Cancer Chemother Pharmacol (2005) 56: 510–520
DOI 10.1007/s00280-005-1028-x

ORIGINAL ARTICLE

**Ian S. Zagon · Jeffrey R. Jaglowski
Michael F. Verderame · Jill P. Smith
Alphonse E. Leure-duPree · Patricia J. McLaughlin**

Combination chemotherapy with gemcitabine and biotherapy with opioid growth factor (OGF) enhances the growth inhibition of pancreatic adenocarcinoma

Received: 30 November 2004 / Accepted: 7 February 2005 / Published online: 10 June 2005

Abstract Gemcitabine is the standard of care for advanced pancreatic neoplasia, and exerts its effect through inhibition of DNA synthesis. However, gemcitabine has limited survival benefits. Opioid growth factor (OGF) is an autocrine-produced peptide that interacts with the nuclear receptor, OGFr, to inhibit cell proliferation but is not cytotoxic or apoptotic. The present study was designed to examine whether a combination of chemotherapy with gemcitabine and biotherapy with OGF is more effective than either agent alone in inhibiting pancreatic cancer growth in vitro and in vivo. The combination of OGF (10^{-6} M) and gemcitabine (10^{-8} M) reduced MIA PaCa-2 cell number from control levels by 46% within 48 h, and resulted in a growth inhibition greater than that of the individual compounds. OGF in combination with 5-fluorouracil also depressed cell growth more than either agent alone. The action of OGF, but not gemcitabine, was mediated by a naloxone-sensitive receptor, and was completely reversible. OGF, but no other endogenous or exogenous opioids, altered pancreatic cancer growth in tissue culture. The combination of OGF and gemcitabine also repressed the growth of another pancreatic cancer cell line, PANC-1. MIA PaCa-2 cells transplanted into athymic mice received 10 mg/kg OGF daily, 120 mg/kg gemcitabine every 3 days; 10 mg/kg OGF daily and 120 mg/kg gemcitabine every 3rd day, or 0.1 ml of sterile saline daily. Tumor incidence, and latency times to tumor appearance, of mice receiving combined therapy with OGF and gemcitabine, were significantly decreased from those of the control, OGF, and gemcitabine groups. Tumor volumes in the OGF, gemcitabine, and OGF/gemcitabine groups were markedly decreased from controls beginning on days 14, 12, and 8, respectively, after tumor cell inoculation. Tumor weight and tumor volume were reduced from control levels by 36–85% in the OGF and/or gemcitabine groups on day 45 (date of termination), and the group of mice exposed to a combination of OGF and gemcitabine had decreases in tumor size of 70% and 63% from the OGF or the gemcitabine alone groups, respectively. This preclinical evidence shows that combined chemotherapy (e.g. gemcitabine) and biotherapy (OGF) provides an enhanced therapeutic benefit for pancreatic cancer.

Keywords [Met^5]-enkephalin · Opioid growth factor · Neoplasia · Gemcitabine · 5-Fluorouracil · Pancreatic cancer

I. S. Zagon (✉) · J. R. Jaglowski · A. E. Leure-duPree
P. J. McLaughlin
Department of Neural and Behavioral Sciences, H109,
Milton S. Hershey Medical Center,
The Pennsylvania State University, 500 University Drive,
Room C3729, Hershey, PA 17033, USA
E-mail: isz1@psu.edu
Tel.: +717-531-6409
Fax: +717-531-5003

M. F. Verderame · J. P. Smith
Department of Medicine, H109,
Milton S. Hershey Medical Center,
The Pennsylvania State University,
Hershey, PA 17033, USA

Introduction

Pancreatic cancer accounts for approximately 2% of new cases, and with a 98% mortality rate accounts for 6% of cancer deaths annually in the United States [1]. Globally, pancreatic cancer occurs in more than 168,000 individuals each year, and is the 9th leading cause of mortality from cancer [2]. The 5-year survival rate in the United States is 4% for patients with cancer of the pancreas and has not changed appreciably in the last 4 decades [1]. With a median survival of less than 6 months, a diagnosis of pancreatic cancer carries one of the most dismal prognoses in all of medicine [3]. The problem of treating pancreatic cancer is compounded by a lack of early detection tools, resulting in 80–90% of the patients having unresectable cancer at the time of initial diagnosis [4, 5].

Chemotherapy for advanced pancreatic cancer is palliative and not curative for metastatic disease [5, 6]. Gemcitabine (2′-deoxy-2′, 2′-difluorocytidine monohydrochloride; Gemzar, Eli Lilly and Co., Indianapolis, IN, USA) is currently the standard of care for metastatic pancreatic cancer [5–8]. Gemcitabine is a nucleoside (deoxycytidine) analog with structural and metabolic similarities to cytarabine. As a prodrug, gemcitabine is phosphorylated intracellularly to both diphosphate and triphosphate derivatives by deoxycytidine kinase. The diphosphate form inhibits ribonucleotide reductase and depletes the intracellular pools of deoxynucleotide triphosphates needed for DNA synthesis. The triphosphate form may be incorporated into an elongating DNA chain and leads to premature chain termination, as well as impeding normal DNA repair [9]. Gemcitabine is also self-potentiating, resulting in greater intracellular concentrations than other nucleoside analogs [10]. In a perspective multicenter randomized trial comparing gemcitabine and 5-fluorouracil (5-FU), Burris and colleagues [11] found that gemcitabine improved survival slightly compared to 5-FU treated patients. Clinically meaningful effects on disease-related symptoms (pain, weight, performance status) were seen more often in gemcitabine-exposed patients than those receiving 5-FU. Thus, despite limited patient benefits, gemcitabine is now accepted as a standard systemic chemotherapy for advanced pancreatic cancer. For added efficacy, gemcitabine has been used in combination with other cytotoxic agents in more recent studies (e.g. docetaxel, cisplatin) [12, 13].

[Met^5]-enkephalin, termed opioid growth factor (OGF), is an endogenous opioid peptide that has been shown to be an important regulator of the growth of pancreatic cancer [14–17]. OGF is a constitutively expressed native opioid that interacts with the OGF receptor (OGFr) to inhibit the growth of pancreatic cancer in vivo and in vitro [14–16, 18], and inhibits anchorage-independent growth of human pancreatic cancer [14]. The action of OGF is tonic, stereo-specific, reversible, non-cytotoxic, non-apoptotic inducing, independent of serum, and occurs at physiologically relevant concentrations in a wide variety of pancreatic cancer cells that includes poorly and well-differentiated human cell lines [16, 19]. The only opioid peptide, natural or synthetic, that influences the growth of pancreatic cancer cells is OGF [16]. The action of this opioid in these neoplasias is targeted to DNA synthesis and is directed toward the G_0/G_1 interface of the cell cycle [17]. Gene expression and protein expression of OGF and OGFr, as well as binding activity of OGFr, have been identified and characterized in pancreatic cancer [15, 16, 18, 20]. Exogenous administration of OGF has a profound antitumor action on xenografts of pancreatic cancer that includes delaying tumor appearance and reducing tumor size [15]. OGF has been utilized in patients with advanced pancreatic cancer, and has successfully completed Phase I clinical trials [21]; Phase II trials for advanced-stage pancreatic cancer are now being conducted (personal communication).

Given the promising nature of OGF (biotherapy) as an antitumor agent in pancreatic cancer, and the lack of preclinical data regarding the simultaneous use of OGF with a chemotherapeutic agent (gemcitabine), the present study was designed to explore the therapeutic potential of a combination of these modalities. Using a tissue culture model of human pancreatic adenocarcinoma, the effect of concomitant exposure to both OGF and gemcitabine was characterized on growth (e.g. reversibility, receptor mediation, specificity). The relationship of another chemotherapy treatment (i.e. 5-FU) and OGF in regard to pancreatic cancer, as well as the ubiquity of combined therapy on other pancreatic cancer cell lines, were evaluated. Finally, the present report addresses the question of whether a combination of OGF and gemcitabine influences growth of human pancreatic cancer in vivo, and does so beyond the efficacy of each compound. The effects of OGF and/or gemcitabine on tumor incidence, appearance, and size, as well as metastasis, were examined in a xenograft model of pancreatic cancer.

Material and methods

Cell lines

Two human pancreatic adenocarcinoma cancer cell lines: MIA PaCa-2 and PANC-1, were chosen for study. Both of these cell lines: i) are markedly depressed in growth by OGF [16], ii) have OGF receptor binding [18], iii) express the OGFr gene [20], iv) demonstrate anchorage independent effects of OGF [14], and v) exhibit OGF and OGFr immunoreactivity [16]. The cell lines were purchased from the American Type Culture Collection (Manasass, VA, USA). MIA PaCa-2 cells were derived from an undifferentiated epithelial carcinoma occurring in the body and tail of the pancreas in a 65-year-old man [22]. The PANC-1 cells were derived from an undifferentiated carcinoma from the head of the pancreas in a 56-year-old man [23]. MIA PaCa-2 and PANC-1 cells were grown in Dulbecco's MEM (modified) media; media was supplemented with 10% fetal calf serum, 1.2% sodium bicarbonate, and antibiotics (5,000 Units/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin), and the cells were maintained in a humidified atmosphere of 7% CO_2/93% air at 37°C.

Growth assays

MIA PaCa-2 cells were seeded at equivalent amounts into either 75 cm^2 flasks, 6-well plates, or 96-well plates (Falcon) and counted 24 h later to determine plating efficiency. Growth assays for PANC-1 cells were conducted in 6-well plates (Falcon). Compounds or sterile

water were added beginning 24 h after seeding (=0 h), and both media and compounds were replaced daily. For cultures receiving OGF and gemcitabine or 5-FU, drugs were added to the cells simultaneously. All drugs were prepared in sterile water and dilutions represent final concentrations of the compounds.

Cell number was recorded either by using a mitogenic bioassay, the MTS assay (Cell Titer 96 One Solution, Promega, Madison, WI, USA), and measuring absorbency after 4 h on a Biorad (Model 3550) plate reader at 490 nm, or by counting cells. For manual counts, cells were harvested with a solution of 0.25% trypsin/0.53 mM EDTA, centrifuged, and counted with a hemacytometer. Cell viability was determined by trypan blue staining. At least two aliquots per flask or 4–10 wells/treatment were counted each time.

Apoptosis

Caspase-3-FITC positive staining was used to characterize early stages of apoptosis [24]. MIA PaCa-2 cells were seeded into 6-well plates and treated with drugs beginning 24 h later; drugs and media were replaced daily. Cells were harvested after 1, 3 and 6 days of drug treatment, and prepared according to the manufacturer's recommendations for FACS analysis (FACS cell sorter with a 15 mW argon ion laser at 488 nm; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For caspase-3 identification, the APO-ACTIVE 3 antibody detection kit (Cell Technology, Mountain View, CA, USA) was used. Three samples from each treatment were analyzed at each time point. The gated percent which the cells recorded by flow cytometry was considered caspase positive.

Animals and tumor cell implantation

Male 4-week-old BALB/c-nu/nu nude mice purchased from Harlan Laboratories (Indianapolis, IN, USA) were housed in pathogen-free isolators in the Department of Comparative Medicine at the Penn State University College of Medicine. All procedures were approved by the IACUC committee of the Penn State University College of Medicine and conformed to the guidelines established by NIH. Mice were allowed 48 h to acclimate prior to the beginning of experimentation.

MIA PaCa-2 cells (10^6 cells/mouse) were inoculated into nude mice by subcutaneous injection into the right scapular region; mice were not anesthetized for this procedure.

Drug treatment

Four groups of mice ($n=12$) were randomly assigned to receive intraperitoneal injections of 10 mg/kg OGF daily, 120 mg/kg gemcitabine every 3 days; 10 mg/kg OGF daily and 120 mg/kg gemcitabine every 3rd day, or 0.1 ml of sterile saline daily [15, 25]. On days when both drugs were given together, OGF was administered first and followed immediately by gemcitabine. All drugs were dissolved in saline and prepared weekly. Initial injections were given within 1 h of tumor cell inoculation.

Tumor growth and metastases

Mice were weighed weekly throughout the experiment, and observed daily for the presence of tumors. The latency for a visible tumor to appear, and the time until tumors were measurable (i.e. 62.5 mm^3) were recorded. Tumors were measured using calipers every day after tumor appearance. Tumor volume was calculated using the formula $w^2 \times l \times \pi/6$, where the length is the longest dimension, and width is the dimension perpendicular to length [26].

Termination day measurements

According to IACUC guidelines, the mice were terminated when tumors became ulcerated, or tumors grew to 2 cm in diameter. Forty-five days following tumor cell inoculation, all mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and killed by cervical dislocation; mice (with tumors) were weighed. Tumors and spleens were removed and weighed, and the lymph nodes, liver, and spleen were examined for metastases.

Plasma levels of [Met^5]-enkephalin (OGF)

At the time of termination (i.e. 24 h after the last drug injection), trunk blood was collected from some mice in each group. Plasma was separated and OGF levels were measured by standard radioimmunoassay procedures using a [Met^5]-enkephalin kit from Peninsula Laboratories (Belmont, CA, USA).

Chemicals

The following compounds were obtained from Sigma Chemicals (St. Louis, MO, USA): [Met^5]-enkephalin (OGF), [D-$Pen^{2,5}$]-enkephalin (DPDPE), [D-Ala^2, Me-Phe^4, $Glyol^5$]-enkephalin (DAMGO), β-endorphin, naltrexone (NTX), naloxone, dynorphin A1-8, [D-Ala-D-Leu-enkephalin] (DADLE), morphine, endomorphin-1, and endomorphin-2.

Data analysis

Cell numbers and/or absorbencies were analyzed using analysis of variance (ANOVA) (one- or two-factor

where appropriate) with subsequent comparisons made using Newman–Keuls tests. Incidence of tumors was analyzed by Chi-square tests. Latency for tumor appearance and tumor volume were analyzed using either two-tailed *t*-tests or ANOVA with subsequent comparisons made using Newman–Keuls tests. Termination data (i.e. body weight, tumor weight, spleen weight) and OGF plasma levels were compared by ANOVA.

Results

Growth assays with OGF and/or gemcitabine

Growth curves for MIA PaCa-2 cell cultures treated with 10^{-6} M OGF (a dosage known to inhibit proliferation of MIA PaCa-2 cells) [16], 10^{-8} M gemcitabine (a dosage selected because our preliminary experiments revealed no logarithmic growth with a dosage of 10^{-7} M; also see [27, 28], 10^{-8} M gemcitabine and 10^{-6} M OGF, or sterile water (controls) are presented in Fig. 1. OGF alone inhibited growth at 48, 72, and 96 h relative to controls, with decreases in cell number of 16%, 18%, and 17%, respectively, noted. Gemcitabine alone decreased cell number relative to controls at 48, 72, and 96 h by 22%, 21%, and 32%, respectively. Cells treated with a combination of OGF and gemcitabine were decreased in number relative to controls by 26%, 49%, and 46% at 48, 72, and 96 h, respectively. At 72 h, cell number in cultures receiving the combined therapy of gemcitabine and OGF was reduced ($P < 0.001$) from that of cells exposed only to OGF or gemcitabine by 38% and 36%, respectively. Moreover, at 96 h, the combined therapy of gemcitabine and OGF reduced ($P < 0.001$) MIA PaCa-2 cell number by 35% and 20% from that of cultures receiving only OGF or gemcitabine, respectively.

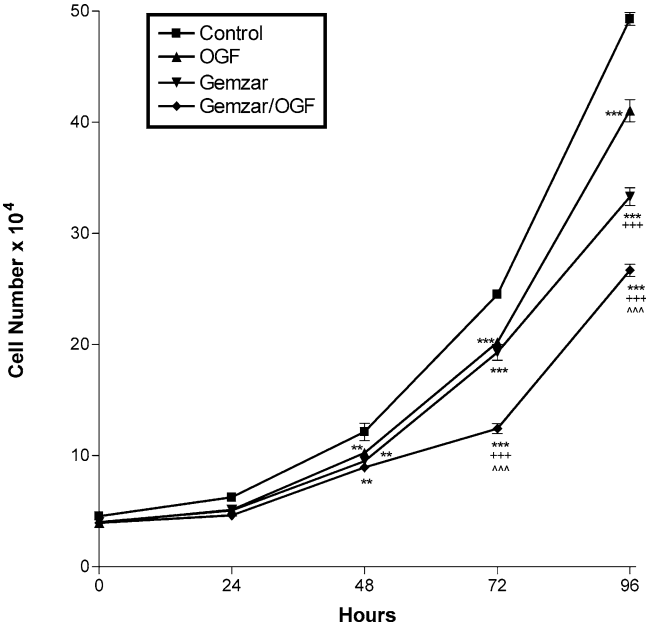

Fig. 1 Cell proliferation assays of MIA PaCa-2 cells subjected to OGF (10^{-6} M) and/or gemcitabine (10^{-8}) (Gemzar) for 96 h. Drugs or an equivalent volume of sterile water (controls) were added 24 h (0 h) after seeding in 6-well plates; media and drugs were replaced daily. Data represent means ± SEM for at least 4 wells/treatment at each time point. Significantly different from controls at $P < 0.01$ (**) and $P < 0.001$ (***). Significantly different from OGF-treated cultures at $P < 0.001$ (+ + +). Significantly different from cultures treated with gemcitabine alone at $P < 0.001$ (^^^)

Growth assays with 5-fluorouracil

To examine whether OGF could enhance the inhibitory effects of other chemotherapies commonly used to treat pancreatic cancer, MIA PaCa-2 cell cultures were exposed to 5-fluorouracil (5-FU) at a concentration of 10^{-6} M for 4 days (Fig. 2); this dosage was selected based on the report of Shi et al. [28]. MIA PaCa-2 cell number in the 5-FU group was reduced from 11% to 15% from control levels at 48, 72, and 96 h. Combination therapy of 5-FU (10^{-6} M) and OGF (10^{-6} M) reduced cell number from control values at 24, 48, 72, and 96 h by 13–30%. At all time points examined, the combined therapy of 5-FU and OGF reduced MIA PaCa-2 cell number by 6–19% from cultures receiving only OGF, and 10–17% from cultures receiving only 5-FU.

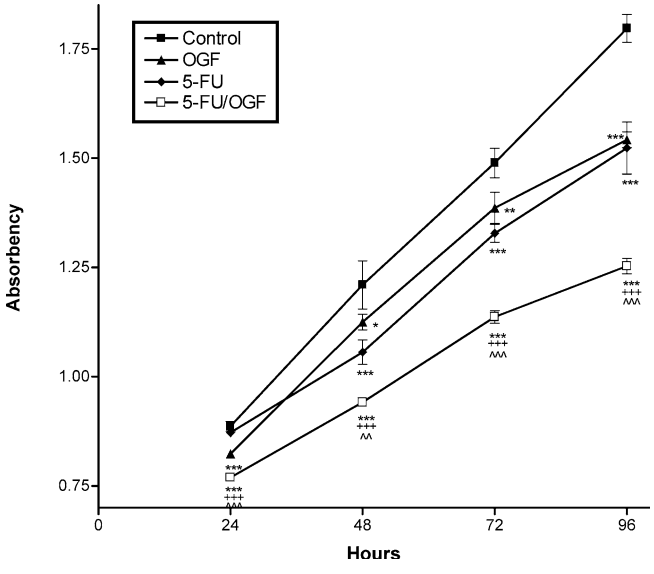

Fig. 2 Growth of MIA PaCa-2 cells treated with 5-FU (10^{-6} M) and/or OGF (10^{-6} M) as measured by the MTS assay (96-well plates). Values represent mean absorbencies ± SEM for 10 wells at each time point. Significantly different from controls at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Significantly different from OGF-treated cultures at $P < 0.001$ (+ + +). Significantly different from 5-FU-treated cultures at $P < 0.01$ (^^) and $P < 0.001$ (^^^)

Receptor mediated effects of OGF and/or gemcitabine

To inquire whether OGF activity was mediated by the OGF receptor, a short-acting opioid antagonist, naloxone, was added at a dosage of 10^{-6} M into cultures receiving 10^{-6} M OGF and/or gemcitabine (10^{-8} M). MIA PaCa-2 cells grown in 96-well plates were treated with 10^{-6} M OGF, 10^{-6} M naloxone, 10^{-8} M gemcitabine, or combinations at the same concentrations: OGF/naloxone, gemcitabine/naloxone, gemcitabine/OGF, and gemcitabine/OGF/naloxone; control cultures received sterile water. Individual plates were read at 96 h after drug addition. Relative to control levels, addition of OGF, gemcitabine, gemcitabine/OGF, and gemcitabine/OGF/naloxone inhibited cell growth from 13–36% (Fig. 3). Addition of naloxone completely blocked the growth inhibitory effects of OGF alone, but had no effect on the growth inhibitory action of gemcitabine alone. Moreover, naloxone partially neutralized the enhanced inhibitory effect of the combination of gemcitabine and OGF; cell number of the gemcitabine/OGF/naloxone group was comparable to that of cells exposed to gemcitabine, but were significantly reduced from control levels. Naloxone alone had no effect on the growth of MIA PaCa-2 cells.

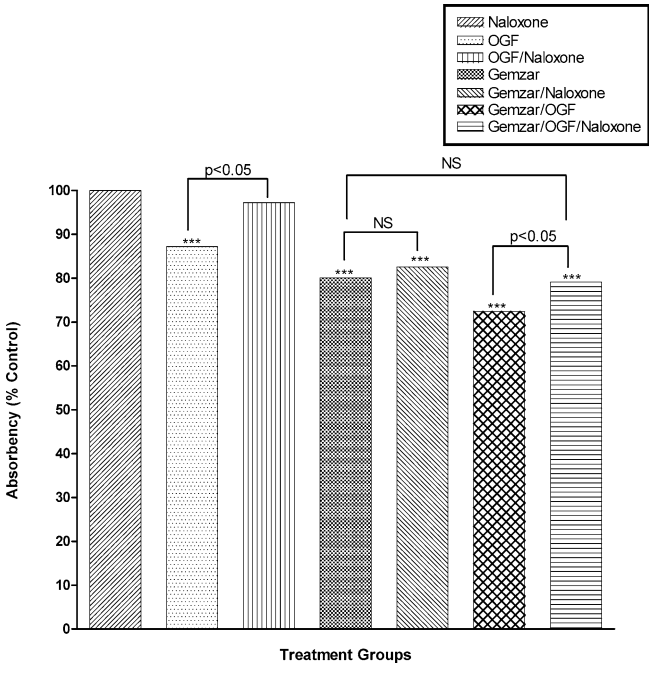

Fig. 3 Receptor mediation of the growth inhibitory effects of gemcitabine and/or OGF in MIA PaCa-2 cells. The number of MIA PaCa-2 cells at 96 h as measured by the MTS assay after being subjected to OGF (10^{-6} M), the opioid antagonist naloxone (10^{-6} M), gemcitabine (Gemzar) (10^{-8} M), or combinations of these compounds; controls were treated with an equivalent volume of sterile water. Compounds and media were replaced every 24 h. Data represent absorbency expressed as percentage of control for 10 wells/treatment at 96 h. Significantly different from controls at $P < 0.001$ (***). NS = not significant

Reversibility of the inhibitory growth effects of OGF and/or gemcitabine

To establish whether the effect of OGF and/or gemcitabine on cell number could be reversed by withdrawing cells from drug exposure, cultures of MIA PaCa-2 cells were exposed for 48 h to 10^{-6} M OGF and/or 10^{-8} M gemcitabine. At 48 h after drug exposure, half of the plates had their media removed and fresh media added with no addition of OGF or gemcitabine (i.e. OGF-reversal; gemcitabine-reversal; gemcitabine/OGF-reversal groups); some cultures continued to receive new media and drugs. At 96 h (i.e. 48 h after reversal), the OGF, gemcitabine, gemcitabine-reversal, gemcitabine/OGF, and the gemcitabine/OGF-reversal groups differed from controls by 21–46% (Fig. 4a, b). The OGF-reversal group had 16% more cells than in the OGF group continuing with OGF exposure. However, the gemcitabine-reversal group did not differ from cell cultures continuing to be treated with gemcitabine. Cell cultures exposed to the combination of OGF and gemcitabine had 7% fewer cells than cultures in the gemcitabine/OGF-reversal group.

Specificity of opioid peptide(s) related to pancreatic cancer cell growth

To determine whether other opioid peptide(s) is(are) related to growth, MIA PaCa-2 cultures (1,000 cells/well) were treated daily with 10^{-6} M concentrations of a variety of natural and synthetic opioid ligands. In some cases, these ligands were specific for other opioid receptors (e.g. μ, δ, or κ receptors). Drugs included OGF, DAMGO, morphine, DPDPE, DADLE, dynorphin A1-8, endomorphin-1, endomorphin-2, and β-endorphin. Cell number was measured on a plate reader after 96 h of treatment (both drug and media were changed daily). OGF inhibited cell number by 16% relative to controls; none of the other drugs utilized had any inhibitory or stimulatory effect on growth (Fig. 5).

Programmed cell death and necrosis

No differences in necrosis could be observed from the analysis of the number of trypan blue positive cells in cultures or supernatants of control cells and those treated with OGF and/or gemcitabine. Using flow cytometry to analyze caspase-3 activity, no differences in the percentages of caspase-3 positive cells were found in any treatment group (i.e. OGF and/or gemcitabine) at 1, 3, or 6 days. The gated percentages of caspase-3 positive MIA PaCa-2 cells for all four groups ranged from 0.94–1.99% on day 1, 0.62–1.66% on day 3, and 0.53–1.00% on day 6.

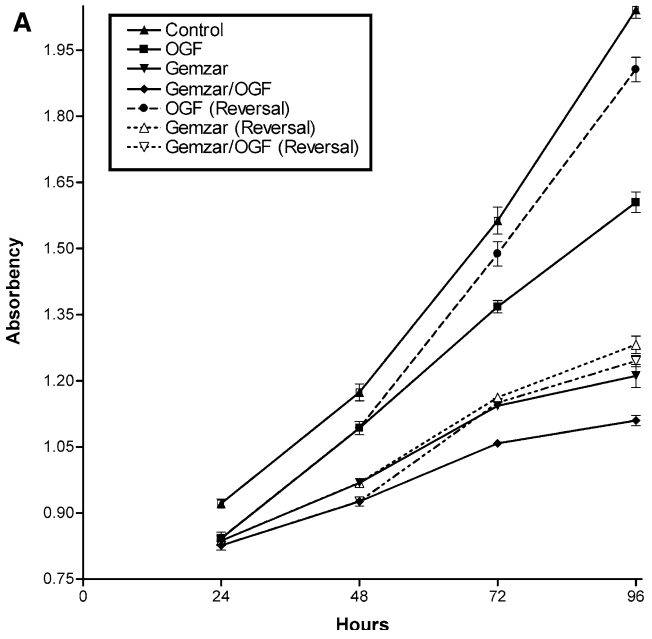

Fig. 4 Reversibility of the growth inhibitory effects on MIA PaCa-2 cells treated with OGF and/or gemcitabine (Gemzar). Cells were seeded into 96-well plates and treated with drugs for 48 h. At this time, half of the plates continued to receive the same drugs for an additional 48 h, and half of the plates were treated with sterile water for 48 h. Control cultures received sterile water throughout the 96 h. Compounds and media were replaced daily. **a** Growth of cells in the reversibility experiments. **b** Cell number at 96 h in the treatment groups. All data represent mean absorbency ± SEM for 10 wells/treatment. Comparisons between cell numbers of cultures maintained with drugs or cultures with drugs replaced by vehicle (reversal) are presented. NS = not significant

Ubiquity of growth inhibition by OGF

To determine whether the growth inhibition observed with MIA PaCa-2 cells following exposure to the combination of gemcitabine and OGF was not a cell-line specific action, another human pancreatic cancer cell line, PANC-1, was tested. After 72 h, exposure of PANC-1 cells to either OGF (10^{-6} M), gemcitabine (10^{-8} M), OGF (10^{-6} M) and gemcitabine (10^{-8} M) revealed 31%, 31%, and 54%, respectively, fewer cells than in control cultures (Fig. 6). These differences in cell growth with exposure to OGF and/or gemcitabine differed significantly ($P < 0.001$) from control levels, and the combination of OGF and gemcitabine differed from the OGF alone and the gemcitabine alone cultures at $P < 0.01$.

MIA PaCa-2 tumor appearance and growth

To investigate the effects of OGF and/or gemcitabine on pancreatic tumor growth in vivo, nude mice were injected with MIA PaCa-2 cells and treated with drugs. On day 10, when 80% of the mice in the saline-injected control group had measurable tumors, and 60% of the OGF and 75% of the gemcitabine-treated animals had

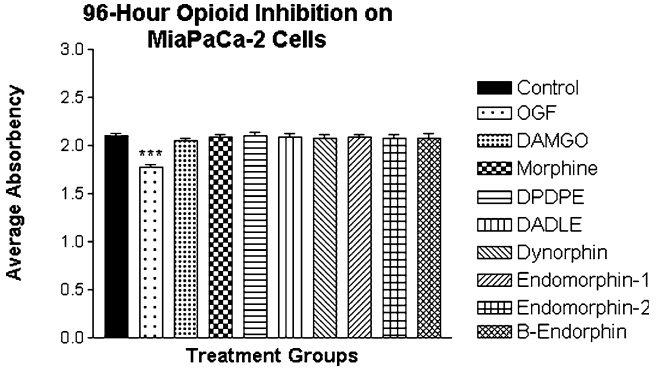

Fig. 5 Growth of MIA PaCa-2 cells grown in 96-well plates treated with a variety of endogenous and exogenous opioids at a concentration of 10^{-6} M. Data represent mean absorbency values ± SEM for 10 wells/treatment. Significantly different from controls at $P < 0.001$ (***)

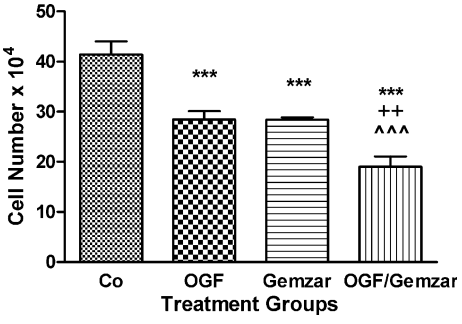

Fig. 6 Effects of gemcitabine (10^{-8} M) (Gemzar) and/or OGF (10^{-6} M) on PANC-1 cells grown in 6-well plates. Data represent means ± SEM for 4 wells at 72 h of treatment. Significantly different from controls at $P < 0.001$ (***), from OGF at $P < 0.01$ (++), and from the respective dosages of gemcitabine at $P < 0.001$ (^^^)

Table 1 Incidence and latency for tumor appearance of MIA PaCa-2 pancreatic carcinoma cells in nude mice treated with OGF and/or gemcitabine (Gemzar)

Parameter	Control	OGF	Gemzar	Gemzar/OGF
N	10	10	12	12
Tumor incidence, day 10	8/10	6/10	9/12	0/10[a]
Tumor incidence, day 16	10/10	10/10	11/12	9/12
Latency to visible tumor, d	10.1 ± 1.8	10.7 ± 0.8	11.1 ± 1.1	16.2 ± 1.2*
Latency to measurable tumor, d	13.2 ± 1.8	14.2 ± 0.8	13.1 ± 1.0	19.5 ± 1.1*

Values represent means ± SEM
[a] Significantly different from every group by Chi-square analyses at $P < 0.001$
Significantly different from controls at $P < 0.05$ (*) using ANOVA

tumors, no mouse in the gemcitabine/OGF group had a measurable tumor; the group receiving combination therapy of gemcitabine and OGF differed significantly from all other groups at $P < 0.001$ (Table 1). On day 16, no differences in the incidence of measurable tumors could be detected between groups, and all animals had a tumor by day 17. The latency time for the appearance of a visible tumor in mice of the gemcitabine/OGF group was delayed by approximately 5–6 days from animals in the control, OGF, and gemcitabine groups; this delay for the gemcitabine/OGF group differed significantly from that of all other groups at $P < 0.05$. The mean latency time for measurable tumor appearance in mice of the gemcitabine/OGF group was delayed ($P < 0.05$) by approximately 6 days from animals in the control, OGF, and gemcitabine groups.

Changes in tumor volume over the 45 days of the experiment were analyzed (Fig. 7a, b). The OGF, gemcitabine, and gemcitabine/OGF groups all differed (at least $P < 0.05$) from controls in tumor volume beginning on day 14. Tumor volumes of mice receiving combined therapy (i.e. gemcitabine/OGF) differed ($P < 0.05$) from mice treated with only OGF beginning on day 10, and from gemcitabine alone beginning on day 35. Differences in tumor volumes between groups persisted through the remainder of the experimental period. Rates of growth over the 45-day period of time were analyzed and presented in Fig. 7b. The results demonstrated that the growth rates of tumors for all three treatment groups were markedly reduced ($P < 0.001$) from control levels. Moreover, the rate of growth of tumors in mice treated with a combination of gemcitabine and OGF were significantly decreased ($P < 0.001$) from both the OGF alone and the gemcitabine alone groups.

At the time of termination (i.e. day 45), body weights of all groups of mice did not differ as from statistical evaluation (Table 2). Moreover, autopsy of the animals in each group did not reveal any metastases. However, the weight of the spleen on day 45 for mice in the gemcitabine alone and the gemcitabine/OGF groups

Fig. 7 Growth of MIA PaCa-2 tumors xenografted into nude mice. Animals were injected with either 10 mg/kg OGF daily, 120 mg/kg gemcitabine every 3 days (Gemzar); 10 mg/kg OGF daily and 120 mg/kg gemcitabine every 3rd day (Gemzar/OGF), or 0.1 ml of sterile saline daily (Control). **a** Tumor volumes monitored for the 45 days of the experiment. Values represent means ± SEM for all mice in the group (see Results for statistical comparisons). **b** Rates of tumor growth for the 45-day experimental period. Tumor volumes were log-transformed and slopes of the lines were calculated. Significantly different from controls at $P < 0.001$(***), from OGF at $P < 0.001$ (+++), and from gemcitabine at $P < 0.001$ (^^^)

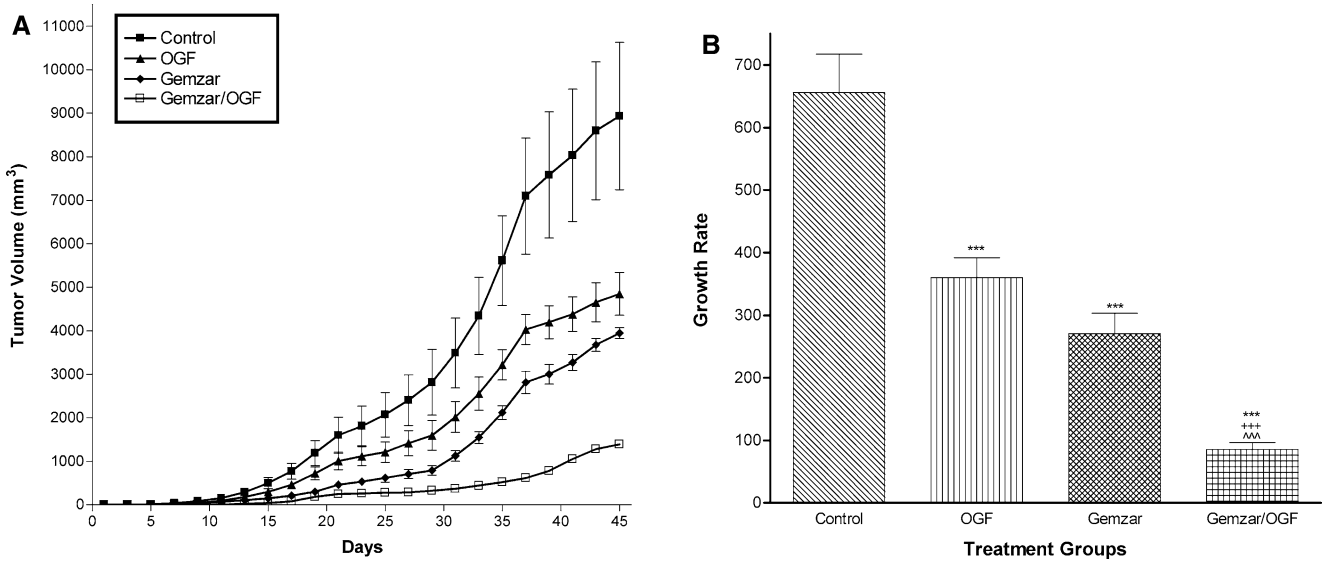

Table 2 Characteristics of nude mice 45 days after subcutaneous inoculation of MIA PaCa-2 pancreatic cancer cells and treatment with OGF and/or gemcitabine (Gemzar)

Parameter	Controls	OGF	Gemzar	Gemzar/OGF
Body weight, g	33.3 ± 1.0	31.4 ± 1.6	27.4 ± 0.55	30.6 ± 0.7
Tumor weight, g	5.5 ± 1.0	3.5 ± 0.5*	2.4 ± 0.1***	0.8 ± 0.1***[bc]
Tumor volume, mm^3	8935 ± 1694	4849 ± 490***	3963 ± 123***	1477 ± 53***[ac]
Spleen weight, mg	761 ± 61	606 ± 121	454 ± 49*	437 ± 62*

Data represent means ± SEM
Significantly different from controls at $*P < 0.05$ and $***P < 0.001$
[a]Significantly different from OGF group at $P < 0.05$
[b]Significantly different from OGF group at $P < 0.01$
[c]Significantly different from the Gemzar-treated mice at $P < 0.05$

were decreased approximately 40% from control values; no changes in spleen weight of the OGF group in comparison to control levels were noted (Table 2). The weights of tumors on the termination day for the OGF alone, gemcitabine alone, and gemcitabine/OGF groups were decreased 36%, 56%, and 85%, respectively, from control levels (Table 2). Tumor volumes on day 45 for the OGF alone, gemcitabine alone, and gemcitabine/OGF groups were decreased 46%, 56%, and 83%, respectively, from control values (Table 2).

Plasma levels of OGF

OGF levels in the plasma of nude mice bearing MIA PaCa-2 tumors ranged from 129–289 pg/ml. No differences were noted between control mice and those treated with OGF alone, gemcitabine alone, or gemcitabine/OGF.

Discussion

The results in this study demonstrate that the combination of OGF and gemcitabine has a potent inhibitory effect on growth in vitro of at least two cell lines of human pancreatic cancer. The antigrowth action of the combination of OGF and gemcitabine was always greater than the individual drugs. In a number of instances the effect of the combination of drugs exceeded that of the sum of the individual drugs, suggesting that the action of a combination of OGF and gemcitabine was supra-additive. The repressive effects on growth in vitro of pancreatic cancer cells observed with OGF and with gemcitabine individually were consonant with previous results [e.g. 16, 29, 30]. The action of OGF on cell growth was mediated by a naloxone-sensitive receptor. This naloxone-sensitive receptor is presumed to be OGFr because synthetic and natural opioids selective for classical opioid receptors such as μ, δ, and κ did not influence growth of pancreatic cancer cells in the present report and earlier [16]. OGF also was discovered to have a reversible action on the replication of MIA PaCa-2 cells, supporting the results from earlier studies showing that treatment with this compound does not lead to cytotoxicity or cell death [16, 19]. On the other hand, the effects of gemcitabine on MIA PaCa-2 cells were neither blocked by naloxone nor could they be reversed, indicating that the characteristics of this drug's effects on MIA PaCa-2 cells are markedly different from that of OGF. Thus, this is the first report of the efficacy of using a combination of the biotherapeutic agent, OGF, and the chemotherapeutic agent, gemcitabine, to retard the growth of human pancreatic cancer.

Although this report concentrated on the effects of OGF and gemcitabine on MIA PaCa-2 cells, it is known that OGF, and gemcitabine, influence the growth of a variety of human pancreatic cancer cell lines [16, 29, 30]. The present investigation demonstrates that not only does OGF and gemcitabine in combination rather than individually have a more marked effect on MIA PaCa-2 cell growth, but a similar pattern can be found with another human pancreatic cancer cell line, PANC-1. Thus, it might be reasonable to conclude that the effects of combination therapy with OGF and gemcitabine observed herein also extend to other human pancreatic cancer cell lines.

To address the question of whether OGF could be combined with chemotherapeutic agents other than gemcitabine, a preliminary study was conducted with the combination of OGF and 5-FU. This allowed a contrast between an antimetabolite (5-FU) and a cytosine analog [31]. The mechanism of 5-FU, a pyrimidine analog, is to inhibit thymidylate synthase (an enzyme involved in de novo synthesis of pyrimidines) by the active metabolite 5-fluoro-deoxyuridine-monophosphate. In addition, the active triphosphate metabolites, 5-fluoro-deoxyuridine-triphosphate and 5-fluoro-uridine-triphosphate, disrupt nucleic acid functions [32]. The present results are the first to show that the effects of a combination of 5-FU and OGF has potent inhibitory properties with respect to human pancreatic cancer. As in the case of gemcitabine and OGF, the effect of a combination of 5-FU and OGF on pancreatic cancer cells was markedly greater than that of each drug and was often additive in nature. Presumably, these results would indicate that OGF could be used in combination with a variety of chemotherapeutic agents.

Although the combination of OGF and gemcitabine markedly delayed the growth of human pancreatic

cancer cells in tissue culture, the efficacy of this therapy on pancreatic tumor growth in vivo required documentation. The results of this study show that the antigrowth properties of OGF and gemcitabine are enhanced beyond the inhibitory effects of each drug alone. These data were most evident for tumor incidence, latency to a visible or measurable tumor, tumor weight, and tumor volume. Thus, the results of in vivo studies are consonant with observations conducted in vitro. Even though the tumor transplantation investigation focused on one human pancreatic cancer cell line, it is known that OGF or gemcitabine influences the growth of a variety of human pancreatic cancer cell lines in vivo [15, 30, 33, 34]. Therefore, the effects of combination therapy with OGF and gemcitabine shown in this study may extend to other human pancreatic cancer cell lines in vivo.

The mechanism of enhanced antitumor activity by a combination of OGF and gemcitabine in our studies is not related to the induction of apoptosis or necrosis, at least at the dosages used herein. These results are in agreement with earlier reports showing that OGF, at a concentration that depresses growth, does not alter cell survival in MIA PaCa-2 cells [19]. Moreover, pancreatic tumor cells are known to be relatively resistant to induction of apoptosis by chemotherapeutic agents [35, 36], and exposure to gemcitabine has been shown to be non-apoptotic inducing in MIA PaCa-2 cells despite the use of drug concentrations that suppress cell growth [30]. However, it is known that OGF is targeted to the G_0/G_1 phase of the cell cycle of human pancreatic cancer cells [17], whereas gemcitabine exerts its action in a cell-phase specific manner by arresting cells in S phase [30]. Therefore, at least in vitro, the antiproliferative effects of the combination of OGF and gemcitabine may be related to altering the cell cycle, and not by inducing apoptotic cell death. Whether the primary effect of the drug combination on the cell cycle leads to senescence, ultimately resulting in cell death indirectly or a slow cell death [37], needs to be elucidated.

Gemcitabine is the standard of care for metastatic cancer [5–8, 38], and is used in clinical trials as a single-agent chemotherapeutic for locally advanced pancreatic cancer [4]. Treatment with gemcitabine is not curative for metastatic disease, and the palliative benefits of this agent must be examined in the face of such factors as toxicity [4, 5]. Given the urgent need for advancement in the treatment of pancreatic cancer, combinations of drug therapies in which many involve a new agent plus gemcitabine for pancreatic cancer have gained attention [5–7, 39]. The present report raises the exciting potential of combining chemotherapy and biotherapy into a novel treatment modality for human pancreatic cancer. OGF is not toxic, avoids problems related to drug resistance, has easy accessibility, and can be integrated into the chronic use of chemotherapeutic agents. Moreover, it introduces the possibility of using chemotherapeutic agents at less toxic concentrations and/or in chronic regimens (metronomic chemotherapy) [see 40, 41] in combination with a biotherapy. OGF used as a single-agent has been successful in a Phase I clinical trial with patients with advanced unresectable pancreatic adenocarcinoma [21]. During the chronic experiments in this study by Smith and colleagues [21], mean survival from the time of diagnosis was 8.7–9.5 months, depending on the route of drug administration, with some patients living as long as 23 months. With the preclinical information in this report showing that a combination of OGF and gemcitabine has marked effects on pancreatic cancer in tissue culture and in xenografts, and the data from the Phase I clinical trial with OGF reporting a lack of toxicity and suggesting efficacy, the prospect of clinical studies using combination drug therapy with OGF and gemcitabine appears to be warranted.

The observations in this study showing that the combination of OGF with gemcitabine has a potent inhibitory action on human pancreatic cancer, both in vitro and in vivo, are consistent with reports for OGF in combination with chemotherapy for treatment of squamous cell carcinoma of the head and neck (SCCHN) [42, 43]. Using tissue culture, McLaughlin and colleagues [43] demonstrated that OGF in combination with either paclitaxel or carboplatin has a profound repressive influence on the growth of SCCHN. Jaglowski et al. [42] has reported that OGF in combination with paclitaxel markedly inhibited tumor growth in xenografts of SCCHN. In both in vitro and in vivo investigations, the effect of the combination of OGF and chemotherapy was greater than that for the individual compounds. In addition to pancreatic cell carcinoma [15, 16] and SCCHN [43–45], OGF has been shown to influence the growth (in vitro and/or in vivo) of a wide variety of cancers including neuroblastoma [46], renal cancer [47], and colon cancer [48]. These data may suggest that combined chemotherapy (e.g. gemcitabine, paclitaxel) and biotherapy (OGF) for a variety of cancers warrants investigation.

Acknowledgements Portions of this research were included as partial fulfillment for the Master's Degree in Anatomy (Jeffery Jaglowski).This research was supported in part by Philip Morris USA Inc. and Philip Morris International, and The Pennsylvania Department of Health. The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions.

References

1. Jemal A, Murray T, Ward E, Samuels A., Twari RC, Ghafoor A., Feuer EJ, Thun MJ (2005) Cancer statistics, 2005. CA Cancer J Clin 55:10–30
2. Parkin DM, Pisani P, Ferlay J (1999) Global cancer statistics. CA Cancer J Clin 49:33–64
3. Warshaw AL, Fernandez-del Castillo C (1992) Pancreatic carcinoma. N Eng J Med 326:455–465
4. Akerele CE, Rybalova I, Kaufman HL, Mani S (2003) Current approaches to novel therapeutics in pancreatic cancer. Invest New Drugs 21:113–129
5. Li D, Xie K, Wolff R, Abbruzzese (2004) Pancreatic cancer. Lancet 363:1049–1057

6. Diaz-Rubio E (2004) New chemotherapeutic advances in pancreatic, colorectal, and gastric cancers. Oncologist 9:282–294
7. Pasetto LM, Jirillo A, Stefani M, Monfardini S (2004) Old and new drugs in systemic therapy of pancreatic cancer. Crit Rev Oncol Hematol 49:135–151
8. Zagon IS, Smith JP (2004) Treatment options in pancreatic cancer. Hospital Pharmacy Europe Nov/Dec:1–2
9. Huang P, Chubb S, Hertel L, Grindley GB, Plunkett W (1991) Action of 2′, 2′-difluorodeoxycitine on DNA synthesis. Cancer Res 51:6110–6117
10. Hertel LW, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC, Grindey GB (1990) Evaluation of the antitumor activity of gemcitabine (2′2′-difluoro-2′-deoxycytidine). Cancer Res 50:4417–4422
11. Burris HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassof P, Nelson R, Dorr FA, Stephens CD, von Hoff DD (1997) Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreatic cancer. A randomized trial. J Clin Oncol 15:2403–2413
12. Philip PA (2002) Gemcitabine and platinum combinations in pancreatic cancer. Cancer 95:908–911
13. Ryan DP, Kulke MH, Fuchs CS, Grossbard ML, Grossman SR, Morgan JA, Earle CC, Shivdasani R, Kim H, Mayer RJ, Clark JW (2002) A phase II study of gemcitabine and docetaxel in patients with metastatic pancreatic carcinoma. Cancer 94:97–103
14. Zagon IS, McLaughlin PJ (2004) Opioid growth factor (OGF) inhibits anchorage-independent growth in human cancer cells. Int J Oncol 24:1443–1448
15. Zagon IS, Hytrek SD, Smith JP, McLaughlin PJ (1997) Opioid growth factor (OGF) inhibits human pancreatic cancer transplanted into nude mice. Cancer Lett 112:167–175
16. Zagon IS, Smith JP, McLaughlin PJ (1999) Human pancreatic cancer cell proliferation in tissue culture is tonically inhibited by opioid growth factor. Int J Oncol 14:577–584
17. Zagon IS, Roesener CD, Verderame MF, Ohlsson-Wilhelm BM, Levin RJ, McLaughlin PJ (2000) Opioid growth factor regulates the cell cycle of human neoplasias. Int J Oncol 17:1053–1061
18. Zagon IS, Smith JP, Conter R, McLaughlin PJ (2000) Identification and characterization of opioid growth factor receptor in human pancreatic adenocarcinoma. Int J Mol Med 5:77–84
19. Zagon IS, McLaughlin PJ (2003) Opioids and the apoptotic pathway in human cancer cells. Neuropeptides 37:79–88
20. Zagon IS, Verderame MF, Allen SS, McLaughlin PJ (2000) Cloning, sequencing, chromosomal location, and function of a cDNA encoding the opioid growth factor receptor (OGFr) in humans. Brain Res 856:75–83
21. Smith JP, Conter RL, Bingaman SI, Harvey HA, Mauger DT, Ahmad M, Demers LM, Stanley WB, McLaughlin PJ, Zagon IS (2004) Treatment of advanced pancreatic cancer with opioid growth factor: Phase I. Anticancer Drugs 15:203–209
22. Yunis AA, Arimura GK, Russin DJ (1977) Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. Int J Cancer 19:128–135
23. Lieber M, Mazzetta J, Nelson-Rees W, Kaplan M, Todaro G (1975) Establishment of a continuous tumor-cell line (PANC-1) from a human carcinoma of the exocrine pancreas. Int J Cancer 15:741–747
24. Sato N, Ohta T, Kitagawa H, Kayahara M, Ninomiya I, Fushida S, Fujimura T, Nishimura G, Shimizu K, Miwa K (2004) FR901228, a novel histone deacetylase inhibitor, induces cell cycle arrest and subsequent apoptosis in refractory human pancreatic cancer cells. Int J Oncol 24:679–685
25. Schwarz RE, McCarty TM, Peralta EA, Diamond DJ, Ellenhorn JD (1999) An orthotopic in vivo model of human pancreatic cancer. Surgery 126:562–567
26. Shim WSN, Teh M, Mack POP, Ge R (2001) Inhibition of angiopoietin-1 expression in tumor cells by antisense RNA approach inhibited xenograft tumor growth in immunodeficient mice. Int J Cancer 94:6–15
27. Fueger BJ, Hamilton G, Raderer M, Pangerl T, Traub T, Angelberger P, Baumgartner G, Dudczak R, Virgolini I (2001) Effects of chemotherapeutic agents on expression of somatostatin receptors in pancreatic tumor cells. J Nucl Med 42:1856–1862
28. Shi X, Liu S, Kleeff J, Friess H, Buchler MW (2002) Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. Oncology 62:354–362
29. Faivre S, Raymond E, Woynarowski JM, Cvitkovic E (1999) Supraadditive effect of 2′,2″-difluorodeoxycitidine (gemcitabine) in combination with oxaliplatin in human cancer cell lines. Cancer Chemother Pharmacol 44:117–123
30. Yip-Schneider MT, Sweeney CJ, Jun S-H, Crowell PL, Marshall MS (2001) Cell cycle effects of nonsteroidal anti-inflammatory drugs and enhanced growth inhibition in combination with gemcitabine in pancreatic carcinoma cells. J Pharmacol Exp Ther 298:976–985
31. Shore S, Raraty GT, Ghaneh P, Neoptolemos JP (2003) Review article: chemotherapy for pancreatic cancer. Aliment Pharmacol Ther 18:1049–1069
32. Di Paolo A, Danesi R, Del Tacca M (2004) Pharmacogenetics of neoplastic diseases: new trends. Pharmacol Res 49:331–342
33. Buchsbaum DJ, Bonner JA, Grizzle WE, Stackhouse MA, Carpenter M, Hicklin DJ, Bohlen P, Raisch KP (2002) Treatment of pancreatic cancer xenografts with Erbitux (IMC-C225) anti-EGFR antibody, gemcitabine, and radiation. Int J Radiat Oncol Biol Phys 54:1180–1193
34. Schultz RM, Merriman RL, Toth JE, Zimmermann JE, Hertel LW, Andis SL, Dudley DE, Rutherford PG, Tanzer LR, Grindey GB (1993) Evaluation of new anticancer agents against the MIA PaCa-2 and PANC-2 human pancreatic carcinoma xenografts. Oncol Res 5:223–228
35. Bold RJ, Virudachalam S, McConkey DJ (2001) Chemosensitization of pancreatic cancer by inhibition of the 26S proteasome. J Surg Res 100:11–17
36. Schniewind B, Christgen M, Kurdow R, Haye S, Kremer B, Kalthoff H, Ungefroren H (2004) Resistance of pancreatic cancer to gemcitabine treatment is dependent on mitochondria-mediated apoptosis. Int J Cancer 109:182–188
37. Blagosklonny MV (2000) Cell death beyond apoptosis. Leukemia 14:1502–1508
38. Jacobs AD (2002) Gemcitabine-based therapy in pancreas cancer. Cancer Suppl 85:923–927
39. Corrie P, Mayer A, Shaw J, D'Ath S, Blagden S, Blesing C, Price P, Warner N (2002) Phase II study to evaluate combining gemcitabine with flutamide in advanced pancreatic cancer patients. Br J Cancer 87:716–719
40. Gasparini G (2001) Metronomic scheduling: The future of chemotherapy. Lancet Oncol 2:733–740
41. Kerbel RS, Klement G, Pritchard KI, Kamen B (2002) Continuous low-dose anti-angiogenic/metronomic chemotherapy: from the research laboratory into the oncology clinic. Ann Oncol 13:73–80
42. Jaglowski JR, Zagon IS, Stack BC, Verderame MF, Leure-duPree AE, Manning JD, McLaughlin PJ (2005) Opioid growth factor (OGF) enhances tumor growth inhibition and increases the survival of paclitaxel-treated mice with squamous cell carcinoma of the head and neck. Cancer Chemother Pharmacol, (in press)
43. McLaughlin PJ, Jaglowski JR, Verderame MF, Stack BC, Leure-duPree AE, Zagon IS (2005) Enhanced growth inhibition of squamous cell carcinoma of the head and neck by combination therapy of paclitaxel and opioid growth factor. Int J Oncol (in press)
44. McLaughlin PJ, Levin RJ, Zagon IS (1999) Regulation of human head and neck squamous cell carcinoma growth in tissue culture by opioid growth factor. Int J Oncol 14:991–998
45. McLaughlin PJ, Levin RJ, Zagon IS (2003) Opioid growth factor (OGF) inhibits the progression of human squamous cell carcinoma of the head and neck transplanted into nude mice. Cancer Lett 199:209–217

46. McLaughlin PJ, Zagon IS, Skitzki J (1999). Human neuroblastoma cell growth in tissue culture is regulated by opioid growth factor. Int J Oncol 14:373–380
47. Bisignani, GJ, McLaughlin PJ, Ordille SD, Beltz MS, Jarowenko MV, Zagon IS (1999) Human renal cell cancer proliferation in tissue culture is tonically inhibited by opioid growth factor. J Urol 162:218–191
48. Zagon IS, Hytrek SD, Lang CM, Smith JP, McGarrity TJ, Wu Y, McLaughlin PJ (1996) Opioid growth factor ([Met^5]-enkephalin) prevents the incidence and retards the growth of human colon cancer. Am J Physiol 271:R780–R786