Cancer Chemother Pharmacol (2008) 61:883–892
DOI 10.1007/s00280-007-0591-8

ORIGINAL ARTICLE

# Effect of Z-360, a novel orally active CCK-2/gastrin receptor antagonist on tumor growth in human pancreatic adenocarcinoma cell lines in vivo and mode of action determinations in vitro

**Daisuke Kawasaki · Yutaka Emori · Runa Eta · Yuka Iino · Hiroki Hamano · Koji Yoshinaga · Takao Tanaka · Mineo Takei · Susan A. Watson**

Received: 7 July 2007 / Accepted: 1 September 2007 / Published online: 28 September 2007


**Abstract**
*Purpose* Gastrin is known to enhance the growth of pancreatic carcinoma via the cholecystokinin (CCK)-2/gastrin receptor. We investigated the anti-tumor effect of Z-360 (calcium bis [(*R*)-(−)-3-[3-{5-cyclohexyl-1-(3,3-dimethyl-2-oxo-butyl)-2-oxo-2,3,4,5-tetrahydro-1*H*-benzo[*b*][1,4]diazepin-3-yl}ureido]benzoate]), a novel orally active CCK-2 receptor antagonist alone or combined with the chemotherapeutic agent, gemcitabine in human pancreatic adenocarcinoma cell lines.
*Results* Z-360 potently inhibited specific binding of [$^3$H]CCK-8 to the human CCK-2 receptor, with a $K_i$ value of 0.47 nmol/l, and showed antagonistic activity for this receptor. The anti-tumor effect of Z-360 alone or combined with gemcitabine was assessed using subcutaneous xenografts of MiaPaCa2 and PANC-1 and an orthotopic xenograft model (PANC-1). Oral administration of Z-360 significantly inhibited the growth of MiaPaCa2 (41.7% inhibition at 100 mg/kg, $P < 0.01$). Combined administration of Z-360 and gemcitabine significantly inhibited subcutaneous PANC-1 tumor growth compared with either agent alone (27.1% inhibition compared to effect with gemcitabine, $P < 0.05$), and significantly prolonged survival compared with the vehicle control (median survival of 49 days in vehicle compared to 57 days in the combination group, $P < 0.05$). In vitro studies showed that Z-360 significantly inhibited gastrin-induced proliferation of human CCK-2 receptor-expressing cells, and also significantly reduced gastrin-induced PKB/Akt phosphorylation to the level of untreated controls.
*Conclusion* In the present study, we have shown that Z-360 combined with gemcitabine can inhibit pancreatic tumor growth and prolong survival in a pancreatic carcinoma xenograft model, on a possible mode of action being the inhibition of gastrin-induced PKB/Akt phosphorylation through blockade of the CCK-2 receptor. Our results suggest that Z-360 may be a useful adjunct to gemcitabine for the treatment of pancreatic carcinoma and a therapeutic option for patients with advanced pancreatic cancer.

**Keywords** Z-360 · Gastrin · Cholecystokinin (CCK)-2/gastrin receptor · Antagonist · Pancreatic carcinoma · Gemcitabine

**Abbreviations**

| | |
|---|---|
| CCK | Cholecystokinin |
| PKB | Protein kinase B |
| G17 | Human gastrin-17 |
| BrdU | 5-Bromo-2′-deoxyuridine |
| SCID | Severe combined immunodeficiency |
| DMEM | Dulbecco's modified Eagle's medium |
| FBS | Fetal bovine serum |
| CMC | Carboxymethyl cellulose |
| BSA | Bovine serum albumin |
| ELISA | Enzyme-linked immuno sorbent assay |
| SE | Standard error |

D. Kawasaki · Y. Emori · R. Eta · Y. Iino · H. Hamano · K. Yoshinaga (✉) · T. Tanaka · M. Takei
Central Research Laboratories,
Zeria Pharmaceutical Co., Ltd,
2512-1, Numagami, Oshikiri, Kumagaya-city,
Saitama 360-0111, Japan
e-mail: kouji-yoshinaga@zeria.co.jp

S. A. Watson
Division of Pre-Clinical Oncology,
University of Nottingham, Nottingham, UK

## Introduction

Pancreatic carcinoma is one of the most fatal cancers because of the difficulty in making an early diagnosis, resulting in late presentation. Only 10–20% of pancreatic cancers can be curatively resected at the time of diagnosis [15, 23, 25], and the overall 5-year survival rate for all pancreatic carcinoma patients is only 0.4%. Although gemcitabine (2′, 2′-difluorodeoxycytidine), a nucleoside analog used as a first line drug can prolong the survival of these patients, <3% remain alive at 5 years after the initial diagnosis [5], thus, the options for drug treatment are limited.

Gastrin is a polypeptide hormone that is involved in the secretion of gastric acid, and has been suggested to have a role in the growth of normal and malignant gastrointestinal tissues in the stomach [19, 39], colon [39], and pancreas [32, 35]. These cancers have been shown to overexpress the gastrin gene and are sensitive to the trophic effects of gastrin [16, 38]. Gastrin stimulates the proliferation of pancreatic carcinoma by endocrine, autocrine, and paracrine mechanisms after binding to the G-protein-coupled cholecystokinin (CCK)-2/gastrin receptor.

Gastrin may have an important growth effect on various gastrointestinal malignancies because they frequently overexpress or ectopically express CCK-2 receptors [16, 29, 30]. Recent studies revealed that the intracellular signaling pathway in the activation of the CCK2 receptor leads to carcinogenesis [13]. Moreover, ontogenic studies on transgenic mice overexpressing the gastrin or CCK-2 receptors have shown that this hormone is involved in gastric and pancreatic tumor formation [10, 26].

In pre-clinical studies, the role of gastrin and the CCK-2 receptor in human pancreatic carcinogenesis has been investigated in vitro by using human pancreatic carcinoma cell lines [34] and in vivo by using xenograft models [32]. CCK-2 receptor antagonists such as L-365,260 [3, 21] can inhibit the growth of pancreatic carcinoma-derived cell lines in these models [32, 35], suggesting that CCK-2 receptor antagonist therapy might be useful for patients with pancreatic cancers expressing this receptor.

In fact, clinical trials have already been performed to evaluate the efficacy of some CCK-2 receptor antagonists for advanced pancreatic carcinoma. Gastrazole (JB95008), a CCK-2 receptor antagonist, significantly prolonged survival compared with placebo [9], providing evidence that the inhibition of gastrin-dependent pathophysiological changes could be effective for the treatment of pancreatic carcinoma, albeit its administration via protracted venous infusion may limit its clinical utility.

Z-360 (calcium bis [(*R*)-(-)-3-[3-{5-cyclohexyl-1-(3,3-dimethyl-2-oxo-butyl)-2-oxo-2,3,4,5-tetrahydro-1*H*-benzo[*b*][1,4]diazepin-3-yl}ureido]benzoate]) is a potent and orally active CCK-2 receptor antagonist (Fig. 1) that was developed by Zeria Pharmaceutical Co., Ltd. We previously reported that Z-360 inhibits pentagastrin-stimulated acid secretion in acute fistula rats and Heidenhain pouch dogs, with $ID_{50}$ values of 0.17 mg/kg (i.d.) and 0.28 mg/kg (p.o.), respectively [27]. Furthermore Z-360 is expected to show clinical benefit in gastrin-sensitive pancreatic carcinoma on the basis of the current evidence that gastrin can play a crucial role for gastrointestinal tumor growth. Currently a Phase Ib/IIa clinical trial is being conducted in Europe to clarify the interaction between Z-360 and gemcitabine for unresectable advanced pancreatic cancer.

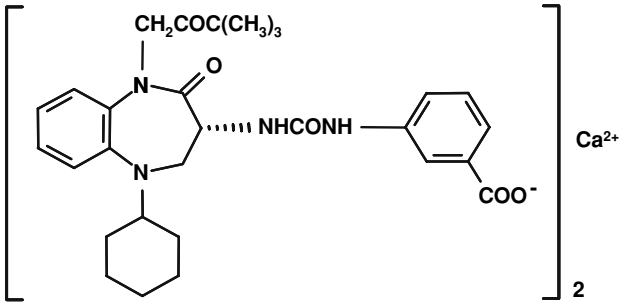


**Fig. 1** Chemical structure of Z-360

In the present study, we have investigated the anti-tumor effect of Z-360 and its combined effect with gemcitabine in xenograft murine models of human pancreatic carcinoma. In addition, we have also examined gastrin-induced cell proliferation and protein kinase B (PKB)/Akt phosphorylation using human CCK-2 receptor-expressing cell lines in vitro to determine a possible mode of action.

## Materials and methods

### Reagents

Z-360 and its calcium-free form (Z-360F) and YF476 were synthesized at the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd (Saitama, Japan). Gemcitabine hydrochloride (gemcitabine) was purchased from Eli Lilly Japan (Hyogo, Japan). L-365,260 and L-364,718 were also synthesized at Zeria, as above. Human gastrin-17 (G17) and CCK-8 sulfate (CCK-8) were purchased from the Peptide Institute (Osaka, Japan). [Propionyl-$^{3}$H]CCK-8 sulfate ([$^{3}$H]CCK-8) was purchased from GE Healthcare (Buckinghamshire, UK). Human CCK-1 and CCK-2 receptor cDNAs were obtained from a human pancreas cDNA library (Clontech, Mountain View, CA). 5-bromo-2′-deoxyuridine (BrdU) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Cell Proliferation ELISA kit (BrdU, colorimetric) was from Roche Applied Science (Tokyo, Japan) and a FACE® AKT ELISA kit was purchased from Active Motif (Tokyo, Japan).

## Mice

Seven-week-old female nude mice (BALB/c-nu/nuSlc) were purchased from Japan SLC (Shizuoka, Japan) and 7-week-old female severe combined immunodeficiency (SCID) mice (C.B-17/lcr-scid/scidJcl) were purchased from Clea Japan (Tokyo, Japan). Six-week-old male mice (Crlj:CD1, ICR) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). All animal experiments were approved by the Animal Care and Use Committee of the Central Research Laboratories of Zeria Pharmaceutical Co., Ltd.

## Cell lines

MiaPaCa2 (a human pancreatic carcinoma cell line), HT-29 (a human colorectal carcinoma cell line), and CHO-K1 (a Chinese hamster ovarian cell line) were purchased from the American Type Culture Collection (Manassas, VA). PANC-1 (a human pancreatic carcinoma cell line) was purchased from the European Collection of Cell Cultures (Wiltshire, UK). MiaPaCa2, PANC-1, and HT-29 cells were cultured using Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Tokyo, Japan), while CHO-K1 cells were maintained in Ham's F-12 nutrient mixture (F-12) (Invitrogen). All of the media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; BenchMark™, Gemini Bio-Products, West Sacramento, CA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

## Human CCK-1 and CCK-2 receptor expression

The amplification and cloning of human CCK-1 and CCK-2 receptor cDNAs were performed by a modified method of Miyake et al. [28] CHO-K1 cells were transfected with plasmids encoding human CCK-1 receptor cDNA (CHO-K1/CCK-1) or human CCK-2 receptor cDNA (CHO-K1/CCK-2), while HT-29 cells were transfected with plasmids encoding human CCK-2 receptor cDNA (HT-29/CCK-2). Stable transfectants were obtained by culture for more than 2 weeks in the presence of G418 (400 μg/ml, Invitrogen).

## Radioligand binding assay

The radioligand binding assay was performed by a modified method of Miyake et al. [28] Briefly, CHO-K1/CCK-1 cells ($5 \times 10^4$/well) or CHO-K1/CCK-2 cells ($1.67 \times 10^4$/well) were cultured overnight in F-12 medium containing 10% FBS and 10 mmol/l HEPES. These cells were suspended in F-12 medium containing 0.1% (w/v) BSA and 10 mmol/l HEPES, and then incubated at 37°C for 1 h in the presence of [$^3$H]CCK-8 with or without Z-360F, L-365,260, or L-364,718. The radioactivity of bound [$^3$H]CCK-8 was determined with a liquid scintillation counter (2000CA, Perkin Elmer Japan, Kanagawa, Japan). Nonspecific binding was measured in the presence of 1 μmol/l CCK-8. The $K_i$ was calculated using Graph Pad Prism™ (computer software for data analysis, Ver. 3.0, GraphPad Software). [$^3$H]CCK-8 concentrations for the calculation of $K_i$ values were 1 nmol/l (CHO-K1/CCK-1) and 0.5 nmol/l (CHO-K1/CCK-2), while the $K_d$ values were $0.98 \pm 0.07$ nmol/l for the CCK-1 receptor and $0.23 \pm 0.02$ nmol/l for the CCK-2 receptor.

## Measurement of intracellular $Ca^{2+}$ release

CHO-K1/CCK-2 cells ($4 \times 10^4$/well) were cultured overnight in F-12 medium containing 10% FBS. The cells were incubated at 37°C for 1 h in assay buffer (Hanks/HEPES buffer containing 2.5 mmol/l probenecid and 0.1% BSA, pH 7.4) containing 4.4 μmol/l Fluo-3AM and 0.04% Pluronic F-127 (both were from Molecular Probes, Eugene, OR). After incubation, the cells were washed 4 times with assay buffer and then placed in buffer containing Z-360F ($10^{-10}$ to $10^{-6}$ mol/l).

Intracellular $Ca^{2+}$ release was measured with a fluid handling integrated fluorescence plate reader (Flex Station; Molecular Devices, Sunnyvale, CA). Briefly, aliquots of CCK-8 (1 nmol/l) were delivered to the sample plate automatically at the preprogrammed times, and then the fluorescence of Fluo-3AM in culture supernatant was read with excitation at 485 nm and emission at 525 nm. The $IC_{50}$ was calculated using GraphPad Prism.

## Subcutaneous xenograft models

Three million MiaPaCa2 cells were injected subcutaneously into the right flank of nude mice. On day 14 after tumor cell injection, the mice were divided into four groups with an approximately equal average tumor size in each group. The animals were orally administered the vehicle or Z-360 at doses of 10, 30, and 100 mg/kg once daily for 21 days. Tumor size was measured on day 3, 7, 11, 14, 18, 21, 25, 29, 32 and 35, These were calculated by using the formula; tumor size ($mm^3$) = 1/2 length × width$^2$. On day 35 after tumor injection, all of the mice were sacrificed, and the tumors carefully removed and weighed.

Three million PANC-1 cells were injected subcutaneously into the right flanks of SCID mice. On day 4 after tumor cell injection, the mice were divided into four groups with an approximately equal average tumor size in each group. The animals were administered oral Z-360 at a dose of 10, 30, and 100 mg/kg once daily for 63 days and/or intravenous gemcitabine at a dose of 100 mg/kg on days 5, 8, and 12 after tumor injection. The vehicle control group

was treated orally with 0.5% carboxymethyl cellulose (CMC)-Na and intravenously with saline as the schedule given above. Tumor size was measured on day 4, 11, 18, 25, 32, 40, 46, 53, 60 and 67. These were calculated by using the formula; tumor size ($mm^3$) = 1/2 length × $width^2$. On day 67 after tumor cell injection, all of the mice were sacrificed, and the tumors were carefully removed and weighed.

### Orthotopic xenograft model

Three million PANC-1 cells were injected subcutaneously into the right flanks of donor SCID mice. Forty days after tumor cell injection, the mice were sacrificed, and the tumors removed and cut into small pieces. Recipient SCID mice were anesthetized by intraperitoneal injection of 0.25 ml of 2.5% (v/v) avertin (2, 2, 2-tribromoethanol). An incision was made in the left abdomen, the pancreas was carefully exposed, and a piece of tumor was attached to the centre of the organ with an absorbable 6–0 suture. The pancreas was returned to the peritoneal cavity and the abdominal wall and the skin were closed with 5-0 sutures. On day 7 after orthotopic tumor implantation, the mice were randomized to four 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 7, 10, and 14 after tumor implantation. Vehicle control group was treated orally with 0.5% CMC-Na and intravenously with saline by respective schedule given above. Survival of the mice was monitored once daily.

### Measurement of cell proliferation

CHO-K1/CCK-2 cells ($6 \times 10^3$/well) were cultured overnight in serum-free F-12 medium. The cells were pretreated with Z-360F ($10^{-9}$ to $10^{-6}$ mol/l) for 30 min, and then G17 ($10^{-7}$ mol/l) was added to each well. On day 4, BrdU (10 μmol/l) was added to each well, and the plate incubated for a further 2 h. Finally, the BrdU uptake of CHO-K1/CCK-2 cells was measured using a cell proliferation ELISA kit.

### Measurement of PKB/Akt phosphorylation

HT-29/CCK-2 cells ($1 \times 10^4$/well) were cultured overnight in DMEM containing 10% FBS. The cells were washed with PBS and preincubated for 2 h with fresh serum-free medium. The cells were then treated with Z-360F ($10^{-6}$ mol/l) for 30 min, after which G17 ($10^{-8}$ mol/l) was added to the cultures. At 30 min after G17 stimulation, phosphorylation of PKB/Akt was measured by using a FACE® AKT ELISA kit.

### Statistical analysis

Results are expressed as the mean ± standard error (SE). Data were analyzed with SAS System Version 8.2 (SAS Institute Japan Ltd., Japan.) using the *t* test, Dunnett's test, Tukey's test or the log-rank test. Differences with a *P* value of less than 0.05 were considered statistically significant.

## Results

### Binding of Z-360 to the CCK-2 receptor

G17 has the same five amino acids on its carboxyl terminus as CCK. These two peptide hormones (G17 and CCK) interact with two distinct receptors, which are known as the CCK-1 and CCK-2 receptors, respectively, and belong to the G-protein-coupled receptor superfamily. Gastrin has a high affinity for the CCK-2 receptor, but not for the CCK-1 receptor. Therefore, the affinity of Z-360 for human CCK-1 and CCK-2 receptors was examined by a radioligand binding assay using [$^3$H]CCK-8.

CHO-K1/CCK-1 or CHO-K1/CCK-2 cells were incubated with [$^3$H]CCK-8 (1 nmol/l) with or without Z-360F, L-365,260 as a selective CCK-2 receptor antagonist [3, 21] and L-364,718 as a selective CCK-1 receptor antagonist [21] at 37°C for 1 h. As shown in Table 1 and Fig. 2, Z-360F potently inhibited specific binding of [$^3$H]CCK-8 to the human CCK-2 receptor with a $K_i$ value of 0.47 nmol/l. This result indicates that Z-360F had a higher affinity for the CCK-2 receptor compared with the CCK-1 receptor (CCK-1/CCK-2 ratio: 615). Regarding CCK-2 receptor, the Hill coefficient of Z-360F was estimated to be 0.79 (0.44–1.15, 95% confidence limits from three separate duplicate determinations) and it was not significantly different from unity ($P > 0.05$). The Hill coefficient analysis indicated that Z-360F recognized a single class of binding site in CCK-2 receptor. Accordingly it was only one class of sites found in

**Table 1** Binding of Z-360F to both the CCK-1 and CCK-2 receptor

| Drug | $K_i$ (nmol/l) | | Selectivity ratio[a] |
|---|---|---|---|
| | CCK-2 receptor | CCK-1 receptor | |
| Z-360F | 0.47 ± 0.03 | 316 ± 81.2 | 672 |
| L-365,260 | 11.9 ± 1.95 | 487 ± 112 | 40.9 |
| L-364,718 | 166 ± 20.4 | 0.25 ± 0.06 | 0.0015 |
| CCK-8[b] | 0.23 ± 0.02 | 0.98 ± 0.07 | |

[a] The selectivity ratio represents the $K_i$ for the CCK-1 receptor divided by the $K_i$ for the CCK-2 receptor. Values are the mean of three separate experiments performed in duplicate

[b] The $K_d$ values (nmol/l) of CCK-8 represented as means ± SE were made by saturation analysis with nine concentrations of the ligand (0.05–7.7 nmol/l) from three or six separate duplicate experiments

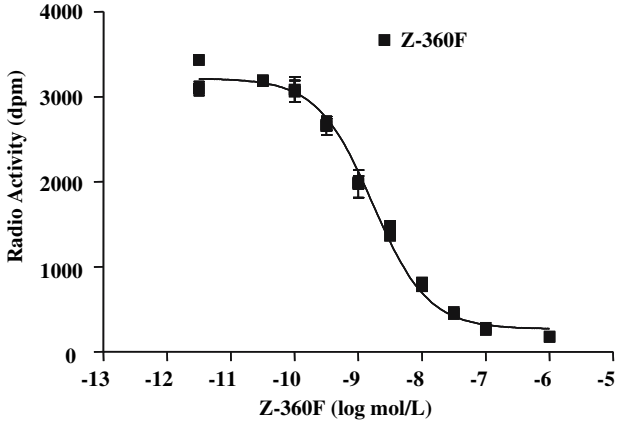


**Fig. 2** Affinity estimates for Z-360F and classical CCK antagonists from radioligand binding studies in CHO-K1/CCK-2 cells and CHO-K1/CCK-1 cells. $K_i$ values represent the mean ± SE from three separate duplicate determinations

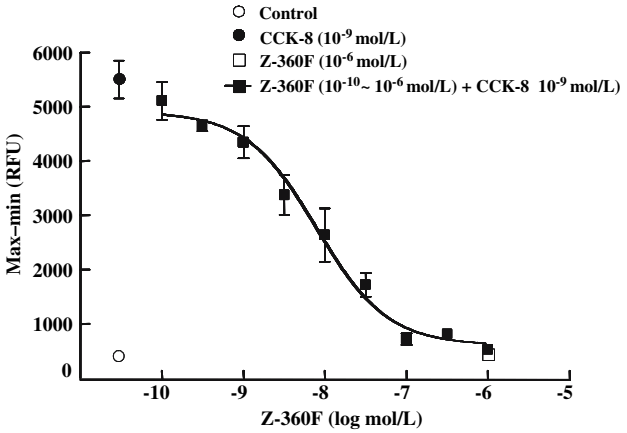


**Fig. 3** Inhibitory effect of Z-360F on CCK-8-induced intracellular $Ca^{2+}$ release in CHO-K1/CCK-2 cells. The ability of 1 nmol/l CCK-8 to increase the release of intracellular $Ca^{2+}$ was determined in the presence of various concentrations of Z-360F by using Fura-3AM-loaded CHO-K1/CCK-2 cells. Figure shows typical results from one of the three independent experiments

this study. The Hill coefficient of Z-360F for CCK-1 receptor was estimated to be 1.05 (0.66–1.43, 95% confidence limits from three separate duplicate determinations) and it was not significantly different from unity ($P > 0.05$). The Hill coefficient analysis indicated that Z-360 recognized a single class of binding site in CCK-1 receptor. On the other hand, YF476, another CCK-2 receptor antagonist, inhibited specific binding to human CCK-2 receptor with a $K_i$ value of 0.21 nmol/l.

## Antagonistic activity for the CCK-2 receptor by Z-360

To determine the antagonistic effect of Z-360 on the CCK-2 receptor, the intracellular $Ca^{2+}$ release was measured. Although it has been reported that CCK-8 and gastrin are not equally acting on the CCK-2 receptor [24], in our preliminary experiments using CHO-K1/CCK-2 cells loaded Fluo-3AM, the degree of the $Ca^{2+}$ release induced by the two kinds of ligand showed the almost same. Therefore we mainly used CCK-8 throughout the study, similarly in receptor binding assay. Whereas 1 nmol/l CCK-8 increased intracellular $Ca^{2+}$ release by CHO-K1/CCK-2 cells, pretreatment with Z-360F ($10^{-11}$ to $10^{-5}$ mol/l) inhibited CCK-8-induced intracellular $Ca^{2+}$ release in a concentration-dependent manner ($IC_{50}$ value: 11 nmol/l, Fig. 3). However, Z-360F at $10^{-5}$ mol/l showed no effect on $Ca^{2+}$ release in CHO-K1/CCK-2 cells without CCK-8 stimulation. As the degree of the $Ca^{2+}$ release from unstimulated CHO-K1/CCK-2 cells is similar to untransfected cells (intact CHO-K1 cells), we defined as basal activity using unstimulated CHO-K1/CCK-2 cells. As Z-360F alone did not increased calcium release in CHO-K1/CCK-2 cells comparing to basal activity, we considered that Z-360 did not act as agonist activity for classical CCK-2 receptor, at least at range of $10^{-5}$ M from $10^{-11}$ M. These results demonstrated that Z-360 shows selective antagonistic activity for the human CCK-2 receptor without an agonistic activity for CCK-2 receptor. On the other hand, YF476 showed the inhibition of CCK-8-induced $Ca^{2+}$ release with $IC_{50}$ value of 1.6 nmol/l.

## Inhibitory effect of Z-360 on tumor growth in xenograft models of pancreatic carcinoma

Since an antagonistic effect of Z-360 on the human CCK-2 receptor was demonstrated, the inhibitory effect of Z-360 was next examined on the tumor growth of two human pancreatic adenocarcinoma cell lines, MiaPaCa2 and PANC-1, both of which have been previously reported to express the CCK-2 receptor [34].

MiaPaCa2 cells were injected subcutaneously into the right flanks of nude mice and allowed to establish for 14 days. Z-360 was then administered orally to the mice at doses of 10, 30, or 100 mg/kg once daily for 21 days. Z-360 significantly inhibited tumor growth as measured by both tumor size (Fig. 4a) and tumor weight (Fig. 4b) in a dose-dependent manner. As shown in Fig. 4b, administration of Z-360 at 10, 30, and 100 mg/kg resulted in 16.5, 39.6, and 41.7% inhibition of final tumor weight, respectively. This dose-dependent inhibitory activity was significant at doses of 30 mg/kg ($P < 0.05$) and 100 mg/kg ($P < 0.01$).

PANC-1 cells were injected subcutaneously into the right flanks of SCID mice. On day 4 after tumor inoculation, Z-360 was administered orally to the mice at doses of 10, 30, and 100 mg/kg once daily for 63 days. As shown in Fig. 5a, in contrast to MiaPaCa2, of Z-360 did not show an inhibitory effect on PANC-1 growth.

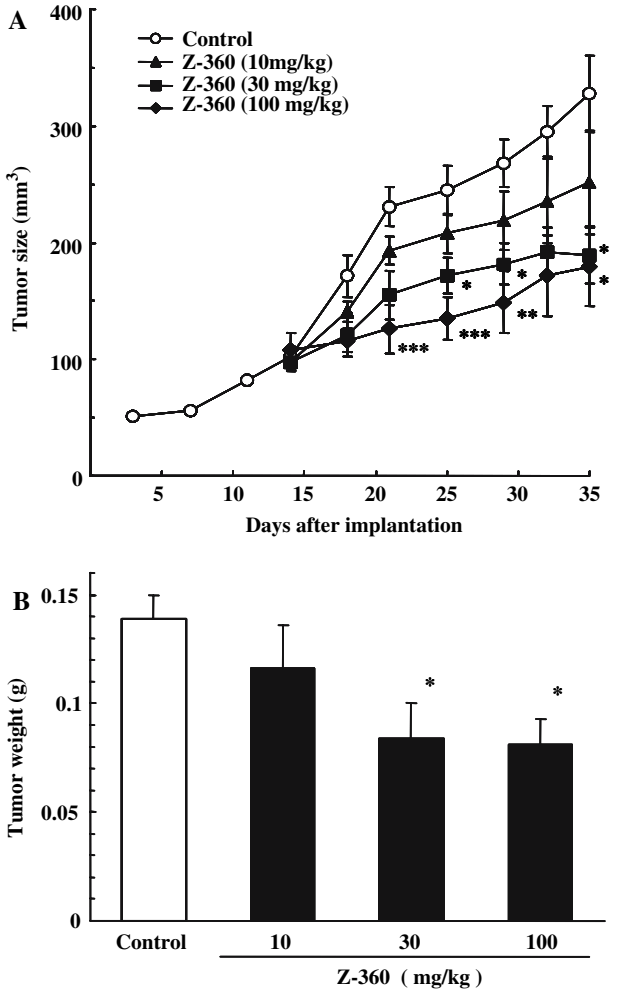


**Fig. 4** Inhibitory effect of Z-360 on tumor growth in the MiaPaCa2 xenograft model. MiaPaCa2 cells ($3 \times 10^6$ cells) were injected subcutaneously into the right flanks of nude mice, after which Z-360 was administered orally at doses of 10, 30, and 100 mg/kg once daily for 21 days. **a** Tumor size was measured on day 3–35, and calculated by using the formula; tumor size ($mm^3$) = 1/2 length × $width^2$. **b** Tumor weight was measured on day 35. Values represent the mean ± SE of eight mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the vehicle control (Dunnett's test)

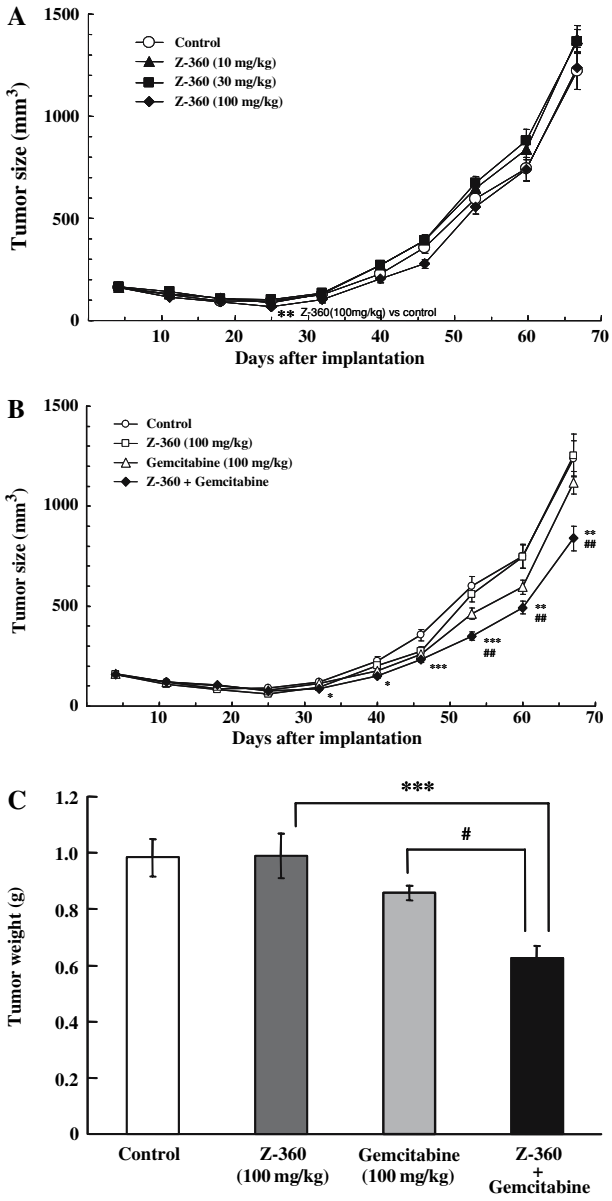


**Fig. 5** Inhibitory effect of Z-360 either alone or combined with gemcitabine on tumor growth in the PANC-1 xenograft model. **a** PANC-1 cells ($3 \times 10^6$ cells) were injected subcutaneously into the right flanks of SCID mice, after which Z-360 was administered orally at a dose of 10, 30, and 100 mg/kg once daily for 63 days. **b** and **c** PANC-1 cells ($3 \times 10^6$ cells) were injected subcutaneously into the right flanks of SCID mice, after which Z-360 was administered orally at a dose of 100 mg/kg once daily for 63 days, and/or gemcitabine was injected intravenously at a dose of 100 mg/kg on days 5, 8, and 12. Tumor size was measured on days 4–67, and calculated by using the formula; tumor size ($mm^3$) = 1/2 length × $width^2$. Tumor weight was measured on day 67. Values represent the mean ± SE for 10–15 mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the Z-360 alone, # $P < 0.05$, ## $P < 0.01$ compared with the gemcitabine alone (Tukey's test)

Gemcitabine has been widely used for the treatment of pancreatic carcinoma as a first-line drug [15, 25]. Therefore we next examined the combined effect of Z-360 and gemcitabine on the tumor growth of PANC-1, which had previously been shown to be resistant to the inhibitory effect of Z-360 alone.

After implantation of PANC-1 cells, Z-360 was administered orally at a dose of 100 mg/kg once daily for 63 days, with or without gemcitabine, which was injected intravenously at a dose of 100 mg/kg on days 5, 8, and 12 after tumor cell implantation. As shown in Fig. 5b (kinetics of tumor sizes) and 5C (final tumor weights), PANC-1 showed no sensitivity to gemcitabine alone (one cycle given) as seen with Z-360 alone. In contrast, the combination of Z-360 and gemcitabine significantly inhibited tumor growth when compared with either Z-360 alone (36.9% inhibition, $P < 0.001$) or gemcitabine alone (27.1% inhibition, $P < 0.05$).

### Combined therapy with Z-360 and gemcitabine in an orthotopic xenograft model of pancreatic carcinoma

It is important to evaluate the prolongation of survival when assessing the potency of treatments for pancreatic carcinoma. Therefore, the survival benefit of Z-360 when combined with gemcitabine was examined in the orthotopic xenograft model of PANC-1. Small pieces of PANC-1 tumors were implanted into the pancreas' of SCID mice, after which Z-360 was given orally at a dose of 100 mg/kg once daily with/without gemcitabine which was injected intravenously at a dose of 100 mg/kg on days 7, 10, and 14. Survival of the mice was analyzed by the Kaplan–Meier method (Fig. 6). Mice treated with the combination of Z-360 and gemcitabine (50% median survival: 57 days) demonstrated significantly longer survival compared with mice treated by the vehicle control (50% median survival: 49 days) ($P < 0.05$). However, Z-360 alone or gemcitabine alone did not significantly prolong survival.

### The plasma concentration of Z-360 in Mice after single oral administration

Plasma concentration of Z-360 after a single oral administration to CD1 male mice was measured by LS/MS/MS method. The highest plasma concentration of Z-360 appeared at 0.25 h after oral administration and the plasma concentration gradually decreased in a time-dependent manner. At 0.25, 0.5, 1, 2 and 6 h after oral administration of Z-360 at a dose of 30 mg/kg, plasma concentration was 920.0 ± 302.8, 713.7 ± 226.8, 172.9 ± 42.8, 246.6 ± 140.1 and 29.57 ± 5.49 ng/ml, respectively. After 0.25, 0.5, 1, 2 and 6 h oral administration of Z-360 at a dose of 100 mg/kg, plasma concentration was 2227 ± 453, 2053 ± 687, 1406 ± 309, 774.0 ± 235.2 and 100.1 ± 33.5 ng/ml, respectively.

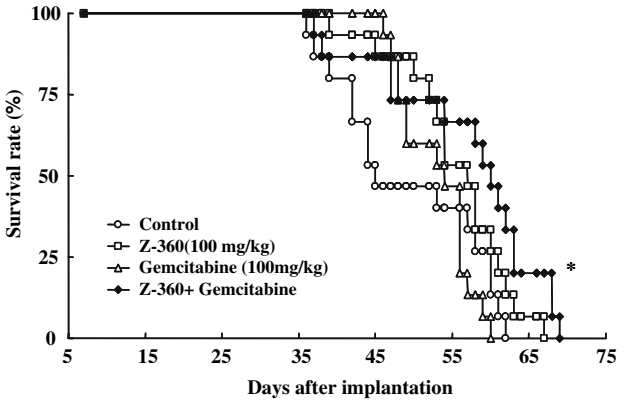


**Fig. 6** Survival benefit of Z-360 combined with gemcitabine in a mouse orthotopic xenograft model ($n = 15$/group). Small pieces of PANC-1 tumor were implanted in the pancreas of SCID mice. Z-360 was administered orally at a dose of 100 mg/kg once daily and/or gemcitabine was injected intravenously at a dose of 100 mg/kg on days 7, 10, and 14 after implantation. Survival of the mice was analyzed by the Kaplan–Meier method. * $P < 0.05$ compared with the vehicle control (log-rank test)

### In vitro studies on possible mode of action of Z-360 on the biological parameters of CCK-2 receptor positive cell lines

Gastrin may have an important role in the proliferation of pancreatic carcinoma cells [7]. In order to investigate the cell growth by gastrin, several studies using CHO-K1/CCK-2 cells has been reported [1, 12, 22]. In assessments of BrdU uptake, the stable and constant proliferation for the response of gastrin after serum starvation was observed in CHO-K1/CCK-2 cells. To determine the inhibitory effect of Z-360 on the cell growth by gastrin-stimulation, the suppression of the gastrin-induced proliferation of cultured CHO-K1/CCK-2 cells was determined. As shown in Fig. 7, G17 significantly enhanced cell proliferation by 1.5-fold compared with the control ($P < 0.001$), whereas Z-360F blocked the enhancement of the cell proliferation by G17 in a concentration-dependent manner ($10^{-8}$ mol/l; $P < 0.05$, $10^{-7}$ mol/l; $P < 0.01$, $10^{-6}$ mol/l; $P < 0.001$).

Gastrin promotes pancreatic tumor cell survival though phosphorylation of PKB/Akt, a kinase known to promote cell survival, via phosphoinositide 3-kinase [11]. Phosphorylated PKB/Akt also inhibits apoptosis by the inactivation of several pro-apoptotic factors [6, 8]. Regarding Akt

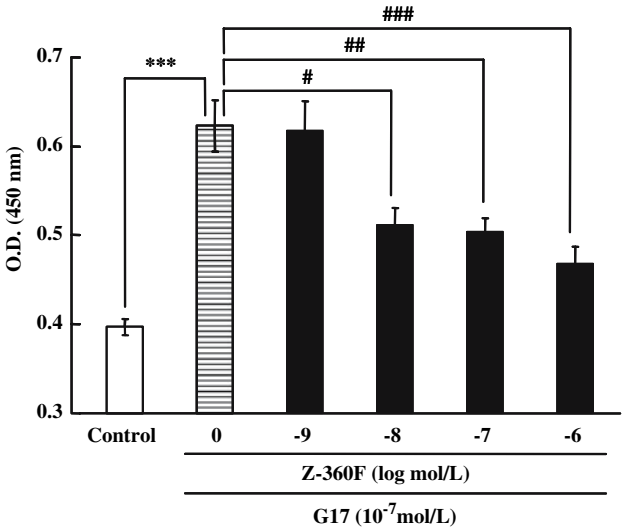


**Fig. 7** Inhibitory effect of Z-360F on gastrin-induced proliferation of CHO-K1/CCK-2 cells. CHO-K1/CCK-2 cells were pretreated with Z-360 ($10^{-9}$ to $10^{-6}$ mol/l) for 30 min before stimulation with gastrin ($10^{-7}$ mol/l). On day 4, the cells were incubated with BrdU (10 μmol/l) for 2 h, and then BrdU uptake was measured as a parameter of cell proliferation. Values represent the mean ± SE of six replicates. *** $P < 0.001$ compared with the control (*t* test). # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared with gastrin alone (Dunnett's test)

phosphorylation by gastrin, the stable and constant responses were observed in HT-29/CCK-2 cells. To investigate the role of phosphorylated PKB/Akt in the anti-tumor activity of Z-360, the inhibitory effect of Z-360 on gastrin-induced PKB/Akt phosphorylation in HT-29/CCK-2 cells was evaluated. G17 ($10^{-8}$ mol/l) significantly increased PKB/Akt phosphorylation compared with untreated control cells ($P < 0.01$), whereas pretreatment with Z-360F ($10^{-6}$ mol/l) significantly reduced phosphorylation compared with that in cells exposed G17 ($P < 0.05$, Fig. 8). Z-360 alone has no effect on basal PKB/Akt phosphorylation.

## Discussion

The therapeutic effect of Z-360, a potent CCK-2 receptor antagonist, on human pancreatic cancer in murine xenograft models was investigated. Z-360 caused dose-dependent inhibition of subcutaneous tumor growth of MiaPaCa2 but not PANC-1 human pancreatic xenografts.

The K-*ras* gene, which can be activated by G-coupled protein receptors such as CCK receptors but can differentially activate down-stream signaling, dependant on the cell type. For example, with the two pancreatic cell lines used in the current study, both showed reduced proliferation after K-*ras* gene knockdown, but only MiaPaca-2 cells showed increased apoptosis, indicating an increased dependency on k-ras induced signaling [14], which may impact on the sensitivity to Z-360.

Z-360 was shown to have no inhibitory effect in the PANC-1 model which was also resistant to gemcitabine

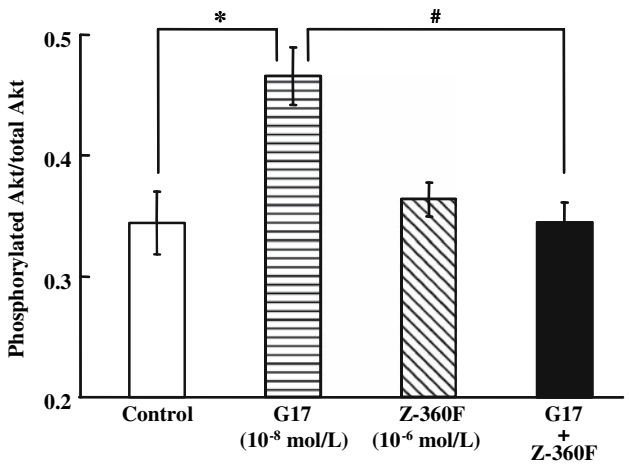


**Fig. 8** Inhibitory effect of Z-360F on gastrin-induced PKB/Akt phosphorylation in HT-29/CCK-2 cells. HT-29/CCK-2R cells ($1 \times 10^4$ cells) were treated with Z-360F ($10^{-6}$ mol/l) for 30 min, and then gastrin ($10^{-8}$ mol/l) was added to the cells. After 30 min of gastrin stimulation, phosphorylation of PKB/Akt was measured. Values represent the mean ± SE of triplicate determinations. ** $P < 0.01$ compared with the control (Dunnett's test). # $P < 0.05$ compared with gastrin alone (*t* test)

therefore this model was used to examine additive effects when combining Z-360 and gemcitabine. The combination of Z-360 and gemcitabine significantly inhibited tumor growth compared with Z-360 alone or gemcitabine alone in a subcutaneous PANC-1 model. Confirmation of an additive combination effect in a pancreatic cancer model with low sensitivity to a standard of care chemotherapeutic agent, such as gemcitabine, may relate to the gain of increased clinical benefit with gemcitabine in pancreatic caner patients through co-administration of Z-360.

Most notably, the combination of Z-360 and gemcitabine was able to prolong survival time compared with vehicle control in the orthotopic PANC-1 model. Although there have been several reports that CCK-2 receptor antagonists can inhibit the growth of pancreatic carcinoma [9, 32, 35], few studies have assessed the inhibitory effect of such agents combined with gemcitabine in a patient-relevant orthotopic survival model.

Gastrin is found in the fetal rat pancreas by radioimmunoassay, but its expression is not detected after birth. In adult rats, gastrin is mainly limited to the G-cells of the gastric antrum [4]. In fetal and neonatal human pancreatic tissues, progastrin was processed to amidated bioactive gastrin to a higher degree than in adult pancreatic tissue [2]. The average level of mRNA encoding the human CCK-2 receptor during fetal life was three or fourfold higher than observed in adults [31]. These observations support the idea of a trophic role of pancreatic gastrin in the early stages of development. However, gastrin has been identified in human pancreatic cancer cells and tissues, but not normal tissues, by radioimmunoassay and immunocytochemistry [32, 33, 35], suggesting that gastrin may be re-expressed in the pancreatic malignant state. Furthermore, treatment of pancreatic cancer cells with an antisense oligonucleotide for gastrin decreases proliferation, suggesting that the growth of these cells might be promoted via an autocrine mechanism [36].

CCK-2 receptor expression is also significantly increased in human pancreatic cancer cells compared with normal pancreatic tissue according to the results of a receptor binding assay using a selective CCK-2 receptor antagonist [33], although optimal measurement of CCK-2 receptors in pancreatic tissue can be problematic as gene expression levels are low [17], splice variants exist [20] and normal CCK-2 receptor-expressing cells, within the pancreatic acini, may lead to artefactual expression levels of the CCK-2 receptor in malignant pancreatic tissue [30]. We found that Z-360 shows a high selectivity for the human CCK-2 receptor compared with the CCK-1 receptor based on our radioligand binding assays. Z-360 also antagonised the human CCK-2 receptor in a functional assay involving measurement of intracellular $Ca^{2+}$ release. Moreover, Z-360 inhibited gastrin-induced proliferation of CHO-K1/CCK-2

cells. These findings suggest that Z-360 potently inhibits the activity of the CCK-2 receptor in pancreatic carcinoma, leading to suppression of tumor growth.

Gastrin has been confirmed as an anti-apoptotic factor acting via the CCK2 receptor [37]. An important downstream consequence of CCK-2 receptor activation is the phosphorylation/activation of PKB/Akt, which is known to promote cell survival by modifying expression of anti-apoptotic proteins [37] and in the present study, Z-360 blocked gastrin-induced PKB/Akt phosphorylation. In addition, Grabowska et al. have revealed that the combination of Z-360 and gemcitabine significantly enhanced the induction of apoptosis in pancreatic carcinoma in vivo compared with gemcitabine alone or Z-360 alone [18]. These results assumed that Z-360 may suppress the expression of anti-apoptotic factors induced by gastrin stimulation in CCK-2 receptor positive cells.

In the present study, we showed that Z-360 combined with gemcitabine can inhibit pancreatic tumor growth and prolong survival time in PANC-1 carcinoma xenograft model. Although the detailed mechanism of this combination effect was not determined, the therapeutic effect of Z-360 in vivo pancreatic cancer model may involve in inhibitory action of gastrin-induced tumor growth via inhibition of PKB/Akt phosphorylation. The elucidation mechanism of combination effect of Z-360 and gemcitabine needs further investigation.

Gemcitabine is well known nucleoside analogue with activity against several solid tumors. It is first-line drug showing clinical efficacy for pancreatic cancer. Survival rates for patients with pancreatic cancer are extremely poor. The disease is often resistant to chemotherapy or radiation therapy, and tends to spread quickly to other tissues of the body. Therefore, treatment of pancreatic cancer requires new drugs for use in combination with gemcitabine to enhance its efficacy. Our results suggest that Z-360 may be a useful adjunct to gemcitabine for the treatment of pancreatic carcinoma and may also be a therapeutic option for patients with advanced pancreatic cancer.

## References


1. Akagi K, Nagao T, Urushidani T (1999) Correlation between Ca2+ oscillation and cell proliferation via CCKB/gastrin receptor. Biochem Biophys Acta 1452:243–253
2. Bardram L, Hilsted L, Rehfeld JF (1990) Progastrin expression in mammalian pancreas. Proc.Natl Acad Sci USA 87:298–302
3. Bock MG, DiPardo RM, Evans BE et al (1989) Benzodiazepine gastrin and brain cholecystokinin receptor ligands: L-365,260. J Med Chem 32:13–16
4. Brand SJ, Fuller PJ (1988) Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development. J Biol Chem 263:5341–5347
5. Burris HA III, Moore MJ, Andersen J et al (1997) Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15:2403–2413
6. Cantley LC, Neel BG (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A 96:4240–4245
7. Caplin M, Savage K, Khan K et al (2000) Expression and processing of gastrin in pancreatic adenocarcinoma. Br J Surg 87:1035–1040
8. Chan TO, Rittenhouse SE, Tsichlis PN (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem 68:965–1014
9. Chau I, Cunningham D, Russell C et al (2006) Gastrazole (JB95008), a novel CCK2/gastrin receptor antagonist, in the treatment of advanced pancreatic cancer: results from two randomised controlled trials. Br J Cancer 94:1107–1115
10. Clerc P, Leung-Theung-Long S, Wang TC, Dockray GJ, Bouisson M, Delisle MB, Vaysse N, Pradayrol L, Fourmy D, Dufresne M (2002) Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferention of acinar cells, and neoplasia. Gastroenterology 122:428–437
11. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM (2000) Serine/threonine protein kinases and apoptosis. Exp Cell Res 256:34–41
12. Dehez S, Bierkamp C, Kowalski-Chauvel A, Daulhac L, Escrieut C, Susini C, Pradayrol L, Fourmy D, Seva C (2002) c-Jun NH2-terminal kinase pathway in growth-promoting effect of the G protein-coupled receptor cholecystokinin B receptor: A protein kinase C/Src-dependent-mechanism. Cell Growth Differ 13:375–385
13. Dufresne M, Seva C, Fourmy D (2006) Cholecystokinin and gastrin receptors. Physiol Rev 86:805–847
14. Fleming JB, Shen GL, Holloway SE, Davis M, Brekken RA (2005) Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: justification for K-ras-directed therapy. Mol Cancer Res3:413–423
15. Gilliam AD, Watson SA (2002) Emerging biological therapies for pancreatic carcinoma. Eur J Surg Oncol 28:370–378
16. Goetze JP, Nielsen FC, Burcharth F, Rehfeld JF (2000) Cloning the gastrin loop in pancreatic carcinoma. Cancer 88:2487–2494
17. Grabowska A, Clarke PA, Watson SA (2004) Development of a specific RT-PCR method to detect expression of CCK-2 receptor isoforms. Regul pept 122:A152
18. Grabowska AM, Morris TM, McKenzie AJ, Kumari R, Hamano H, Yoshinaga K, Emori Y, Watson SA (2007) Pre-clinical evaluation of a new orally active CCK-2 receptor antagonist, Z-360 in gastro-intestinal cancer models. Regul Pept (in press)
19. Hakanson R, Axelson J, Ihse I, Sundler F (1991) Trophic effects of gastrin and cholecystokinin. Z Gastroenterol Verh 26:265–269
20. Hellmich MR, Rui XL, Hellmich HL, Fleming RY, Evers BM, Townsend CM Jr (2000) Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates cell growth. J Biol Chem 275:32122–32128
21. Huang SC, Zhang L, Chiang HC et al (1989) Benzodiazepine analogues L365,260 and L364,718 as gastrin and pancreatic CCK receptor antagonists. Am J Physiol 257:G169–174
22. Ito M, Matsui T, Taniguchi T, Tsukamoto T, Murayama T, Arima N, Nakata H, Chiba T, Chihara K (1993) Functional characterization of a human brain cholecystokinin-B receptor. J Biol Chem 268:18300–18305
23. Jemal A, Siegel R, Ward E et al (2006) Cancer statistics, 2006. CA Cancer J Clin 56:106–130
24. Lallement JC, Oiry C, Lima-Leite AC, Lignon MF, Fulcrand P, Galleyrand JC, Martinez J (1995) Cholecystokinin and gastrin are not equally sensitive to GTP gamma S at CCKB receptors: Importance of the sulphated tyrosine. Eur J Pharmacol 290:61–67

25. Li D, Xie K, Wolff R, Abbruzzese JL (2004) Pancreatic cancer. Lancet 363:1049–1057
26. Mathieu A, Clerc P, Portolan G, Bierkamp C, Lulka H, Pradayrol L, Seva C, Fourmy D, Dufresne M (2005) Transgenic expression of CCK2 receptors sensitizes murine pancreatic acinar cells to carcinogen-induced preneoplastic lesions formation. Int J Cancer 115:46–54
27. Miura N, Yoneta T, Ukawa H, Fukuda Y, Eta R, Mera Y, Omata T, Kinomoto T, Kurimoto T, Ito Z (2001) Pharmacological profiles of Z-360, a novel CCK-B/Gastrin (CCK2) receptor antagonist with excellent oral potency. Gastroenterology 120:A311
28. Miyake A, Mochizuki S, Kawashima H (1994) Characterization of cloned human cholecystokinin-B receptor as gastrin receptor. Biochem Pharmacol 47:1339–1343
29. Rehfeld JF, Bardram L, Hilsted L (1989) Gastrin in human bronchogenic carcinomas: constant expression but variable processing of progastrin. Cancer Res 49:2840–2843
30. Reubi JC, Waser B, Gugger M et al (2003) Distribution of CCK1 and CCK2 receptors in normal and diseased human pancreatic tissue. Gastroenterology 125:98–106
31. Saillan-Barreau C, Dufresne M, Clerc P, Sanchez D, Corominola H, Moriscot C, Guy-Crotte O, Escrieut C, Vaysse N, Ramon G, Tarasova N, Fourmy D (1999) Evidence for a functional role of the cholecystokinin-B/gastrin receptor in the human fetal and adult pancreas. Diabetes 48:2015–2021
32. Smith JP, Fantaskey AP, Liu G, Zagon IS (1995) Identification of gastrin as a growth peptide in human pancreatic cancer. Am J Physiol 268(1 Pt 2):R135–R141
33. Smith JP, Hamory MW, Verderame MF, Zagon IS (1998) Quantitative analysis of gastrin mRNA and peptide in normal and cancerous human pancreas. Int J Mol Med 2:309–315
34. Smith JP, Liu G, Soundararajan V, McLaughlin PJ, Zagon IS (1994) Identification and characterization of CCK-B/gastrin receptors in human pancreatic cancer cell lines. Am J Physiol 266(1 Pt 2):R277–R283
35. Smith JP, Shih A, Wu Y, McLaughlin PJ, Zagon IS (1996) Gastrin regulates growth of human pancreatic cancer in a tonic and autocrine fashion. Am J Physiol 270(5 Pt 2):R1078–R1084
36. Smith JP, Verderame MF, Zagon IS (1999) Antisense oligonucleotides to gastrin inhibit growth of human pancreatic cancer. Cancer Lett 135:107–112
37. Todisco A, Ramamoorthy S, Witham T et al (2001) Molecular mechanisms for the antiapoptotic action of gastrin. Am J Physiol Gastrointest Liver Physiol 280:G298–G307
38. Watson SA, Clarke PA, Smith AM et al (1998) Expression of CCKB/gastrin receptor isoforms in gastro-intestinal tumor cells. Int J Cancer 77:572–577
39. Watson SA, Durrant LG, Morris DL (1988) Growth-promoting action of gastrin on human colonic and gastric tumor cells cultured in vitro. Br J Surg 75:342–345