images of KLM1 xenograft tumors untreated (Control) and treated with cyclodextrin conjugated α-bisabolol (CD-BSB) or Gemcitabine (GEM). n=5 for each group. (b) The graphs show the average volume of the xenograft tumors in each experimental group. Control: ■, CD-BSB: △, GEM: ●. ∗p<0.05. (c) Histological analysis of the xenograft tumors of each experimental group was performed by hematoxylin-eosin staining. Ki-67 expression in the xenograft tumors of each experimental group was determined by immunohistochemistry. (d) The expression of FAK and pFAK1 (925) was detected of the xenograft tumors of each experimental group was performed by Western blot analysis. β-actin was used as an internal loading control.*

difference concerning tumor volume between the CD-BSB and GEM-treated groups, whereas Ki67-positive cells in CD-BSB treated group were lower than the GEM-treated groups. Unexpectedly, there were no significant differences concerning the suppression of FAK phosphorylation in xenograft tumor among the control group, CD-BSB-treated group, and Gemcitabine-treated group (Figure 4D).

Discussion

In this study, we clarified the efficiency of cyclodextrin conjugated α-bisabolol (CD-BSB) in xenograft tumors using intravenous administration. The dissolution rate of α-bisabolol is reported to be approximately 2.5% using ethanol, therefore it is not possible to administer this compound intravenously (15). Cyclodextrin is a substance, which is frequently used to encapsulate hydrophobic molecules to increase their solubility in water (10). CD-BSB improved its solubility and allowed intravenous administration without the risk of embolism. Intravenous administration of CD-BSB was able to suppress the growth of tumors, similarly to the oral intake in a previous study.

Gemcitabine is one of anti-cancer drugs, which is widely used for pancreatic cancer (16). *In vivo* study, we compared the antitumor effect between Gemcitabine and solubilized α-bisabolol. Unexpectedly, there was no difference regarding tumor volume between the CD-BSB and GEM-treated groups, whereas Ki67-positive cells in the CD-BSB treated group were lower than the GEM-treated groups. CD-BSB was administrated continuously and intravenously to mice using the infusion pump. In contrast, Gemcitabine was injected to mice intraperitoneally twice a week. The administration methods of the two compounds may have affected the difference between the tumor volume and Ki67 expression of the xenograft tumor. Unfortunately, there were no significant differences concerning the suppression of FAK phosphorylation in xenograft tumor among the control group, CD-BSB-treated group, and Gemcitabine - treated group (Figure 4D). As mice were sacrificed a week after the final administration of CD-BSB, α-bisabolol may not have been able to suppress the FAKs phosphorylation within the tumor.

In addition, we revealed that CD-BSB suppressed FAK phosphorylation in pancreatic cancer cells. FAK is a nonreceptor cytoplasmic protein tyrosine kinase, which is implicated in a wide array of cellular processes (17). It is also involved in the production of matrix metalloproteinases (MMPs) which degrade extracellular matrix (ECM) components. The degradation of the ECM is an initial step for the invasion and metastasis of cancer cells (17). Some researchers have reported that the FAK/PI-3K/Rac1 signaling regulated the rapid and potent actin remodeling in malignant cells (18). We have previously reported that α-bisabolol suppressed the invasiveness and motility of a pancreatic cancer cell line (4). Our data show that α-bisabolol suppresses FAK phosphorylation and impairs cytoskeletal polymerization.

Interestingly, CD-BSB differentially suppressed the FAK phosphorylated sites among the different pancreatic cancer cell lines. pFAK 397 was inhibited by CD-BSB in a concentration-dependent manner in KP4, KLM1 and Panc1

cells. In contrast, the suppression of pFAK577 and pFAK925 was different among the pancreas cancer cell lines. Integrin or several growth factors trigger the autophosphorylation of tyrosine 397, thereafter followed by phosphorylation of other phosphorylation sites (12). Mon *et al*. reported that Tyr 397 and Tyr 925 of six major tyrosine phosphorylation sites of FAK are important for FAK-dependent signaling and phosphorylated Tyr 397 induced phosphorylation of Tyr 925 in a time dependent manner (17, 19, 20). Our results, at least in part, demonstrate the importance of FAK Tyr 397 in the antitumor effect of CD-BSB for pancreatic cancer.

Conclusion

In summary, this study demonstrated the efficacy of solubilized α-bisabolol for pancreatic cancer both *in vitro* and *in vivo*. Further investigations are required to reveal the precise mechanisms of the antitumor effects of solubilized α-bisabolol to facilitate its clinical application as a novel cancer treatment. Our data indicate that solubilized α-bisabolol has therapeutic potential and could improve the prognosis of cancer patients.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

Mikiko Takebayashi Kano, Toshio Kokuryo, Tomoki Ebata and Masato Nagino conceived and designed this study. Kimitoshi Yamazaki, Taisuke Baba, Junpei Yamaguchi, Masaki Sunagawa, Kazushi Miyata and Atsushi Ogura performed experiments and acquired the data. Nobuyuki Watanabe, Shunsuke Onoe and Takashi Mizuno analyzed the data. Mikiko Takebayashi Kano, Toshio Kokuryo, Kay Uehara, Tsuyoshi Igami and Yukihiro Yokoyama wrote the manuscript. All Authors read and approved the final manuscript.

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