Significance of combination therapy of zoledronic acid and gemcitabine on pancreatic cancer

趙, 茗
九州大学大学院医学研究院臨床腫瘍外科

https://doi.org/10.15017/26344

出版情報：Kyushu University, 2012, 博士（医学）, 課程博士
バージョン：

Significance of combination therapy of zoledronic acid and gemcitabine on pancreatic cancer

Ming Zhao[1], Yohei Tominaga[1], Kenoki Ohuchida[1,2], Kazuhiro Mizumoto[1,3], Lin Cui[1], Shingo Kozono[1], Hayato Fujita[1], Ryo Maeyama[1], Hiroki Toma[1], Masao Tanaka[1]

[1]*Department of Surgery and Oncology,*

[2]*Department of Advanced Medical Initiatives, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

[3]*Kyushu University Hospital Cancer Center, Fukuoka, Japan*

Correspondence to: Yohei Tominaga or Kenoki Ohuchida, Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Fukuoka 812-8582, Japan.

E-mail: tominaga@cd6.so-net.ne.jp or kenoki@surg1.med.kyushu-u.ac.jp

Tel: +81-92-642-5440; Fax: +81-92-642-5458

Word count: 5409 words

Running title: Combination therapy of ZOL and GEM

Keywords: combination therapy; zoledronic acid; gemcitabine; pancreatic cancer

Abstract

In this study, we examined the cytotoxic effects of combination therapy with zoledronic acid (ZOL) and gemcitabine (GEM) on pancreatic cancer cells *in vitro* and *in vivo*. Four human pancreatic cancer cell lines were treated with ZOL, GEM or a combination of both, and the effects of the respective drug regimens on cell proliferation, invasion and matrix metalloproteinase (MMP) expression were examined. A pancreatic cancer cell line was also intrasplenically or orthotopically implanted into athymic mice and the effects of these drugs on tumor metastasis and growth *in vivo* were evaluated by histologic and immunohistochemical analyses. Combination treatment with low doses of ZOL and GEM efficiently inhibited the proliferation ($P < 0.001$) and invasion ($P < 0.001$) of pancreatic cancer cells *in vitro*. Western blotting assay revealed that MMP-2 and MMP-9 expression levels were decreased after ZOL treatment. *In vivo*, combined treatment significantly inhibited tumor growth ($P < 0.05$) and the development of liver metastasis ($P < 0.05$). These data revealed that, when used in combination, ZOL and GEM have significant antitumor, anti-metastatic and anti-angiogenic effects on pancreatic cancer cells. In the present study, we first reported the significance of the combination treatment of ZOL and GEM in pancreatic cancer using an *in vivo* model. These data show promise for the future application of this drug regimen in patients with pancreatic cancer.

Introduction

Pancreatic cancer has the potential for rapid growth and metastasis and is one of the most fatal human cancers. In Japan, pancreatic cancer is the fifth leading cause of cancer-related death, with almost 25,000 patients dying of pancreatic cancer in 2007 [1]. Despite developments in diagnostic imaging, for the majority of patients the diagnosis of pancreatic cancer occurs during the advanced stage of the disease. Consequently, the overall 5-year survival rate for pancreatic cancer is only 6.7% [1], and most patients treated by curative resection develop incurable local relapses, liver metastases and/or peritoneal disseminated disease.

Conventional adjuvant therapies such as radiotherapy and chemotherapy (or a combination of both) have had little beneficial net impact on this aggressive neoplasm. Of the chemotherapeutic agents available, gemcitabine (GEM), a deoxycytidine analogue, is the most common first-line treatment for pancreatic cancer [2]. Intracellular phosphorylated GEM (dFdCTP) incorporates into the DNA and potently inhibits the proliferation of pancreatic cancer cells by inhibiting DNA synthesis [3]. Moreover, GEM also inhibits ribonucleotide reductase, leading to a depletion in cellular deoxynucleotides [4]. Recent studies have shown that in patients with advanced disease, combining GEM with other systemic agents can improve patient outcomes relative to the outcomes obtained with standard GEM monotherapy [5]. However, the survival benefit of GEM for patients with advanced pancreatic cancer remains limited [6, 7]. To improve the prognosis for patients with pancreatic cancer, especially those with advanced stage disease, other strategies are urgently required.

Bisphosphonates (BPs) are currently the most important class of inhibitors of osteoclast-mediated bone resorption [8] and are used extensively for the treatment of skeletal diseases, such as Paget's disease [9], postmenopausal osteoporosis [10] and tumor-induced osteolysis [11]. Previous research has shown that zoledronic acid (ZOL) demonstrates antitumor activity in several human neoplasms such as myeloma [12], breast [13], prostate [14], colon [15] and pancreatic cancers [16]. Further, ZOL has been reported to inhibit proliferation and induce apoptosis of tumor cells through the mevalonate pathway [17] by preventing the translocation of small GTPase Ras to the

plasma membrane [18]. Recently, Li *et al* published a report showing the ability of ZOL to inhibit invasion and migration through downregulation of vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) in human nasopharyngeal carcinoma cells *in vitro* [19]. Other research on *in vivo* models has also shown that ZOL impairs the growth of cervical carcinomas in transgenic HPV/E2 mice by suppressing tumor-associated angiogenesis [20]. In this study, ZOL increased apoptosis in neoplastic epithelial and endothelial cells and suppressed the expression of the pro-angiogenic MMP-9 in infiltrating macrophages, which are implicated in the mobilization of the VEGF [20]. These data suggest that chemotherapy with ZOL may be an effective treatment with the potential to inhibit proliferation, invasion and angiogenesis in pancreatic cancer.

In the present study, we analyzed the therapeutic effects of ZOL, GEM and a combination of both agents in pancreatic cancer using *in vitro* and *in vivo* models. The *in vitro* results revealed that GEM exerts a strong anti-proliferative effect but is relatively ineffective against the invasive ability of pancreatic cancer. In contrast, low-dose ZOL had no anti-proliferative effect but did show a potent anti-invasive effect. *In vivo* experiments also revealed that intraperitoneal injection of ZOL combined with weekly GEM treatment significantly reduced tumor growth, angiogenesis and metastasis of human pancreatic cancer orthotopically implanted into nude mice. The present data suggest that combination treatment with ZOL plus GEM may be a novel therapy for controlling pancreatic cancer.

Materials and methods

Cell culture and materials. We used following four human pancreatic cancer cell lines: SUIT-2 (purchased from Health Science Research Resources Bank, Osaka, Japan), CFPAC-1 (American Type Culture Collection, Rockville, MD), Panc-1 and KP-2 (generously provided by Dr. H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan). The cells were maintained as described previously [21]. Both GEM (2', 2'-difluorodeoxycytidine; Eli Lilly & Company, Indianapolis, IN, USA) and ZOL (Novartis Pharma, Basel, Switzerland) were obtained commercially. The drugs were dissolved and diluted in phosphate-buffered saline (PBS) and then added to the cell culture medium or injected intraperitoneally (i.p.) into the nude mice at the indicated concentrations.

Cell proliferation assay. To analyze cell proliferation, cell populations were evaluated by measuring the fluorescence intensity of propidium iodide (PI) at specified time using Infinite F200 (TECAN, Mannedorf, Switzerland) as described previously [22]. The pancreatic cancer cells were seeded in 24-well tissue culture plates at a density of 1.5×10^4 cells/well and cultured for 24 h. After determining the initial cell population, fresh medium containing ZOL (0, 1, 2, 5 and 10 μM), GEM (0, 1, 2.5, 5 and 10 nM) and different combinations of doses of ZOL and GEM at the indicated concentrations was added to the cells. Control experiments were also conducted in parallel, whereby cells were not treated with the drugs. All experiments were performed in triplicate wells and were repeated at least three times.

Invasion assay. The invasion of the pancreatic cancer cells was measured based on the number of cells invading through Matrigel (20 μg/well, Becton Dickinson, San Jose, CA)-coated transwell inserts (BD Biosciences, Franklin Lakes, NJ) with 8-μM pores as reported previously [21]. In brief, the cells were suspended in medium supplemented with 10% FBS in the presence or absence of ZOL, GEM and different-dose combinations of ZOL and GEM, and were seeded into the upper

chambers at a density of 1×10^5 cells/cm^2. The SUIT-2, CFPAC-1 and KP2 cells were incubated for 48 h, whereas Panc-1 cells were incubated for 24 h. The number of invaded cells was counted in five randomly selected fields under light microscopy.

Western blotting analysis. The SUIT-2 and Panc-1 cells were cultured in DMEM supplemented with 10% FBS and treated with 2 and 10 μM of ZOL or an equal volume of PBS as a control for 48 h. The cells were directly lysed in PRO-PREP (iNtRON Biotechnology, Seongnam, Korea). Cell lysate proteins (25 μg) were fractionated through electrophoresis using 12% sodium dodecyl sulfate polyacrylamide gels and wet-transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was then incubated overnight at 4 °C with anti-MMP2 (sc-10736; 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MMP9 (EP1254; 1:1000, Millipore), anti-MMP14 (sc-12367-R; 1:200, Santa Cruz Biotechnology) or anti-β-actin (sc-1616; 1:2000, Santa Cruz Biotechnology) antibodies and then probed with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Immunoblots were detected by enhanced chemiluminescence with a ChemiDoc XRS System (Bio-Rad Laboratories, USA).

***In vivo* experiments**. Five-week-old female athymic nude mice (BALB/c nu/nu) were obtained from Kyudo Co (Saga, Japan). All the animals were bred in laminar-flow cabinets under specific pathogen-free conditions. All experiments with mice were conducted and approved by the Ethics Committee of Kyushu University (protocol number, A020-018-0). Prior to implanting the SUIT-2 cells, the cells were briefly treated with trypsin-EDTA and washed twice with serum-free medium. The mice were anesthetized with ether, and cell suspensions containing 1×10^6 cells/100 μl were implanted by open injection into the spleen or pancreas. To prevent any leakage of tumor cells, a cotton swab was held over the injection site for 1 min. Following the procedure, the mice were closely monitored to ensure the procedure was well tolerated.

Treatment with ZOL in intrasplenic implantation mouse model. The mice that received an intrasplenic implantation of SUIT-2 cells were treated with 0.08, 0.16 and 0.32 mg/kg of ZOL. The ZOL was diluted in PBS and delivered to the animals via a 100 μl i.p. injection, commencing on Day 3 after implantation of the cells, followed by Day 10, Day 17 and Day 24. A control group who received an equal volume of PBS was run in parallel. Each group contained five mice. Four weeks (28 days) after implantation, all mice were sacrificed, and the presence of liver metastasis, liver weight and body weight were evaluated.

Treatment with ZOL in combination with GEM in orthotopic implantation mouse model. The mice orthotopically implanted with the SUIT-2 cells were composed of two groups. For the first cohort, the mice (n = 9 mice/ group) were treated i.p. with ZOL (0.08 mg/kg), GEM (40 mg/kg) or ZOL + GEM at Days 3, 10, 17, 24 and 31 after cell implantation. A control group who received PBS was also run in parallel. After 5 weeks, the mice were sacrificed and their body weights were determined. The pancreases were then excised and weighed, and the volume of primary pancreatic tumor, the presence of peritoneal dissemination and liver metastasis were evaluated. Pancreatic tumor diameter was measured with calipers. Pancreatic tumor volume was then calculated according to the following formula: tumor volume = $ab^2/2$, where a is the longest diameter of the tumor, and b is the shortest diameter. The pancreatic tumors samples were subjected to histological and immunohistochemical analyses. A second group of mice with orthotopically implanted SUIT-2 cells were treated i.p. 7 days later with ZOL (0.08 mg/kg, twice weekly; n = 6), GEM (40 mg/kg, once weekly; n = 6), ZOL + GEM (ZOL 0.08 mg/kg, twice weekly; GEM 40 mg/kg, once weekly; n = 9), or PBS (n = 5). On Day 30 post-implantation, the mice from this second cohort were sacrificed and the number of visible nodules greater than 1 mm in size disseminated within the peritoneal was evaluated.

Immunohistochemical analysis and cell proliferation.

Immunohistochemistry analysis was performed using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). To stain microvessels, the sections were incubated with a mouse monoclonal anti-CD31 antibody JC/70A (1:50, NeoMarkers, Fremont, CA) overnight at 4 °C. Microvessel density (MVD) was assessed in tumor areas showing high-density staining. The number of vessels was counted in 10 fields/section; one field was magnified 200× (0.739 mm^2/field) and the mean counts were recorded. Proliferating cells were detected with a Ki-67 nuclear proliferation-related antigen (mouse mAb, Clone 7B11; pre-diluted; Invitrogen, Japan). To quantify Ki-67 staining in the tumors, the number of positive cells was counted in five random fields/section at 200× magnification. Apoptotic cells within tumor nodules were detected using a TUNEL assay (In Situ Apoptosis Detection Kit; Takara, Shiga, Japan). The number of positive cells was counted in five random fields/section at 200× magnification.

Statistical analyses. Statistical analyses were performed using JMP version 8.0 software (SAS Institute, Cary, NC, USA). Values are expressed as the mean ± SD. Comparisons between the two groups were performed using a student *t* test. Statistical significance was defined as $P<0.05$ or $P<0.001$.

Results

In vitro inhibitory effect of ZOL or GEM on invasion or proliferation of pancreatic cancer cells. To test the cytotoxic potential of ZOL or GEM on pancreatic cancer cells, SUIT-2 cells were incubated with or without ZOL or GEM at the indicated concentrations for 4 days and then number of cells in each group was determined. Treatment with ZOL (1–10 μM) slightly inhibited the proliferation of SUIT-2 cells. For the cells treated with 10 μM ZOL, the number of viable cells decreased by 26% compared with untreated control groups (Fig.1A, left). However, for the cells treated with 10 nM GEM, the number of viable cells decreased by 60% compared with the control group (Fig. 1A, right). Treatment with GEM also inhibited the proliferation of SUIT-2 cells in a dose-dependent manner.

To investigate the effect of ZOL or GEM on cell invasion, the SUIT-2 cells were cultured in Matrigel invasion chambers in the absence or presence of ZOL or GEM at the indicated concentrations (Fig. 1B). At 10 nM, GEM did not have a large impact on reducing the number of SUIT-2 cells penetrating the Matrigel membrane, which was consistent with previous reports [23]. However, invasion of SUIT-2 cells was inhibited by ZOL at the indicated concentrations in a dose-dependent manner, whereby even low doses of ZOL (2 μM) inhibited SUIT-2 cell invasion by 58%, and at a higher dose (10 μM) ZOL induced an 80% inhibition of invasion (Fig. 1C).

Effect of combination with ZOL and GEM on proliferation and invasion in pancreatic cancer cells in vitro. To investigate the effect of combination treatment with ZOL and GEM on cell proliferation, SUIT-2 cells were incubated with 2 μM ZOL, 10 nM GEM or a combination of both for 3 days, and the cell number in each group was determined. Simultaneous treatment with ZOL at 2 μM plus 10 nM GEM did not show any additive effect on GEM-induced inhibition of cell proliferation, indicating that the antiproliferative effects were induced by GEM alone (Fig. 2A).

To investigate the effect of combination treatment with ZOL+ GEM on cell

invasion, SUIT-2 cells were cultured in Matrigel invasion chambers with ZOL, GEM or the combination of both (at the same concentrations). Cellular invasion was not affected by GEM treatment alone and an additive effect was not detected in the combination treatment group. These data indicated that the anti-invasion effect was induced by ZOL alone (Fig. 2B).

These *in vitro* results indicate that combined treatment with ZOL + GEM inhibits both proliferation and invasion of SUIT-2 cells. The effects of ZOL or/and GEM on cell invasion were also examined in the other pancreatic cancer cell lines: Panc-1, CFPAC-1 and KP-2. However, we found only minor effects of GEM on invasion, similar to the SUIT-2 cells. Moreover, we did not find an additive effect on anti-invasion in the combined treatment group. Combined treatment with ZOL + GEM showed the same inhibitory effects of ZOL alone on invasion in all three-cell lines (Fig. 2C). Taken together, these data suggest that the effects of combined GEM with low-dose ZOL are due to the anti-proliferative effects induced by GEM plus the anti-invasion effects induced by ZOL.

ZOL inhibits the invasion of pancreatic cancer cells by impairing the expression of MMP-2 and MMP-9. The MMPs are the most prominent family of proteinases associated with tumorigenesis. In addition to their role in extracellular matrix turnover and cancer cell migration, MMPs regulate the signaling pathways that control cell growth, inflammation or angiogenesis and may even work in a non-proteolytic manner. We performed western blots for MMP-2, MMP-9 and MMP-14, which are known to be involved in the invasion and angiogenesis of pancreatic cancer [24]. At 10 μM, ZOL significantly decreased MMP-2 and MMP-9 expression levels in two of the pancreatic cancer cell lines, although 2 μM ZOL was not as potent as that of 10 μM (Fig. 3A and B). There was no difference in the expression levels of MMP-14 between the ZOL-treated or untreated groups (Fig. 3A and B). These results indicate that ZOL may inhibit pancreatic cancer cell invasion by disrupting the expression of MMP-2 and MMP-9.

***In vivo* inhibitory effect of ZOL monotherapy on liver metastasis of human pancreatic cancer.** The therapeutic effect of ZOL alone was evaluated *in vivo* whereby on Day 3 after the intrasplenic implantation of tumor cells, the mice were treated i.p. with 0.08, 0.16 or 0.32 mg/kg ZOL (or PBS in the control group) followed by treatment once weekly for 3 weeks. On Day 28 after the cells were implanted, gross examination revealed the presence of liver metastases in all groups (Fig. 4). The implanted SUIT-2 cells exhibited an aggressive and malignant phenotype *in vivo* and intrasplenically implanted cells consistently produced metastatic nodules such that in the control group, the livers were almost completely covered by metastatic tumors. The presence of liver metastases and the numbers of liver metastatic nodules were significantly decreased by ZOL treatment in a dose-dependent manner (Table 1). Moreover, the liver weights were significantly lower in mice treated with ZOL than those in control mice (Table 1). These data indicate that ZOL can suppress tumor growth in metastatic sites at the indicated doses. We found no treatment-induced cumulative toxicity or changes in body weight.

Inhibition of growth and metastasis of human pancreatic cancer in an orthotopic mouse model. The therapeutic efficacy of ZOL + GEM was investigated in an orthotopic model of pancreatic cancer. In order to investigate whether a synergistic effect could be detected, the doses of ZOL and GEM were determined as previously described [13, 14 20, 23 25] with following modifications: at 3 days after orthotopic implantation the mice were treated i.p. with ZOL (1.6 μg/0.1 ml), GEM (0.8 mg/0.1 ml), ZOL + GEM, or PBS followed by treatment once weekly for 4 weeks. At 35 days after implantation, peritoneal dissemination was examined in the retroperitoneum, mesenterium and hepatic hilum (Fig. 5A), and gross evaluation of pancreatic tumors was also investigated in all groups (Fig. 5B, top panel). Liver metastases were detected in 55% of the control mice. The presence of liver metastases was decreased by treatment with ZOL or GEM alone and combination therapy completely suppressed liver metastases (Table 2). We found no treatment-induced cumulative toxicity or changes to body weight (Table 2). Pancreatic tumor volume

was significantly smaller in the mice treated with GEM alone. Moreover, the tumor volumes of the mice treated with ZOL + GEM were significantly less than those treated with GEM($P < 0.05$) or ZOL($P < 0.05$) or the control group ($P < 0.05$)(Fig. 5B, bottom panel). The presence of peritoneal dissemination was reduced by treatment with ZOL or GEM, and combination therapy showed the strongest inhibitory effect on peritoneal dissemination ($P < 0.05$) (Fig. 5A, Table 2). We also performed additional *in vivo* experiments using orthotopic implantation models with higher dose of ZOL and GEM. On day 30 after implantation, visible peritoneal disseminations greater than 1 mm in diameter were evaluated. The extent of visible peritoneal dissemination was significantly less in the mice treated with GEM or ZOL alone compared with the control mice (Fig. 5C, D). Moreover, the extent of peritoneal disseminations in mice treated with ZOL + GEM was significantly less than in the mice treated with GEM or ZOL alone ($P < 0.05$) or the control group ($P < 0.05$)(Fig. 5D).

Histological and immunohistochemical analysis. Histology revealed that the primary tumors in all groups were moderate to poorly differentiated adenocarcinomas. Necrotic lesions were identified in the center of the tumor region in the control mice and the mice treated with the GEM or ZOL monotherapy. Visual-examination of the H&E stained sections revealed tumor areas in the mice treated with ZOL in combination with GEM that were smaller than the tumor areas in the mice treated with ZOL or GEM monotherapy, or the control group.

Tumor cell proliferation was evaluated as the percentage of Ki67-positive cells, and a significant inhibition was observed in tumors from the mice treated with GEM (44%) or GEM + ZOL (36%) compared with the percentage in the control group (72%). No significant differences in the number of Ki67-positive cells between the ZOL and control groups were noted (Fig. 6A and B). The apoptotic tumor cells, as determined by the number of TUNEL-positive cells / high power field (hpf), was significantly greater in the lesions of mice treated with either ZOL or GEM, and the highest number of apoptotic tumor cells was observed in the combination therapy group (Fig. 6A and C). The analysis of MVD, as evaluated by anti-CD31 staining,

revealed that MVD was significantly reduced in the tumors from the mice treated with ZOL (7.9/hpf) or ZOL + GEM (2/hpf) compared with MVD in the control group (14.3/hpf) (Fig. 6A and D). No significant difference in tumor MVD between the GEM-treated and control mice was found. These results indicate that combination therapy induces apoptosis in cancer cells and suppresses cancer cell proliferation and invasion and tumor angiogenesis.

Discussion

This study presents findings outlining the novel application of an amino-bisphosphonate compound, ZOL combined with GEM on the proliferation and development of metastasis of pancreatic cancer *in vitro* and *in vivo*. We showed that intraperitoneal injection of ZOL, in combination with GEM, strongly inhibited the growth and development of peritoneal dissemination and liver metastasis of human pancreatic cancer cells implanted into the pancreas of athymic mice. *In vivo* analysis revealed that the antitumor effect of ZOL was mediated in part by inhibition of angiogenesis and mild induction of apoptosis. In contrast, the antitumor effect of GEM was due to its antiproliferative effect, and GEM had no influence on angiogenesis. Thus, the high rate of apoptosis in the combination therapy was possibly induced by GEM and anti-angiogenesis induced by ZOL. *In vitro*, low doses of ZOL potently inhibited the invasion of the cancer cells and had no effect on proliferation. In contrast, GEM had a very limited effect on invasive ability but showed powerful antiproliferative effects, similar to that observed *in vivo*. This powerful antiproliferative effect was induced by phosphorylated GEM incorporating into the DNA and inhibiting DNA synthesis by self-potentiation and masked chain termination [3]. These data suggest that the potent antitumor effects observed *in vivo* might be due to the inhibitory effects of the combination therapy on proliferation and invasion of cancer cells, and due to an effect on angiogenesis.

Recent clinical studies have demonstrated that BPs have beneficial effects on bone metastases in breast cancer patients [26-28]. On the other hand, the effects of BPs on non-bone metastases are still controversial [29-31]. In the present study, we demonstrated that ZOL, one of the most potent BPs currently available, at the dosages indicated, significantly inhibited tumor growth at metastatic sites. Of importance, higher dose of ZOL treatment significantly suppressed metastasis of SUIT-2 pancreatic cancer cells to the liver, which is consistent with the results found in previous reports in breast cancer [13].

It is known that SUIT-2 cells constitutively express many MMPs, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-14 [32]. While these

enzymes may participate in the process of matrix degradation, the degradation of basement membrane type IV collagen is a critical early event in tumor invasion, suggesting that MMPs, especially MMP-2 and MMP-9, may be particularly important [33]. In our *in vitro* study, 10 μM ZOL significantly decreased MMP-2 and MMP-9 expression, but 2 μM ZOL was not as potent as 10 μM. Whereas 2 μM ZOL substantially inhibited the invasion of pancreatic cancer cells, this phenomenon may have been induced by some mechanisms other than the digestion of the basement membrane by MMP-2 or MMP-9. For example, small GTPases play a key role during integrin activation, and ZOL has been shown to inhibit tumor cell adhesion, migration and invasion by inhibiting geranylgeranylation (which is required for the prenylation of small GTPases), thereby suggesting that ZOL could inhibit integrin activation [34, 35]. Furthermore, ZOL may inhibit chemokine CXCL-12-induced tumor cell migration by decreasing the cell surface expression of CXCR-4 [36]. These data may suggest that ZOL inhibits cancer invasion via multiple mechanisms; however, further research is required to firmly validate this hypothesis.

In our *in vivo* study, ZOL inhibited neovascularization in the tumors. The MMP-9-expressing stromal cells are essential to angiogenesis and progressive growth of various cancers in mouse models [20, 37]. In our study, ZOL also inhibited the angiogenic response induced by bFGF and VEGF *in vivo* [38]. These data indicate that ZOL may inhibit angiogenesis *in vivo* via multiple pathways. According to previous reports, the ZOL-induced antiproliferative and apoptotic effects are due to cleavage/activation of caspase-9 and poly(ADP)-ribose polymerase as well as interference of growth and survival pathways downstream to $p21^{ras}$ in human pancreatic cancer cells *in vitro* [16]. However, our *in vitro* findings demonstrated that ZOL, at a low dosage, had mild antiproliferative effects. However, *in vivo*, ZOL had no effect on proliferation but induced apoptosis. These results are consistent with previous reports in breast, prostate and lung cancers [39, 40].

Clinical trials of GEM combined with several antitumor agents have been conducted on pancreatic cancer [41]. However, in the majority of these therapies, cytotoxic drugs, such as docetaxel, 5FU, cisplatin and irinotecan were used [42-46].

Although these cytotoxic drugs also exert antitumor effects (via different mechanisms), the applicable dose of each drug is limited due to cumulative systemic toxicity. In such clinical trials, combination therapy may result in more adverse side effects despite the expected additive antitumor effects. However, ZOL, a nitrogen-containing bisphosphonate (N-BP) that is approved by the FDA to reduce skeletal complications of bone metastasis in patients with multiple myeloma and several solid tumor types with minimal side effects, has been reported to act synergistically with other anticancer agents against various types of cancer both *in vitro* and *in vivo* [47-51]. In the present study, the combination of ZOL, plus a sufficient dose of GEM, had antitumor effects but did not lead to escalating toxicity. The present study is based on data from a mouse model, thus further investigation on the dosage and the course of treatment in clinical patients are needed.

In conclusion, our results confirm the findings that the ZOL influences metastasis not only to bone, but also to visceral organs. These effects of ZOL combined with GEM may be attributed to an inhibition of invasion and proliferation of cancer cells. The effects of combination therapy with ZOL and GEM show great promise for the treatment of pancreatic cancer, and further studies examining the clinical efficacy of this drug regimen are warranted.

Acknowledgments

We appreciate M. Ohmori and E. Manabe (Department of Surgery and Oncology, Kyushu University Hospital) for their expert technical assistance. This work was supported in part by the following: a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and grants from the Japanese Society of Gastroenterology and the Pancreas Research Foundation of Japan.

Disclosure Statement

The authors have no competing interests to declare.

References

[1] Low SK, Kuchiba A, Zembutsu H, et al. Genome-wide association study of pancreatic cancer in Japanese population. PLoS One. 5: e11824.
[2] Burris HA 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997; 15: 2403-13.
[3] Ueno H, Kiyosawa K, Kaniwa N. Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy? Br J Cancer. 2007; 97: 145-51.
[4] Fruscella E, Gallo D, Ferrandina G, D'Agostino G, Scambia G. Gemcitabine: current role and future options in the treatment of ovarian cancer. Crit Rev Oncol Hematol. 2003; 48: 81-8.
[5] Ducreux M, Boige V, Malka D. Treatment of advanced pancreatic cancer. Semin Oncol. 2007; 34: S25-30.
[6] Boeck S, Ankerst DP, Heinemann V. The role of adjuvant chemotherapy for patients with resected pancreatic cancer: systematic review of randomized controlled trials and meta-analysis. Oncology. 2007; 72: 314-21.
[7] Saif MW. Pancreatic cancer: highlights from the 42nd annual meeting of the American Society of Clinical Oncology, 2006. JOP. 2006; 7: 337-48.
[8] Heymann D, Ory B, Gouin F, Green JR, Redini F. Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol Med. 2004; 10: 337-43.
[9] Silverman SL. Paget disease of bone: therapeutic options. J Clin Rheumatol. 2008; 14: 299-305.
[10] Black DM, Delmas PD, Eastell R, et al. Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med. 2007; 356: 1809-22.
[11] Coleman RE. Bisphosphonates: clinical experience. Oncologist. 2004; 9 Suppl 4: 14-27.
[12] Green JR. Antitumor effects of bisphosphonates. Cancer. 2003; 97: 840-7.
[13] Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res. 2004; 10: 4559-67.
[14] Corey E, Brown LG, Quinn JE, et al. Zoledronic acid exhibits inhibitory effects on osteoblastic and osteolytic metastases of prostate cancer. Clin Cancer Res. 2003; 9: 295-306.
[15] Sewing L, Steinberg F, Schmidt H, Goke R. The bisphosphonate zoledronic acid inhibits the growth of HCT-116 colon carcinoma cells and induces tumor cell apoptosis. Apoptosis. 2008; 13: 782-9.
[16] Tassone P, Tagliaferri P, Viscomi C, et al. Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells in vitro. Br J Cancer. 2003; 88: 1971-8.
[17] Benford HL, Frith JC, Auriola S, Monkkonen J, Rogers MJ. Farnesol and geranylgeraniol prevent activation of caspases by aminobisphosphonates:

biochemical evidence for two distinct pharmacological classes of bisphosphonate drugs. Mol Pharmacol. 1999; 56: 131-40.
[18] Dunford JE, Thompson K, Coxon FP, et al. Structure-activity relationships for inhibition of farnesyl diphosphate synthase in vitro and inhibition of bone resorption in vivo by nitrogen-containing bisphosphonates. J Pharmacol Exp Ther. 2001; 296: 235-42.
[19] Li XY, Lin YC, Huang WL, et al. Zoledronic acid inhibits proliferation and impairs migration and invasion through downregulating VEGF and MMPs expression in human nasopharyngeal carcinoma cells. Med Oncol.
[20] Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J Clin Invest. 2004; 114: 623-33.
[21] Ohuchida K, Mizumoto K, Murakami M, et al. Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. Cancer Res. 2004; 64: 3215-22.
[22] Zhang L, Mizumoto K, Sato N, et al. Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin. Cancer Lett. 1999; 142: 129-37.
[23] Ogura Y, Mizumoto K, Nagai E, et al. Peritumoral injection of adenovirus vector expressing NK4 combined with gemcitabine treatment suppresses growth and metastasis of human pancreatic cancer cells implanted orthotopically in nude mice and prolongs survival. Cancer Gene Ther. 2006; 13: 520-9.
[24] Yang X, Staren ED, Howard JM, Iwamura T, Bartsch JE, Appert HE. Invasiveness and MMP expression in pancreatic carcinoma. J Surg Res. 2001; 98: 33-9.
[25] Hirbe AC, Roelofs AJ, Floyd DH, et al. The bisphosphonate zoledronic acid decreases tumor growth in bone in mice with defective osteoclasts. Bone. 2009; 44: 908-16.
[26] Hortobagyi GN, Theriault RL, Porter L, et al. Efficacy of pamidronate in reducing skeletal complications in patients with breast cancer and lytic bone metastases. Protocol 19 Aredia Breast Cancer Study Group. N Engl J Med. 1996; 335: 1785-91.
[27] Kanis JA, Powles T, Paterson AH, McCloskey EV, Ashley S. Clodronate decreases the frequency of skeletal metastases in women with breast cancer. Bone. 1996; 19: 663-7.
[28] Theriault RL, Lipton A, Hortobagyi GN, et al. Pamidronate reduces skeletal morbidity in women with advanced breast cancer and lytic bone lesions: a randomized, placebo-controlled trial. Protocol 18 Aredia Breast Cancer Study Group. J Clin Oncol. 1999; 17: 846-54.
[29] Diel IJ, Solomayer EF, Costa SD, et al. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med. 1998; 339: 357-63.
[30] Powles T, Paterson S, Kanis JA, et al. Randomized, placebo-controlled trial of clodronate in patients with primary operable breast cancer. J Clin Oncol. 2002; 20: 3219-24.

[31] Saarto T, Blomqvist C, Virkkunen P, Elomaa I. Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomized controlled trial. J Clin Oncol. 2001; 19: 10-7.
[32] Kitamura N, Iwamura T, Taniguchi S, et al. High collagenolytic activity in spontaneously highly metastatic variants derived from a human pancreatic cancer cell line (SUIT-2) in nude mice. Clin Exp Metastasis. 2000; 18: 561-71.
[33] Evans JD, Ghaneh P, Kawesha A, Neoptolemos JP. Role of matrix metalloproteinases and their inhibitors in pancreatic cancer. Digestion. 1997; 58: 520-8.
[34] Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. Cancer Res. 2005; 65: 4971-4.
[35] Coxon JP, Oades GM, Kirby RS, Colston KW. Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation. BJU Int. 2004; 94: 164-70.
[36] Denoyelle C, Hong L, Vannier JP, Soria J, Soria C. New insights into the actions of bisphosphonate zoledronic acid in breast cancer cells by dual RhoA-dependent and -independent effects. Br J Cancer. 2003; 88: 1631-40.
[37] Nakamura T, Kuwai T, Kim JS, Fan D, Kim SJ, Fidler IJ. Stromal metalloproteinase-9 is essential to angiogenesis and progressive growth of orthotopic human pancreatic cancer in parabiont nude mice. Neoplasia. 2007; 9: 979-86.
[38] Wood J, Bonjean K, Ruetz S, et al. Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. J Pharmacol Exp Ther. 2002; 302: 1055-61.
[39] Boissier S, Ferreras M, Peyruchaud O, et al. Bisphosphonates inhibit breast and prostate carcinoma cell invasion, an early event in the formation of bone metastases. Cancer Res. 2000; 60: 2949-54.
[40] Yamada T, Muguruma H, Yano S, et al. Intensification therapy with anti-parathyroid hormone-related protein antibody plus zoledronic acid for bone metastases of small cell lung cancer cells in severe combined immunodeficient mice. Mol Cancer Ther. 2009; 8: 119-26.
[41] Jacobs AD. Gemcitabine-based therapy in pancreas cancer: gemcitabine-docetaxel and other novel combinations. Cancer. 2002; 95: 923-7.
[42] Berlin JD, Catalano P, Thomas JP, Kugler JW, Haller DG, Benson AB, 3rd. Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. J Clin Oncol. 2002; 20: 3270-5.
[43] Colucci G, Giuliani F, Gebbia V, et al. Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale. Cancer. 2002; 94: 902-10.
[44] Philip PA, Zalupski MM, Vaitkevicius VK, et al. Phase II study of gemcitabine and cisplatin in the treatment of patients with advanced pancreatic carcinoma.

Cancer. 2001; 92: 569-77.
[45] Rocha Lima CM, Savarese D, Bruckner H, et al. Irinotecan plus gemcitabine induces both radiographic and CA 19-9 tumor marker responses in patients with previously untreated advanced pancreatic cancer. J Clin Oncol. 2002; 20: 1182-91.
[46] Ryan DP, Kulke MH, Fuchs CS, et al. A Phase II study of gemcitabine and docetaxel in patients with metastatic pancreatic carcinoma. Cancer. 2002; 94: 97-103.
[47] Budman DR, Calabro A. Zoledronic acid (Zometa) enhances the cytotoxic effect of gemcitabine and fluvastatin: in vitro isobologram studies with conventional and nonconventional cytotoxic agents. Oncology. 2006; 70: 147-53.
[48] Horie N, Murata H, Kimura S, et al. Combined effects of a third-generation bisphosphonate, zoledronic acid with other anticancer agents against murine osteosarcoma. Br J Cancer. 2007; 96: 255-61.
[49] Matsumoto S, Kimura S, Segawa H, et al. Efficacy of the third-generation bisphosphonate, zoledronic acid alone and combined with anti-cancer agents against small cell lung cancer cell lines. Lung Cancer. 2005; 47: 31-9.
[50] Melisi D, Caputo R, Damiano V, et al. Zoledronic acid cooperates with a cyclooxygenase-2 inhibitor and gefitinib in inhibiting breast and prostate cancer. Endocr Relat Cancer. 2005; 12: 1051-8.
[51] Ottewell PD, Monkkonen H, Jones M, Lefley DV, Coleman RE, Holen I. Antitumor effects of doxorubicin followed by zoledronic acid in a mouse model of breast cancer. J Natl Cancer Inst. 2008; 100: 1167-78.

Figure legends

Fig 1. Comparison of ZOL or GEM on the proliferation and invasion of pancreatic cancer cells. (A) The ability of ZOL or GEM to inhibit the proliferation of SUIT-2 cells was determined using a PI assay. The cells were treated with ZOL (left) or GEM (right) at indicated concentrations for 72 h. (B) The appearance of SUIT-2 cells invading through the Matrigel and filter membrane. The ability of ZOL and GEM to inhibit the invasion of SUIT-2 cells was determined by invasion assay. The cells were treated with ZOL (upper) or GEM (lower) at the indicated concentrations for 48 h. (C) The anti-invasive effect of ZOL or GEM in SUIT-2 cells relative to the control. ZOL significantly suppressed invasion compared with control at indicated dosages (**$P < 0.001$).

Fig 2. The effects of ZOL combined with GEM on the growth and invasion of pancreatic cancer cells. (A) The ability of ZOL, GEM or ZOL + GEM to inhibit the proliferation of SUIT-2 cells. (B) The ability of ZOL, GEM or ZOL + GEM to inhibit the invasion of SUIT-2 cells. In the upper panel, the appearance of SUIT-2 cells invading the Matrigel and filter membrane is shown. The lower panel shows that ZOL in combination with GEM significantly suppressed cellular invasion compared with GEM alone, or in the control group (**$P < 0.001$). (C) The ability of ZOL, GEM or ZOL + GEM to inhibit the invasion of Panc-1, CFPAC-1 and KP2 cells. The upper panel shows the appearance of Panc-1, CFPAC-1and KP2 cells invading the Matrigel and filter membrane. In the lower panel, ZOL + GEM significantly suppressed invasion compared with GEM alone, or the control cells, in all of the cell lines (**$P < 0.001$).

Fig 3. The expression of MMP-2, MMP-9 and MMP-14 in SUIT-2 and Panc-1 after treatment with ZOL. Western blot analysis revealed that ZOL treatment decreased the expression of MMP-2 and MMP-9 proteins in both SUIT-2 (A) and Panc-1 (B) cells. However, MMP-14 synthesis was not suppressed by ZOL administration.

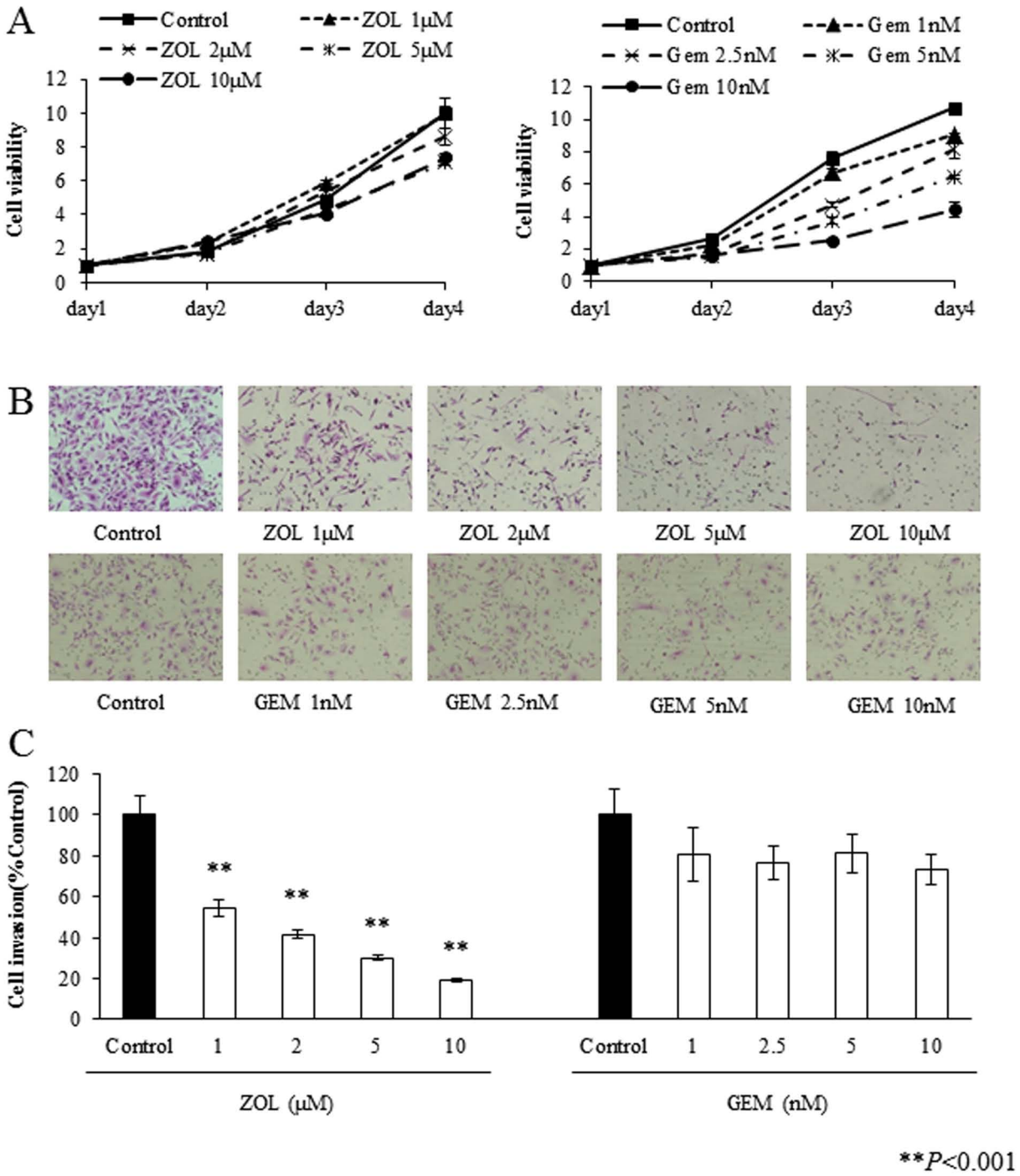
Fig 4. Suppression of metastatic liver tumors by i.p. injection of ZOL. PBS or the indicated doses of ZOL were injected i.p. on Day 3 after intrasplenic implantation of SUIT-2 cells. The upper panel shows the macroscopic appearance of metastatic liver tumors after treatment with (a) PBS, (b) ZOL at 0.08 mg/kg/week, (c) ZOL 0.16 mg/kg/week or (d) ZOL at 0.32 mg/kg/week on Day 28 after intrasplenic implantation of SUIT-2 cells (n = 5/group). The lower panel shows magnification of the upper panel metastatic liver tumors.

Fig 5. Therapeutic effect of ZOL + GEM on orthotopically implanted pancreatic tumors. (A) The gross appearance of the pancreatic tumors and peritoneal dissemination after treatment with PBS (control) or treatment with ZOL + GEM. (B) The upper panel shows the gross appearance of pancreatic tumors after treatment with (a) PBS (control), (b) ZOL, (c) GEM or (d) ZOL in combination with GEM on Day 35 after the orthotopic implantation of SUIT-2 cells (n = 9/group). The lower panel shows that therapy with GEM alone significantly reduced the mean primary pancreatic tumor volume compared with volume of control tumors (#$P < 0.05$). Combination therapy (ZOL + GEM) significantly decreased mean pancreatic tumor volume compared with the volume of tumors in the control (#$P < 0.05$) and ZOL or GEM monotherapy groups (*$P < 0.05$). (C) Representative images of the metastasis in the peritoneal cavity of mice after indicated treatments. The arrowheads indicate peritoneal disseminations. (D) The number of visible peritoneal disseminations was counted. GEM, ZOL and GEM + ZOL versus control (#P <0.05); GEM, ZOL versus GEM + ZOL (*$P < 0.05$). Bars represent mean ± S.D.

Fig 6. (A) H&E and immunohistochemical staining for cell proliferation (Ki67), cell death (TUNEL) and angiogenesis (CD31) in the primary pancreatic tumors from an orthotopic mouse model following treatment with ZOL, GEM or ZOL + GEM. (B) Ki67-positive cells were counted in three microscopic fields per tumor section at 200× magnification. The total number of cells/field and number of Ki67-positive cells in

each field were counted to calculate median percentage in each section. The proliferation index was significantly lower in the tumors treated with GEM alone or ZOL + GEM compared with tumors in the control samples (** $P < 0.001$). (C) TUNEL-positive cells were counted in three microscopic fields per tumor section at 200× magnification. The total number of cells/field and number of TUNEL-positive cells in each field were counted to calculate median percentage in each tumor section. The number of apoptotic cells per high power field (hpf) was significantly greater in the tumors treated with ZOL (*$P < 0.05$), GEM (**$P < 0.001$), or with ZOL + GEM (**$P < 0.001$) compared with that of the controls. The difference between GEM and combination therapy was not significant. (D) The mean number of blood vessels per (hpf) was significantly lower in the tumors treated with ZOL or ZOL + GEM compared with tumors from the control group (*$P < 0.05$) or tumors from the GEM only group (*$P < 0.05$). The difference between ZOL and combination therapy was not significant. Bars represent mean ± S.D.

Figure 1



**$P<0.001$

Figure 2

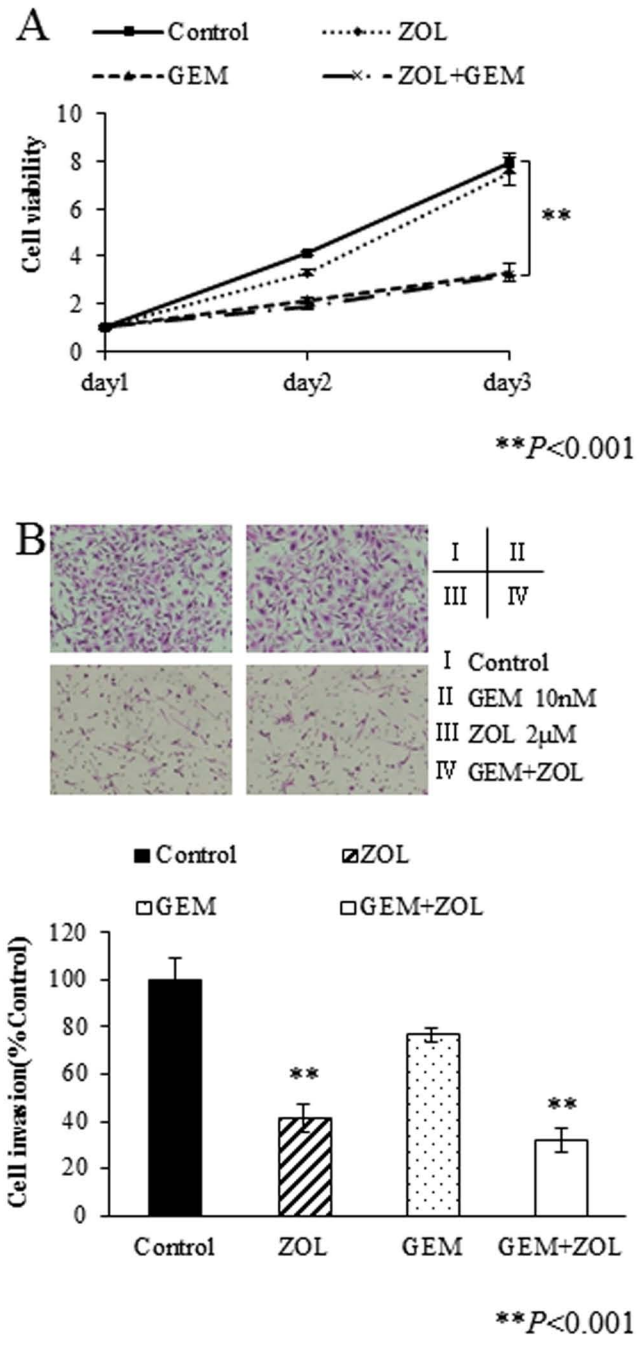

A
Control
ZOL
GEM
ZOL+GEM
Cell viability
10
8
6
4
2
0
day1
day2
day3
**
**P<0.001
B
I
II
III
IV
I Control
II GEM 10nM
III ZOL 2μM
IV GEM+ZOL
Control
ZOL
GEM
GEM+ZOL
Cell invasion(%Control)
120
100
80
60
40
20
0
**
**
Control
ZOL
GEM
GEM+ZOL
**P<0.001
C
Control
GEM
ZOL
GEM+ZOL
Panc-1
CFPAC-1
KP-2
Control
GEM 10nM
ZOL 2μM
ZOL+GEM
Cell invasion(%Control)
120
100
80
60
40
20
0
Panc-1
CFPAC-1
KP-2

Figure 3

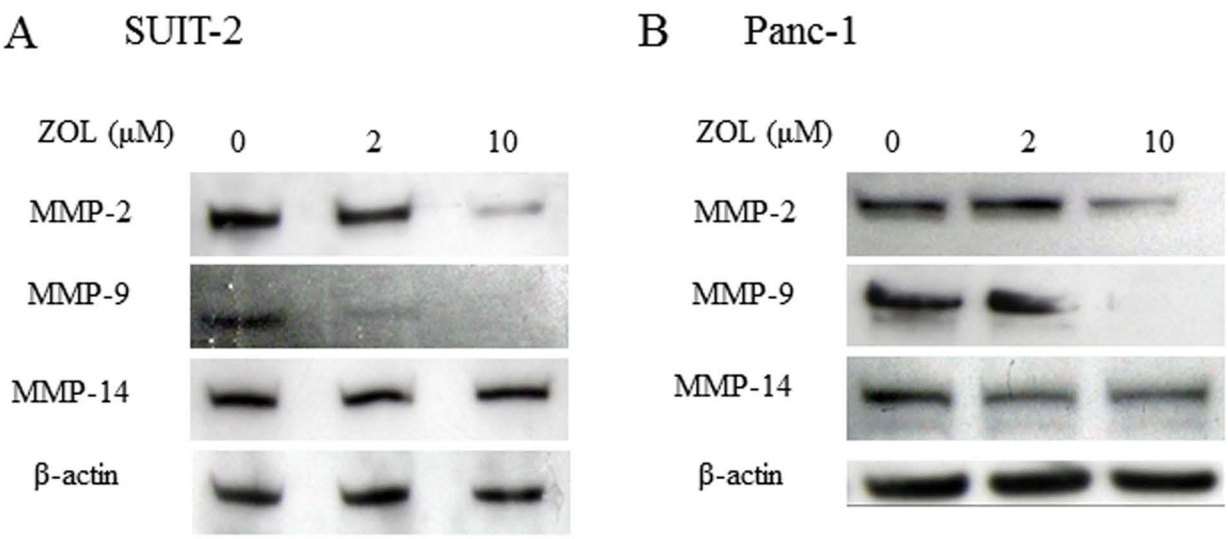

A
SUIT-2
ZOL (μM)
0
2
10
MMP-2
MMP-9
MMP-14
β-actin
B
Panc-1
ZOL (μM)
0
2
10
MMP-2
MMP-9
MMP-14
β-actin

Figure 4

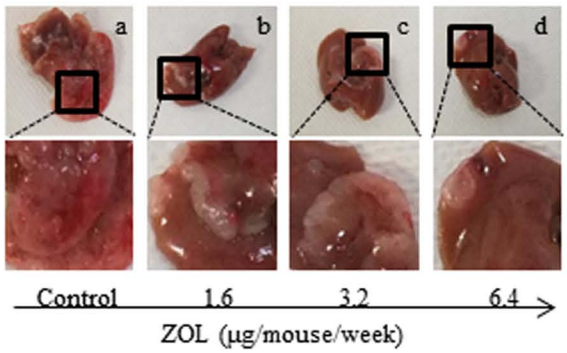


Figure 5

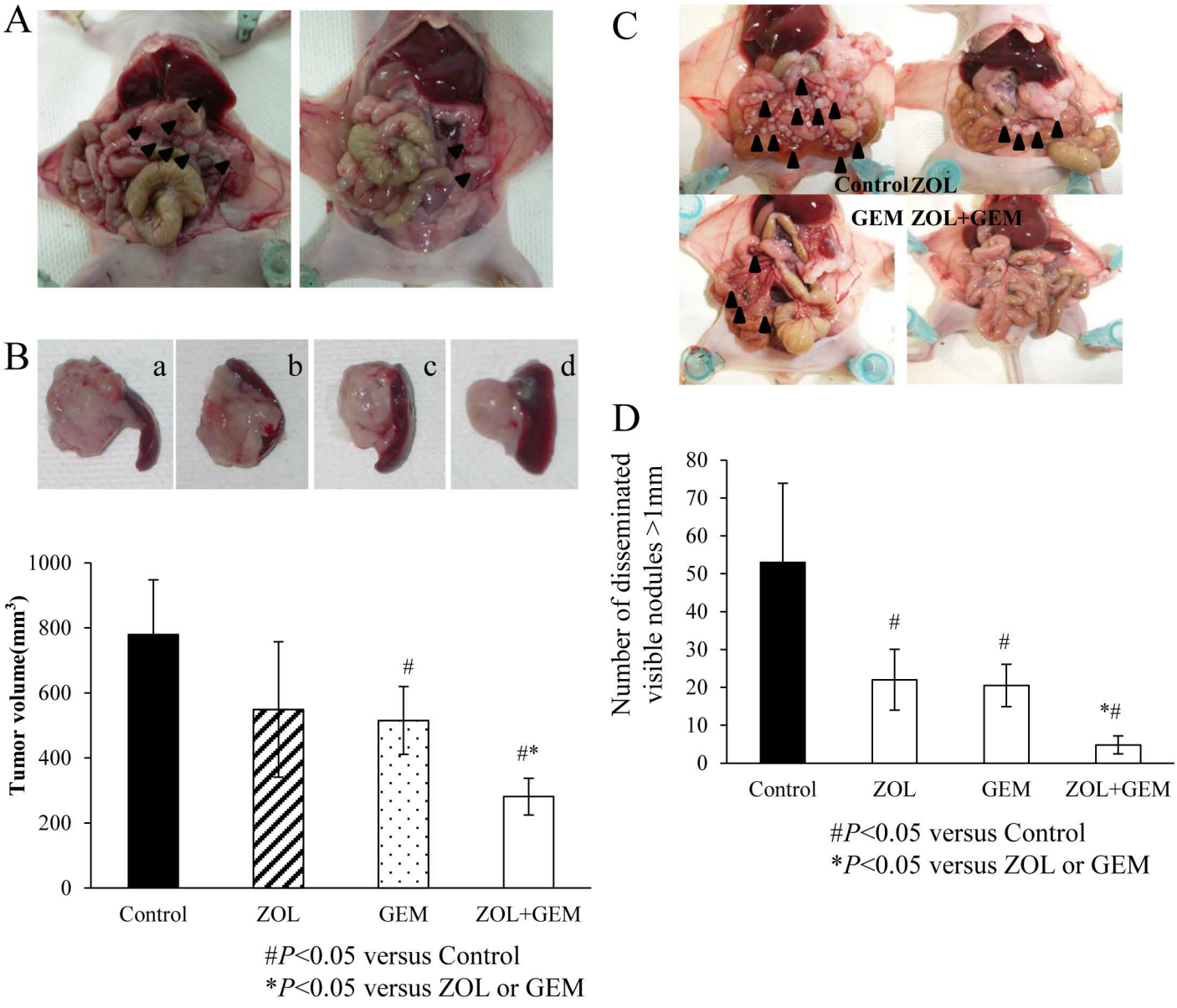
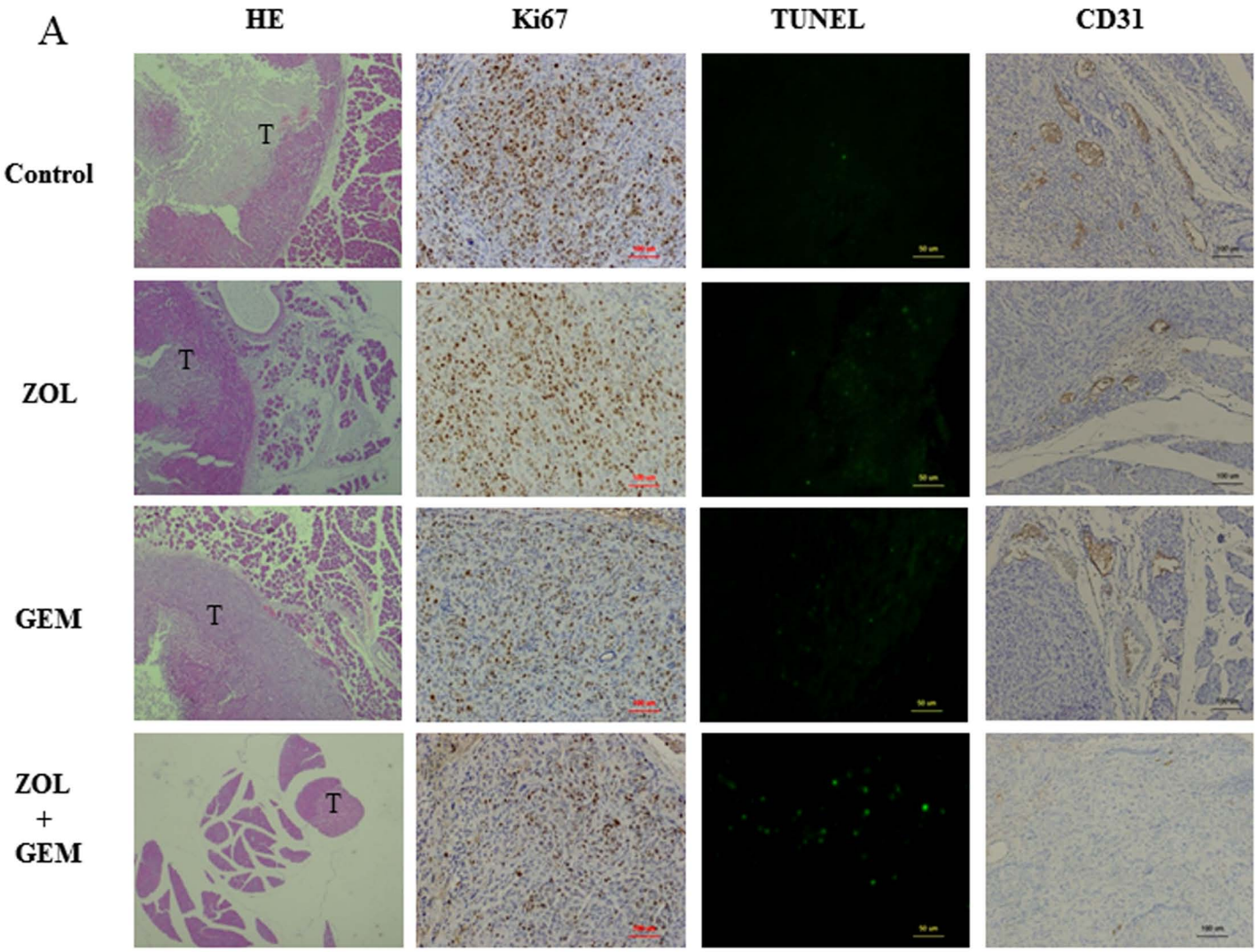
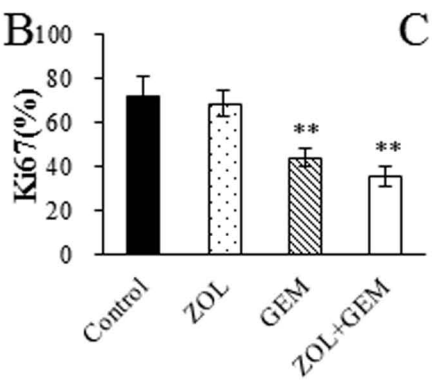
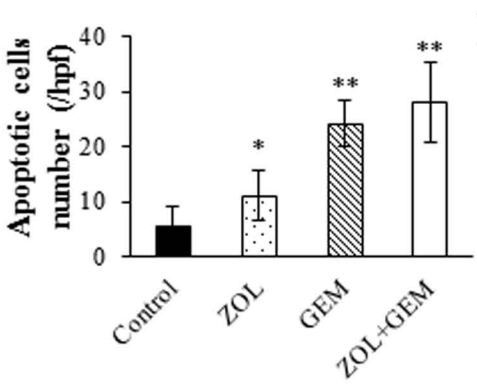
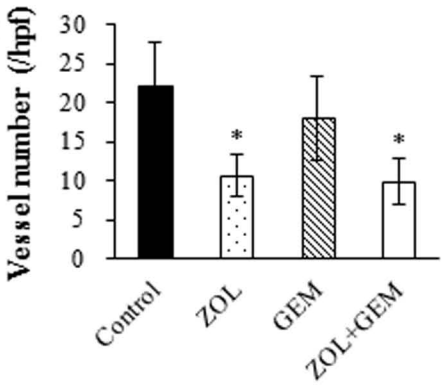

A
B
a
b
c
d
Tumor volume(mm3)
1000
800
600
400
200
0
Control
ZOL
GEM
ZOL+GEM
#
#*
#P<0.05 versus Control
*P<0.05 versus ZOL or GEM
C
Control
ZOL
GEM
ZOL+GEM
D
Number of disseminated visible nodules >1mm
80
70
60
50
40
30
20
10
0
Control
ZOL
GEM
ZOL+GEM
#
#
*#
#P<0.05 versus Control
*P<0.05 versus ZOL or GEM

Figure 6









*P<0.05
**P<0.001

Table 1 Effect of Zoledronic acid on pancreatic cancers implanted intrasplenically in nude mice

Zoledronic acid (mg/kg)	*Liver metastasis*	*Total liver weight: g(s.d.)*	*Body weight:g(s.d.)*
Control	5/5	1.96±0.14	21.09±0.95
0.08	5/5	1.41±0.09*	21.37±0.91
0.16	3/5	1.41±0.10*	21.88±0.65
0.32	2/5*	1.31±0.13**	20.90±1.65

*P <0.05, **P <0.001

Table 2 Therapies for human pancreatic cancers implanted orthotopically in nude mice

Therapy	*Incidence of macroscopic tumors*			*Total pancreas weight: g (s.d.)*	*Body wight: g (s.d.)*
	Pancreas tumor	*Liver metastasis*	*Peritoneal dissemination*		
Control	9/9	5/9	9/9	0.57±0.05	20.26±2.74
ZOL	9/9	1/9	5/9	0.40±0.12*	18.42±2.58
GEM	9/9	2/9	5/9	0.43±0.01*	20.35±0.60
ZOL+GEM	9/9	0/9*	4/9*	0.32±0.01**	18.94±1.30

*P <0.05 versus Control, **P <0.001 versus Control