Z-360, a Novel Cholecystokinin-2/Gastrin Receptor Antagonist, Inhibits
Gemcitabine-Induced Expression of the Vascular Endothelial Growth
Factor Gene in Human Pancreatic Cancer Cells

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Z-360 is a novel cholecystokinin (CCK)-2/gastrin receptor antagonist that is being developed for the treatment of pancreatic adenocarcinoma in combination with gemcitabine. A previous study shows that the co-administration of Z-360 with gemcitabine significantly prolonged the survival of mice with orthotopically implanted human pancreatic adenocarcinoma cell lines. To clarify the therapeutic effects of Z-360 in combination with gemcitabine, we analyzed gene expression. When gemcitabine was administered, CCK-2/gastrin receptor expression was induced in an orthotopic xenograft model; the result indicating that Z-360 could act on gemcitabine-sensitive cells. Both in vitro and in vivo studies showed that gemcitabine increased the expression of vascular endothelial growth factor A (VEGFA), a prognostic factor for survival in pancreatic cancer, while Z-360 suppressed this induction of VEGFA gene expression. These results help to explain how Z-360 prolongs survival when used in combination with gemcitabine.

Key words Z-360; gastrin; cholecystokinin-2 (CCK)/gastrin receptor; antagonist; pancreatic carcinoma; vascular endothelial growth factor (VEGF)

Z-360, calcium bis([R]-(−)-3-[3-{5-cyclohexyl-1-(3,3-di-methyl-2-oxo-buty1)-2-oxo-2,3,4,5-tetrahydro-1H-benzo[h]-[1,4]diazepin-3-yl}ureido]benzoate), is a novel orally active cholecystokinin (CCK)-2/gastrin receptor antagonist that is being developed for use in combination with the chemotherapy agent gemcitabine for the treatment of pancreatic adenocarcinoma.1,2) Our previous studies show that the oral administration of Z-360 significantly inhibited the growth of subcutaneous xenograft of human pancreatic tumor cells in mice3,4) and that Z-360 combined with gemcitabine inhibited pancreatic tumor growth and prolonged survival of a pancreatic carcinoma orthotopic xenograft model.1

It is known that gastrin stimulates the survival or proliferation of normal cells and gastric,5,6) colorectal,5) or pancreatic cancer cells6,7) by the endocrine, autocrine, and paracrine mechanisms. Watson et al. report that cancer cells overexpress the gastrin gene and are sensitive to the trophic effects of gastrin.8,9) A recent study revealed that the intracellular signaling pathway involved in the activation of the CCK-2/gastrin receptor leads to carcinogenesis.9) Moreover, studies on transgenic mice overexpressing the gastrin or CCK-2/gastrin receptor show that gastrin is involved in the development of gastric and pancreatic tumors.10,11)

In the pre-clinical studies of CCK-2/gastrin receptor antagonists, the role of gastrin and the CCK-2/gastrin receptor in human pancreatic carcinogenesis was investigated in vitro by using human pancreatic carcinoma cell lines8,9) and in vivo by using xenograft models.6) CCK-2/gastrin receptor antagonists such as L-36526010–13) inhibited the growth of pancreatic carcinoma-derived cell lines in these models.6,7) The results suggest that treatment with a CCK-2/gastrin receptor antagonist is useful for patients with pancreatic cancer expressing this receptor. In fact, the efficacy of some CCK-2/gastrin receptor antagonists for advanced pancreatic carcinoma was evaluated in several clinical trials. Gastrazole (JB95008), a CCK-2/gastrin receptor antagonist, significantly prolonged survival compared with placebo,14,15) and this observation provides evidence that the inhibition of gastrin-dependent pathophysiological changes can be effective therapy for pancreatic carcinoma.

Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide and is overexpressed in tumor tissues of patients with some cancers such as pancreatic cancer or nonsmall lung cancer.16–18) Many studies show that the expression of VEGF is correlated with the survival of patients with pancreatic cancer,19,20) and that VEGF is an important prognostic factor for the survival of patients with pancreatic cancer. VEGF is strongly induced by hypoxia, which often occurs in the tumor microenvironment.21) It is now becoming clear that the microenvironment has an influence on both tumorigenesis and metastasis, and VEGF is very important in this microenvironment.

In this study, we investigated factors related to survival, including VEGF, which are relevant to the probability of patients. To elucidate the mechanism underlying the survival advantage of combined therapy with Z-360, which is a novel CCK-2/gastrin antagonist, and gemcitabine, we assessed the influence of Z-360 on the expression of prognostic factors by using the in vitro or in vivo models.

MATERIALS AND METHODS

Materials Z-360, its calcium-free form (Z-360F), YM022 were synthesized at the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd. (Saitama, Japan). Gemcitabine hydrochloride (Gemzar® Injection) was purchased from Eli Lilly Japan (Hyogo, Japan). Gemcitabine hydrochloride (for in vitro analysis) was purchased from Toronto Research Chemicals Inc. (North York, Canada). Carboxymethylcellulose sodium (CMC-Na) was obtained from Maruishi Pharmaceutical Co., Ltd. (Osaka, Japan).

Cell Lines PANCl-1 (a human pancreatic carcinoma cell

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line) was purchased from the European Collection of Cell Cultures (Wiltshire, U.K.). BxPC-3 (a human pancreatic carcinoma cell line) was purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). PANC-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Wako, Tokyo, Japan), while BxPC-3 cells were maintained in RPMI 1640 medium (Wako). Both media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermofisher Scientific, Waltham, MA, U.S.A.), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, U.S.A.). Cells were grown as adherent cultures under a humidified 5% CO2 atmosphere.

**Mice** Seven-week-old female mice with severe combined immunodeficiency (SCID) (C.B-17/lcr-scid/scidJcl) were purchased from Clea Japan (Tokyo, Japan). Animals were housed in a temperature-controlled (23±3 °C) and light-controlled (12 h light/dark; lights on at 7:00 a.m.) room and were provided with standard rodent chow (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. All animal experiments were approved by the Animal Care and Use Committee of the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd.

**Orthotopic Xenograft Model** The orthotopic xenograft model was created as described previously. Three million PANC-1 cells were injected subcutaneously into the right flank of each donor SCID mouse. At 55 or 56 d after tumor cell injection, the mice were killed, the tumors that grew were removed, and these tumors were cut into small pieces. Recipient SCID mice were anesthetized with isoflurane (Mylan Seiyaku Ltd., Tokyo, Japan). An incision was made in the left side of the abdomen, the pancreas was carefully exposed, and a piece of tumor tissue was attached to the center of the organ with a 6-0 absorbable suture (Matsuda Medical Industry, Tokyo, Japan). Then the pancreas was returned into the peritoneal cavity and the abdominal wall and the skin were closed with 5-0 sutures (Matsuda Medical Industry). On day 8 after orthotopic tumor implantation, the mice were randomized to 4 groups on the basis of body weight. Mice were treated orally with Z-360 at a dose of 100 mg/kg once daily and/or intravenously with gemcitabine at a dose of 100 mg/kg on days 8, 11, and 14 after the tumor implantation. The vehicle control group was treated orally with 0.5% DMSO as vehicle at the concentrations indicated in the Figures/legends.

**Gene Expression Analysis of Cultured Cells** PANC-1 was cultured on collagen-coated 6-well-plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) in DMEM supplemented with 10% FBS (Thermofisher Scientific), 100 U/ml of penicillin, and 10 μg/ml of streptomycin (Invitrogen) at 37°C in a humidified atmosphere (5% CO2, 95% air) for 24 h, and the cells were treated with Z-360, gemcitabine, or dimethyl sulfoxide (DMSO) as vehicle at the concentrations indicated in the Figures/legends.

BxPC-3 was cultured on collagen-coated 6-well plates (Becton Dickinson) in RPMI 1640 supplemented with 10% FBS at 37°C in a humid atmosphere (5% CO2, 95% air) for 24 h, and cells were treated with gemcitabine or DMSO (vehicle) at the concentrations indicated in the Figures/legends.

After treatment with compounds for 24 h, the cells were harvested with lysis buffer from an RNasey mini Kit (QIAGEN, Hilden, Germany) and homogenized with a syringe for preparation of total RNA.

**Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)** Total RNA was prepared from tumor tissue or cell lysate with an RNasey mini Kit (QIAGEN). After purification, the amount of RNA was measured by spectrophotometry (OD260) and the quality of the RNA was checked by gel electrophoresis and spectrophotometric analysis (OD260/280). Total RNA (2 μg) was converted into first-strand cDNA by Moloney murine leukemia virus reverse transcriptase (RNase H free) (Promega, Madison, WI, U.S.A.) using oligo (dT) primer (Promega). The first-strand cDNAs were subjected to real time RT-PCR with ABSoluteTM QPCR ROX Mix (Thermofisher Scientific, Waltham, MA, U.S.A.) and TaqMan probes for interleukin-6 (IL-6) (Hs00174131_m1), VEGFA (Hs00900058_m1), matrix metalloproteinase-14 (MMP-14; Hs01037009_g1), and activating transcription factor 3 (ATF3; Hs00231069_m1) with an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.). The program involved 15 min at 95 °C and then 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Data obtained were normalized for the expression of peptidylprolyl isomerase A (PPIA, 4326316E) and analyzed by the comparative CT method (Applied Biosystems).

**ELISA for VEGFA in Vitro or in Vivo** For in vitro, the conditioning media were collected from PANC-1 cultured with Z-360 or YM022 or gemcitabine for 24 h. VEGF levels in conditioned media were quantified using a separate Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) specific to human VEGF (Quantikine Human VEGF Immunoassay kits; R&D Systems, Minneapolis, MN, U.S.A.), according to the manufacturer’s recommended protocols.

For in vivo, tumors were resected and immediately frozen in liquid nitrogen. The tissues were homogenized in 10 ml Lysis buffer (50 mM Tris–HCl buffer pH 7.4 containing 0.25% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Nonidet P-40 (NP-40), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, U.S.A.))/g tumor tissue. The entire procedure was performed with the tissue at 4°C. The total protein level in each sample was determined by the bicinchoninic acid (BCA) protein assay reagent using bovine serum albumin as a standard. Tissue extracts were diluted with the Lysis buffer to yield samples with the same protein concentration. Tissue VEGF levels were quantified by a separate Sandwich ELISA specific to human VEGF, (Quantikine Human VEGF Immunoassay kits; R&D Systems), according to the manufacturer’s recommended protocols.

**Immunohistochemistry for VEGFA in Tumor Tissue** For immunohistochemistry, excised xenografts were fixed with neutral buffered formalin for overnight at 4°C. The xenografts were then processed and embedded in paraffin. The paraffin-embedded tumor tissues were sectioned in 5 μm slices and mounted on slides. After being dried overnight, the slides were deparaffinized, washed, and treated with 0.5% H2O2 in methanol for 20 min. The slides were washed by PBS, blocked by TNBS buffer (0.4% TritonX-100 and 1% horse serum in PBS), incubated in diluted rabbit anti mouse
VEGF antibody (A20, Santa Cruz Biotechnology) for overnight at 4 °C, and again washed by PBS. Then, the slides were incubated for 2 h in a diluted biotinylated goat anti rabbit immunoglobulin G (IgG). After being washed by PBS, signals were visualized with ABC reagent (Vectastain, Vector, Buringame, CA, U.S.A.) and 3,3′-diaminobenzidine (DAB). Counterstain was done with hematoxylin.

**Statistical Analysis** Data were analyzed with SAS System Version 8.2 (SAS Institute Japan Ltd., Tokyo, Japan) using the t-test or Dunnett’s test. The differences were considered statistically significant when a p value was less than 0.05.

**RESULTS**

**Changes of Gene Expression in Cultured Human Pancreatic Cancer Cells Treated with Gemcitabine** Since many researchers describe IL-6, MMP-14, VEGFA, and ATF3 are most likely useful prognostic factors for cancer, the expression of these genes was assessed in gemcitabine-exposed PANC-1 cells. In the presence of FBS, these genes were induced by exposure to 10 μmol/l gemcitabine for 24 h (Fig. 1a). The gemcitabine treatment increased the expression of VEGFA, IL-6, MMP-14, and ATF3 with statistical significance.

In BxPC-3 cells, another human pancreatic adenocarcinoma cell line, the expression of VEGFA was also induced by exposure to gemcitabine with statistical significance (Fig. 1b). The induction was more prominent than that of PANC-1 cells.

**Effect of Z-360 on Gemcitabine-Induced Gene Expressions in Cultured PANC-1 Cells** In addition, we examined whether exposure to Z-360 plus gemcitabine suppressed the gemcitabine-induced expression of various genes. Z-360 suppressed gemcitabine-induced expression of VEGFA with statistical significance (Fig. 2a). On the other hand, the effect of Z-360 on MMP-14 and ATF3 differed from that on VEGFA. That is, gemcitabine-induced expression of MPP-14 was suppressed by about 50% (Fig. 2b), while in case of ATF3, Z-360 had no effect on gemcitabine-induced expression (Fig. 2c). Similarly to Z-360, a typical CCK-2/gastrin receptor antagonist, YM022 suppressed VEGFA mRNA expression induced by gemcitabine with statistical significance in PANC-1 cells (Fig. 3).

**Effect of Z-360 on Gemcitabine-Induced VEGF Protein Levels in Conditioning Media from Cultured PANC-1 Cells** In the conditioning media from cultured PANC-1 cells, the amount of VEGFA protein was measured by ELISA. Z-360 suppressed VEGFA protein levels with statistical significance in the conditioning medium from PANC-1 cells treated with gemcitabine (Fig. 4). Likewise, YM022 suppressed VEGFA protein levels with statistical significance in the conditioning medium from PANC-1 treated with gemcitabine and mRNA expression (Fig. 4).

**Changes of VEGFA Expression in the Orthotopic Xenograft Model** Since Kawasaki et al. report that the combined administration of Z-360 and gemcitabine prolonged survival compared with the control group in the PANC-1 orthotopic xenograft model, gene expression was also analyzed in this model; the result indicated that the injection of gemcitabine induced the expression of VEGFA, MMP-14, IL-6, and ATF3 (Figs. 5a—5d). Although not statistically significant, gemcitabine-induced expression of VEGFA and MMP-14 was down-regulated by the administration of Z-360 (p=0.0723 by Welch’s t-test; p=0.0922 by the t-test) (Figs. 5a, b). The gemcitabine-induced expression of VEGFA was completely inhibited by the administration of Z-360, but that of MMP-14 not suppressed; the result was similar to that in the in vitro study (Figs. 5a, b). Expression of ATF3 and IL-6 was not affected by the administration of Z-360 (Figs. 5c, d).

The amount of VEGFA protein in tumor tissues was measured by ELISA. Z-360 combined with gemcitabine significantly reduced the amount of VEGFA protein in tumor tissue compared with a group to which the vehicle was administered in the PANC-1 orthotopic xenograft model (Fig. 6). In contrast, gemcitabine alone could not significantly reduce VEGFA protein levels. Although immunohistochemical analysis showed that the sole administration of gemcitabine increased the density of area in tumor tissue stained for VEGFA and mRNA expression in mice (Fig. 7), the concomitant administration of Z-360 with gemcitabine reduced...
the density.

The expression of CCK-2/gastrin receptor was also induced by gemcitabine with statistical significance (Fig. 8a). On the other hand, individual variability in the level of gastrin gene expression was so great that there was no statistically significant difference of gastrin expression, but it was dramatically elevated in some tissues (Fig. 8b). Z-360 had no effect on gene expression of CCK-2/gastrin receptor or gastrin.

Z-360 Has no Effect on Tumor Size

The effect of the drugs on tumor size was assessed at day 18 or 40 after implantation. The injection of gemcitabine significantly reduced tumor size compared with the vehicle control group (Table 1). The administration of Z-360 had, however, no effect on tumor size (Table 1) on day 18 or 40. These results

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<td>1105.8±93.2</td>
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<td>983.0±74.7</td>
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<tr>
<td>Gemcitabine/vehicle</td>
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<td>Gemcitabine/Z-360</td>
<td>61.5±5.6**</td>
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Values represent the mean (mg)±S.E. of 8—9 mice on day 18 and 11 mice on day 40. **p<0.01, *p<0.05; compared with control (Dunnett’s test).
indicate that the administration of Z-360 did not undermine the activity of gemcitabine, so that down-regulation of the genes was not related to any change of the activity of gemcitabine.

Fig. 5. Effects of Z-360 on Gemcitabine-Induced Gene Expression in Orthotopic Xenografts

Tumor fragments of PANC-1 human pancreatic carcinoma cells were implanted orthotopically into the pancreatic body in SCID mice. From day 8 after implantation, mice were orally administrated Z-360 (100 mg/kg once a day) and injected with gemcitabine (100 mg/kg) on days 8, 11, and 14 after implantation. On day 15 after implantation, all mice were killed, and then the tumors were carefully removed for gene expression analysis. Gene expression was analyzed by quantitative PCR as described in Materials and Methods. Graphs show the results for (a) VEGFA, (b) MMP-14, (c) IL-6, and (d) ATF3. Graphs represent-fold changes vs. the vehicle control group. Results are expressed as the mean±S.E. (n=8—9). ***p<0.001, *p<0.05; compared with control (Dunnett’s test).

Fig. 6. Effect of Z-360 and Gemcitabine on VEGFA Protein Expression Levels in Orthotopically Implanted PANC-1 Xenograft

Tumor fragments of PANC-1 human pancreatic carcinoma cell were implanted orthotopically into the pancreatic body of SCID mice. From day 8 after implantation, mice were orally administrated Z-360 100 mg/kg once a day and injected gemcitabine 100 mg/kg on days 8, 11, and 14 after implantation. At day 15 after implantation, mice were killed and then the tumors were carefully removed. The amounts of VEGF protein in PANC-1 tumor tissues were measured by ELISA. Results are expressed as mean±S.E. (n=10). *p<0.05; compared with control (Dunnett’s test).

Fig. 7. Immunohistochemistry for VEGFA of in PANC-1 Tumor Xenografts

Tumor fragments of PANC-1 human pancreatic carcinoma cell were implanted orthotopically into the pancreatic body of SCID mice. From day 8 after implantation, mice were orally administrated Z-360 100 mg/kg once a day and injected gemcitabine 100 mg/kg on days 8, 11, and 14 after implantation. At day 18 after implantation, mice were killed and then the tumors were carefully removed. Tumors were fixed by formalin and embedded in paraffin. Formalin-fixed and paraffin-embedded tissues were sectioned into 5 μm. The sections were stained by rabbit anti VEGFA antibody. The detection was done using biotynlated secondary antibodies in combination with horseradish peroxidase coupled with streptavidin and the substrate 3,3′-diaminobenzidine. After immunostain, counterstain was done by hematoxyline. Magnifications, ×200.
Gemcitabine in patients with pancreatic cancer. Some molecular targeting drugs, such as VEGF antibody or receptor kinase inhibitors, are being developed for various cancers and they are useful to decrease expression of VEGF. Our studies show that gemcitabine increases the expression of VEGF, ATF3, and MMP-14; thus, the prolongation of the survival of cancer patients is probably attributable to the inhibition of an increase in the expression of VEGF induced by gemcitabine.

Meng et al. report that chemotherapy stress selectively activates nuclear factor (NF)-kappaB-dependent Akt and VEGF expression in liver cancer-derived endothelial cells.24) That study confirms that gemcitabine increased the production of VEGF or angiogenesis in tumor-derived endothelial cells, but not in normal endothelial cells.24) Although these data support our results that VEGF expression was increased by gemcitabine, the effect of gemcitabine on VEGF expression in pancreatic cancer has never been reported and this is the first report about pancreatic cancer cells.

Z-360 is an antagonist to CCK-2/gastrin receptor but neither inverse agonist nor partial agonist.1) In addition, this compound can bind to the splicing form of CCK-2/gastrin receptor and wild type of the receptor.25) On the basis of report of Chao et al., the overexpression of constitutive active mutant form of CCK-2/gastrin receptor activates hypoxia inducible transcription factor-alpha (HIF-alpha) and increases the expression of VEGF.26) HIF-alpha is known to be a potent inducer of VEGF expression in the presence of hypoxia.27) HIF-alpha activation is mediated by its stabilization via the Akt/Pi3kinase signaling pathway.28) Akt/Pi3kinase is a major kinase involved in the down-stream signaling pathway of the CCK-2/gastrin receptor.29,30) Thus, gastrin is a likely mediator of HIF-alpha activation or VEGF expression. In our study, CCK-2/gastrin receptor mRNA was increased by administration of gemcitabine to orthotopic xenograft mice. Previous studies demonstrate that Z-360 significantly inhibited gastrin-induced proliferation of human CCK-2 receptor-expressing cells and also significantly reduced gastrin-induced proliferation of human CCK-2 receptor-expressing cells.31) In our study, gemcitabine-induced VEGF gene expression was inhibited by addition of an anti-gastrin antibody to the culture medium of PANC1 cells (data not shown). Accordingly, the inhibitory effect of Z-360 on promotion of VEGF expression by gemcitabine is likely to be mediated via the suppression of the Akt signaling pathway due to an autocrine effect of gastrin.

The transcriptional promoter activity of gastrin is regulated by Smads, which are transcriptional factors, in the transforming growth factor-beta (TGF-beta) signaling pathway.31) Our study demonstrates that treatment of cultured PANC-1 cells by gemcitabine increased plasminogen activator inhibitor (PAI-1) and connective tissue growth factor (CTGF), which are known as major target genes of Smads (data not shown). Therefore, the gene expression mechanism related to treatment with gemcitabine is probably mediated by the activation of Smads. On the other hand, Sánchez-Elsner et al. report that overexpression of HIF-1alpha/beta or
Smad3/4 caused a slight increase of VEGFA promoter activity, whereas co-transfection of HIF-lalpha/beta and Smad3/4 had a marked effect; hence, the mechanism of VEGFA mRNA induction by gemcitabine might involve via synergistic co-operation with Smad activation and HIF-lalpha activation by gastrin. Z-360, a CCK-2/gastrin receptor antagonist, most likely suppresses a part of this mechanism through the inhibition of CCK-2/gastrin receptor activation by gastrin, and consequently suppress gemcitabine-induced expression of VEGFA.

Since Hendriksen et al. report that the amount of VEGF protein accumulated in tumor tissue is dependent on the size of tumors, we examined whether VEGF protein levels in tumor tissue by administration of gemcitabine in orthotopic xenograft mice do not change as results of reduction of tumor size by itself. Taking all things into consideration, the amount of VEGF protein in tumor tissue might be based on a balance of both the increase by stimuli and the decrease by tumor size reduction. As a result, the administration of Z-360 in absence of gemcitabine did not affect either VEGF expression or tumor size. These findings suggest that Z-360 decreased an accumulation of VEGF by reducing gemcitabine-induced VEGF expression in tumor tissue.

Grabowska et al. describe that Z-360 combined with gemcitabine significantly reduces the microvessel density in tumor area stained with the endothelial marker, CD34, in mice orthotopically implanted with human pancreatic cells. This report supports our result that the suppressive effects of Z-360 on gemcitabine-induced VEGF expression probably contribute to reduce the microvessel area in tumor tissue.

In conclusion, these findings suggest that suppression of VEGF expression by Z-360 is important for its prolongation of survival during the combination therapy with gemcitabine.

REFERENCES