# Effects of the Epidermal Growth Factor Receptor Inhibitor OSI-774, Tarceva, on Downstream Signaling Pathways and Apoptosis in Human Pancreatic Adenocarcinoma[1]

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## Abstract

**Pancreatic cancer is the fifth leading cause of cancer death in North America. Gemcitabine improves the quality of life of patients but fails to significantly reduce mortality. Our laboratory has demonstrated previously that the phosphatidylinositol 3′-kinase inhibitor wortmannin promotes gemcitabine antitumor activity (S. S. W. Ng *et al.,* Clin. Cancer Res., 7: 3269–3275, 2001). The present study examined the effects of the epidermal growth factor receptor (EGFR) inhibitor OSI-774 ("Tarceva") alone and in combination with wortmannin and/or gemcitabine on downstream signaling molecules, as well as apoptosis in primary pancreatic cancer xenografts implanted orthotopically in severely combined immunodeficient mice. Tumors established from two pancreatic cancer patients [Ontario Cancer Institute Pancreas number (OCIP#) 2 and OCIP#7] were treated with various combinations of the above three drugs and harvested for analyses of the following: the levels of phosphorylated and nonphosphorylated forms of EGFR, protein kinase B (PKB/Akt) and extracellular-regulated kinase (ERK1/2), and the extent of apoptosis using immunofluorescence image analysis and TUNEL assay, respectively. OSI-774 alone significantly inhibited phosphorylation of EGFR in both of the primary xenografts. Phosphorylation of pERK decreased in OCIP#2, but not in OCIP#7. No significant effects on pPKB because of OSI-774 were observed in either tumor type. The extent of apoptosis was significantly increased by 2-fold in OCIP#2 tumors treated with gemcitabine and wortmannin in combination; an additional 2-fold increase in apoptosis was evident in the presence of OSI-774. Although wortmannin failed to enhance gemcitabine-induced apoptosis in OCIP#7 tumors, the extent of apoptosis was significantly increased with the inclusion of OSI-774 in the combination. Taken together, these findings support the use of OSI-774 plus a phosphatidylinositol 3′-kinase inhibitor in combination with gemcitabine in the treatment of pancreatic cancer.**

## Introduction

Pancreatic adenocarcinoma is one of the most lethal of all cancers. The mortality approaches 100% because of the propensity for early metastatic spread, and because the disease is highly resistant to radiation and chemotherapy. Although the first-line agent gemcitabine (2′,2′-difluorodeoxycytidine) has produced clinical benefit response such as reduced pain and weight gain (1), the prognosis remains dismal with a 5-year survival rate of 1–4% and a median survival period of 4–6 months (2). Given that ∼27,000 new cases are diagnosed every year in North America (2), there is an urgent need to develop novel treatment strategies to reduce the mortality of pancreatic cancer patients.

Receptor tyrosine kinases regulate epithelial cell growth, differentiation, and death. On ligand binding, the receptor autophosphorylates and subsequently stimulates a diverse signaling network resulting in cell proliferation and survival. It is well documented that pancreatic adenocarcinomas and dysplasias frequently overexpress receptor tyrosine kinases such as EGFR,[3] HER-2/*neu,* and Met/HGFR (3). Furthermore, ligands such as epidermal growth factor and transforming growth factor α are secreted by both malignant cells and surrounding dysplastic pancreatic ductal cells (4). It has been shown that overexpression of EGFR and its ligands contributes to the malignant phenotype and correlates with the decrease in survival in pancreatic cancer patients (5). These observations suggest that enhanced EGFR signaling via autocrine and/or paracrine loops is pivotal in the biology of pancreatic cancer and that blockade of EGFR represents an attractive strategy in the treatment of the disease.

EGFR can be selectively blocked by a number of small molecule inhibitors such as OSI-774 (Tarceva; formerly known as CP-358,774) or monoclonal antibodies. OSI-774 has been reported to inhibit EGFR autophosphorylation *in vitro* ($IC_{50}$ = 2 nM) and in intact tumor cells ($IC_{50}$ = 20 nM), as well as to induce apoptosis in human colon cancer cells (6). The PI3k-PKB/Akt and Raf-ERK pathways, both of which are downstream of EGFR, have been shown to mediate cell

[3] The abbreviations used are: EGFR, epidermal growth factor receptor; PI3k, phosphatidylinositide 3′-kinase; PKB, protein kinase B; SCID, severely combined immunodeficient; ERK, extracellular signal-regulated kinase; Met/HGFR, hepatocyte growth factor receptor; OCIP#, Ontario Cancer Institute Pancreas number; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling.

survival (7). As demonstrated in our previous studies, inhibition of the PI3k-PKB/Akt pathway by wortmannin promotes gemcitabine-induced apoptosis in human pancreatic cancer (8, 9). It seems logical to postulate that the use of OSI-774, which blocks the Raf-ERK pathway, in combination with wortmannin would be more efficacious in enhancing apoptosis induced by gemcitabine. Furthermore, OSI-774 may additionally inhibit the PI3k-PKB/Akt pathway. The objective of the current study was to examine the effects of OSI-774 alone and in combination with wortmannin and/or gemcitabine on downstream signaling molecules, as well as apoptosis in primary pancreatic cancer xenografts implanted orthotopically in SCID mice.

## Materials and Methods
### Primary Pancreatic Cancer Specimens and Animal Model
Primary pancreatic cancer specimens were obtained from patients undergoing radical pancreatectomy for pancreatic cancer at Toronto General Hospital and Mount Sinai Hospital (Toronto, Ontario) in 1999–2000 according to institutional ethical guidelines, and assigned an OCIP#. Harvested specimens were washed twice in antibiotic-containing RPMI 1640 to prevent possible infection. Necrotic tissues were removed, and the remaining viable tumor tissues were cut into small pieces of $\sim 3 \times 3 \times 3$ mm. Five to 6-week-old male SCID mice, weighing 18–23 g, were anesthetized with isoflurane (Janssen Pharmaceutica, North York, Ontario, Canada). An incision was made in the upper left abdomen, and the pancreas was exposed. Tumor pieces were attached to the pancreas using 4–0 chromic gut suture (Roboz Surgical Instrument Co. Inc., Rockville, MD). The pancreas was then returned to the peritoneum, and the abdominal wall and the skin were closed with 4–0 chromic gut and silk sutures (Roboz Surgical Instrument Co. Inc.), respectively. The animals were allowed to recover. These primary patient xenografts were propagated continuously in SCID mice for *in vivo* drug testing.

### Immunohistochemistry
Five-$\mu$m-thick sections of paraffin-embedded, formalin-fixed s.c. and orthotopic tumor tissues were stained with H&E for histological examination. For detection of EGFR, Met/HGFR, and HER-2/*neu*, sections were stained with a monoclonal anti-EGFR antibody (1:100; Zymed Laboratories Inc., San Francisco, CA), polyclonal anti-Met/HGFR, and anti-Her2/neu antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Antigens were visualized using the streptavidin-biotin-peroxidase method.

### Treatment Protocols
Gemcitabine was obtained from Eli Lilly & Co. (Indianapolis, IN) and dissolved at 20 mg/ml in 0.9% NaCl. Wortmannin was purchased from BioMol (Philadelphia, PA), dissolved at 0.4 mg/ml in DMSO, and diluted with 0.9% NaCl before use. OSI-774 (formerly known as CP-358,774) was a gift from OSI Pharmaceuticals (Uniondale, NY). The compound was dissolved at 10 mg/ml in DMSO.

Tumor-bearing SCID mice were randomly assigned to one of the following eight treatment groups ($n = 4$ each): (*a*) the vehicle control; (*b*) gemcitabine (80 mg/kg, i.v.); (*c*) wortmannin (0.7 mg/kg, i.v.); (*d*) gemcitabine + wortmannin; (*e*) OSI-774 (50 mg/kg, i.p.); (*f*) gemcitabine + OSI-774; (*g*) wortmannin + OSI-774; and (*h*) gemcitabine + wortmannin + OSI-774. For group h, gemcitabine was given at 0 h followed by wortmannin at 48 h and OSI-774 at 51 h. A similar schedule was used in other groups, except that the drug not included in the treatment regimen was substituted with the corresponding vehicle. All of the mice were sacrificed 52 h after the administration of gemcitabine. The schedules for gemcitabine and wortmannin were based on our recent publication (9), and those for OSI-774 was selected with reference to information in Moyer *et al.* (6). Harvested tumors were snap frozen in OCT (Miles Inc., Elkhart, IN) in liquid nitrogen, and subsequently processed for immunofluorescence staining and TUNEL assay.

### Quantification of Phosphorylated Forms of PKB/Akt, ERK1/2, and EGFR
**Indirect Immunofluorescence.** Because the tumors were interspersed with variable amounts of necrotic and nonmalignant tissues, a quantitative immunofluorescence technique was developed to examine levels of the phosphorylated forms of PKB/Akt, ERK1/2, and EGFR in viable tumor areas. Seven serial sections (5-$\mu$m thick) were obtained from each frozen tumor, mounted on glass slides, and then fixed in 4% paraformaldehyde. The first section was stained with H&E for transmitted light microscopy. The remaining sections were incubated with primary antibodies (Cell Signaling Technology, Beverly, MA) directed against the following proteins: phosphoPKB/Akt (serine 473; 1:10), PKB/Akt (1:50), phosphoERK1/2 (1:10), ERK1/2 (1:100), phosphoEGFR (Transduction Laboratories; 1:10), or EGFR (1:100) for 1.5 h. A Cy3-conjugated donkey antirabbit IgG (1:200; Molecular Probes, Eugene, OR) was used as the secondary antibody. Secondary antibody used alone is control for nonspecific background. All of the sections were counterstained with the DNA-specific dye 4′,6-diamidino-2-phenylindole (Molecular Probes) at 1 $\mu$g/ml to outline the nuclear area.

**Computerized Image Analysis.** Composite digital images of the entire H&E sections were captured using a color CCD video camera (Sony DXC 970 MD) mounted on a Zeiss Axioskop transmitted light microscope fitted with an autostage and linked to a MicroComputer Image Device (Imaging Research Inc., St. Catharine's, Ontario, Canada). These images were used to select representative areas of viable tumor tissues for analysis of the fluorescence markers.

Viable tumor tissues labeled with the anti-phosphoPKB/Akt, anti-PKB/Akt, anti-phosphoERK1/2, anti-ERK1/2, anti-phosphoEGFR, or anti-EGFR antibodies were examined using a second MicroComputer Image Device image analysis system equipped with a Xillix MicroImager (Xillix, Vancouver, British Columbia, Canada) mounted on an Olympus BX50 reflected fluorescence microscope. Each individual field was obtained using a $\times 40$ objective. Twenty random 0.149-mm$^2$ fields of the same tumor and
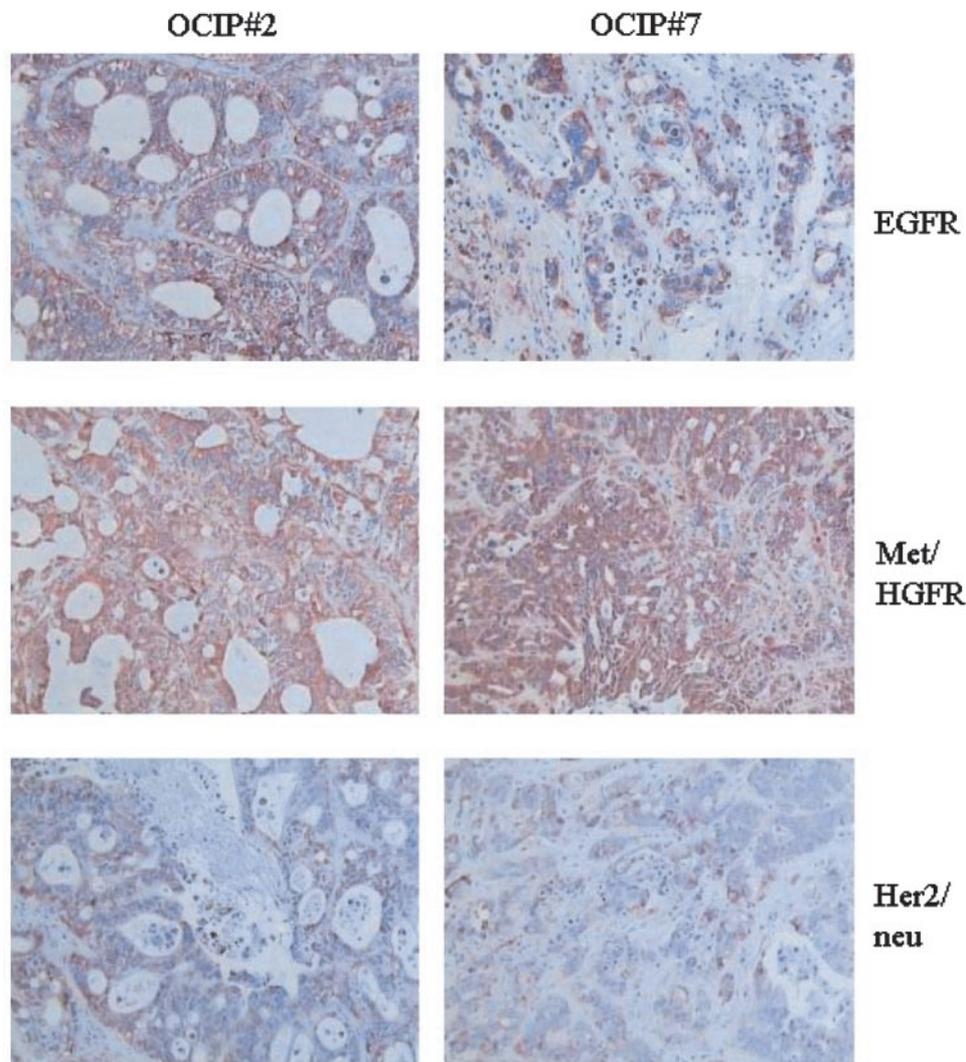
Fig. 1.   Immunohistochemical staining of OCIP#2 and OCIP#7 orthotopic xenografts for EGFR, Met/HGFR, and HER-2/*neu*.

four tumors per group were analyzed. The Cy3-conjugated secondary antibody were excited using a 540-nm band-pass filter with emission collected at 610 nm. For phosphoPKB/Akt and phosphoERK1/2 staining, the nuclear image obtained with 4′,6-diamidino-2-phenylindole was used to create a mask, which was then overlaid on the Cy3 fluorescence image, so that only nuclear phosphoPKB/Akt fluorescence was measured. It has been shown that PKB/Akt and ERK1/2 translocate to the nucleus on phosphorylation (10). For PKB/Akt and ERK1/2 staining, total Cy3 fluorescence was measured. The fluorescence intensities of Cy3 were expressed as mean integrated optical densities, which are measures of the amounts of labeled phosphoPKB/Akt, PKB/Akt, phosphoERK1/2, and ERK1/2 in the tumor sections. The levels of phosphoEGFR and EGFR were expressed as the sum of positively stained areas divided by the microscopic field area.

### Apoptosis Assay

Five serial sections (5-$\mu$m thick) were obtained for each frozen tumor, mounted on glass slides, and then fixed in 1% paraformaldehyde. The first section was processed for H&E staining. TUNEL assay was performed on the remaining four sections using the ApopTag Red kit according to the manufacturer's instructions (Intergen Co., Purchase, NY). Tissue sections processed in the absence of terminal deoxynucleotidyl transferase served as negative controls. The rhodamine-conjugated secondary antibody was excited using a 540-nm bandpass filter with emission collected at 610 nm.

Using the fluorescence image analysis system described above fitted with a Quantix cooled CCD camera (Photometrics Inc., Tucson, AZ), tiled images of tumor sections subjected to TUNEL assay were acquired using a $\times$20 objective. Four sections of the same tumor and four tumors per group were analyzed. Tumors were traced manually with reference to the parallel H&E sections so as to exclude edges and

necrotic and nonmalignant tissues from analysis. Apoptotic nuclei, often consisting of clusters of discrete nuclear fragments, could be readily defined using image analysis criteria so as to reject artifacts. The extent of apoptosis in each tumor, expressed as proportional area, was calculated from the sum of the TUNEL-positive pixel area divided by the total viable tumor area.

### Statistics

All of the values are presented as mean ± SE. Comparisons between control and treatment groups were made with ANOVA followed by Student Newman-Keuls test, with $P <$ 0.05 as the criterion for statistical significance.

### Results

**Characterization of Primary Pancreatic Cancer Xenografts.** H&E staining revealed that OCIP#2 and OCIP#7 are moderately differentiated ductal and ampullary adenocarcinomas, respectively. Palpable tumors were evident 1–2 months after orthotopic implantation of both resected patient tumors. Immunohistochemical staining showed that both primary xenografts expressed moderate and high levels of HER-2/*neu* and Met/HGFR, respectively (Fig. 1). High and moderate levels of EGFR expression were detected in OCIP#2 and OCIP#7, respectively (Fig. 1).

### In Vivo Pharmacodynamics of OSI-774 in Orthotopic Xenografts

**Blockade of EGFR Activation.** Immunofluorescence measurements demonstrated that the levels of EGFR were approximately three times higher in orthotopic xenografts from OCIP#2 than those from OCIP#7 (data not shown). Fig. 2, A and B, illustrate plots of the ratio of phosphorylated EGFR to EGFR *versus* various treatment regimens in both patient xenografts. In OCIP#2 and OCIP#7 tumors, i.p. injection of 50 mg/kg OSI-774 1 h before sacrifice significantly reduced the levels of phosphorylated EGFR by 6–8-fold in the tumors. Treatment with gemcitabine (80 mg/kg for 48 h) or wortmannin (0.7 mg/kg for 4 h) alone, or in combination, neither altered EGFR phosphorylation nor enhanced the effect of OSI-774.

**Effects on PKB/Akt and ERK1/2 Phosphorylation.** Fig. 3, A and B, show bar graphs of the ratio of phosphorylated PKB/Akt to PKB/Akt *versus* different treatment protocols in the two patient xenografts. OSI-774 failed to inhibit PKB/Akt activation relative to the vehicle control. In contrast, wortmannin significantly reduced the levels of phosphorylated PKB/Akt by ~50%. Such inhibition was unchanged by OSI-774. Gemcitabine alone had no effect on PKB/Akt phosphorylation. Total PKB/Akt levels were comparable between control and treated orthotopic tumors established from OCIP#2 and OCIP#7 (data not shown).

In Fig. 4, A and B, the ratios of phosphorylated ERK1/2 to ERK1/2 in both patient xenografts were plotted against treatment regimens. OSI-774 significantly decreased phosphorylated ERK1/2 levels by ~33% ($P <$ 0.05) in OCIP#2 but not in OCIP#7 xenografts. Total ERK1/2 levels were similar between control and treated orthotopic tumors established
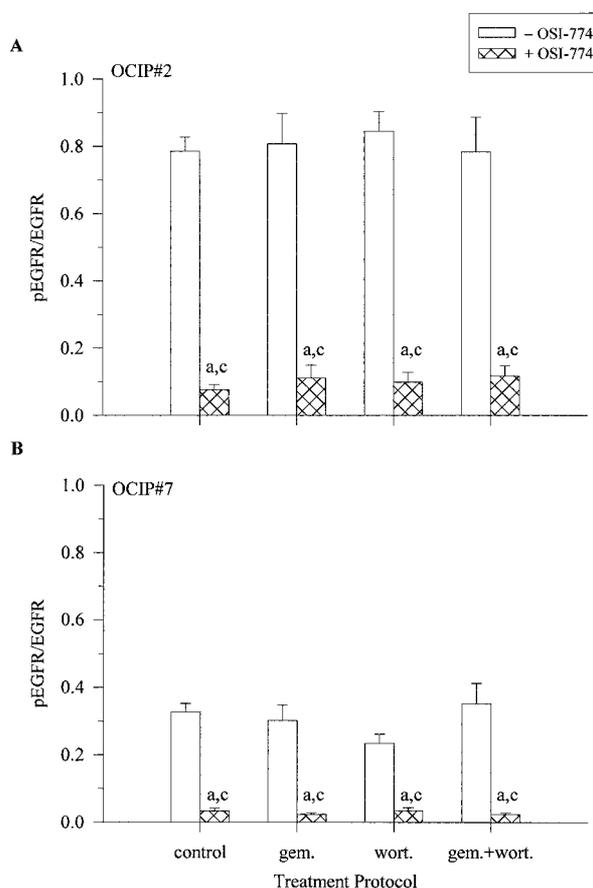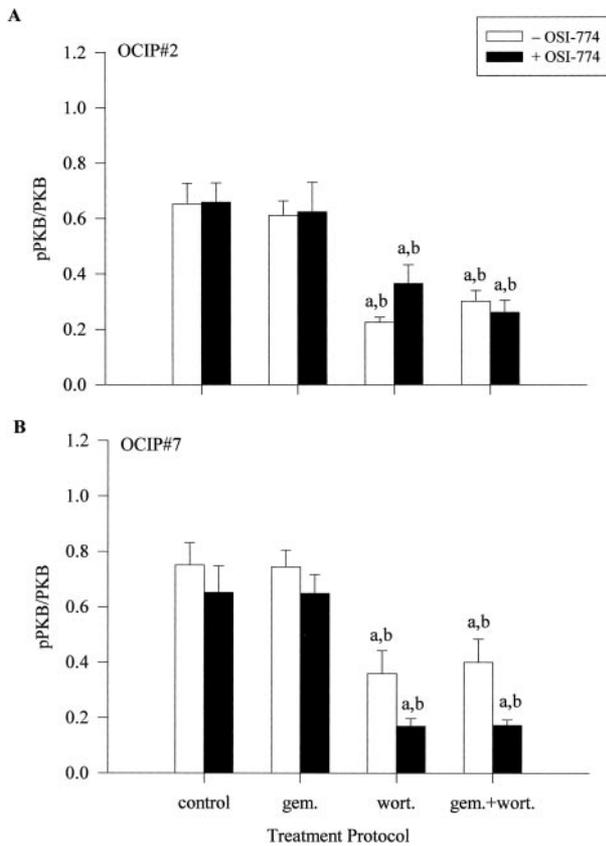


Fig. 2. Bar graphs of the ratio of phosphorylated EGFR (*pEGFR*) to EGFR *versus* different treatment protocols in the absence (☐) or presence (▨) of OSI-774 in OCIP#2 (*A*) and OCIP#7 (*B*) xenografts (*n* = 4/group). *gem.*, gemcitabine; *wort.*, wortmannin. *a,* significantly different from vehicle control; *c,* significantly different from similar treatment without OSI-774; *bars,* ±SE.

from the two patients (data not shown). The effect of OSI-774 was not modified by additional treatment with wortmannin. Gemcitabine or wortmannin alone, or in combination, had no detectable effects on phosphorylated ERK1/2 levels.

**Effects of OSI-774 on Gemcitabine-treated Orthotopic Tumors.** As shown in Fig. 5, A and B, OSI-774 (50 mg/kg for 1 h), gemcitabine (80 mg/kg for 48 h), or wortmannin (0.7 mg/kg for 4 h) alone did not cause significant increase in apoptosis compared with the vehicle control in either of the primary xenografts. However, the extent of apoptosis was significantly increased by 2-fold in OCIP#2 tumors treated with gemcitabine and wortmannin in combination; an additional 2-fold increase in apoptosis was evident with the addition of OSI-774 to this combination. Interestingly, wortmannin failed to enhance gemcitabine-induced apoptosis in OCIP#7 tumors although the extent of apoptosis was significantly increased by 2-fold relative to the vehicle control with the inclusion of OSI-774 in the combination.
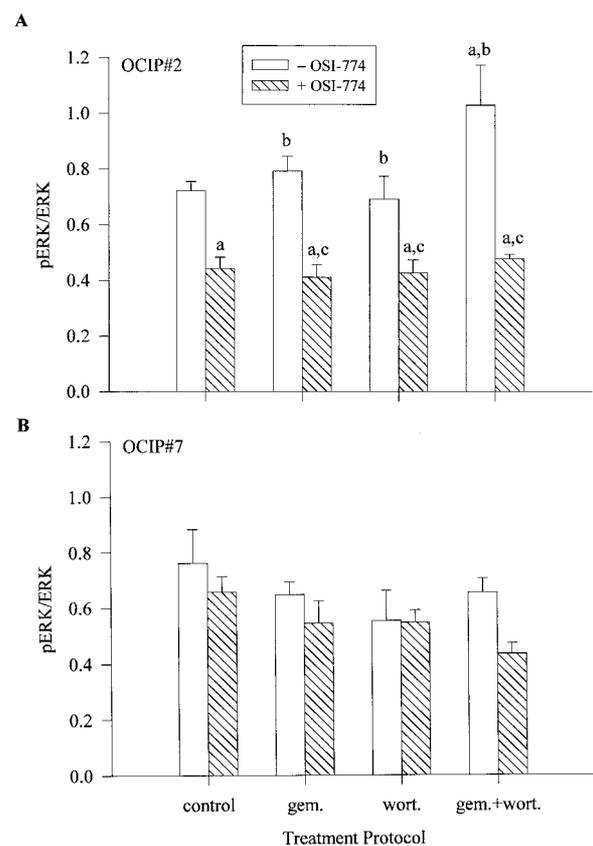
### Discussion

These results showed that OSI-774 inhibits EGFR activation and demonstrates differential suppression of ERK1/2 phos-

**A**



**B**



Fig. 3. Bar graphs of the ratio of phosphorylated PKB/Akt (*pPKB*) to PKB/Akt *versus* different treatment protocols in the absence (☐) or presence (■) of OSI-774 in OCIP#2 (*A*) and OCIP#7 (*B*) xenografts (*n* = 4/group). *gem.,* gemcitabine; *wort.,* wortmannin. *a,* significantly different from vehicle control; *b,* significantly different from OSI-774 alone; *bars,* ±SE.

**A**



**B**



Fig. 4. Bar graphs of the ratio of phosphorylated ERK1/2 (*pERK*) to ERK1/2 *versus* different treatment protocols in the absence (☐) or presence (▨) of OSI-774 in OCIP#2 (*A*) and OCIP#7 (*B*) xenografts (*n* = 4/group). *gem.,* gemcitabine; *wort.,* wortmannin. *a,* significantly different from vehicle control; *b,* significantly different from OSI-774 alone; *c,* significantly different from similar treatment without OSI-774; *bars,* ±SE.

phorylation, as well as enhancement of gemcitabine-induced apoptosis when used alone and in combination with wortmannin in primary pancreatic cancer xenografts. Primary xenografts accumulate fewer additional mutations compared with cell lines in culture; therefore, they are more representative of clinical tumors (11). OCIP#2 and OCIP#7 were chosen based on their differences in EGFR levels. In OCIP#2 tumors, OSI-774 alone significantly inhibited phosphorylation of EGFR and ERK1/2 but failed to block that of PKB/Akt. Similar data were obtained with OCIP#7 tumors except that OSI-774 did not reduce phosphoERK1/2 levels. Because OCIP#2 tumors express higher levels of EGFR than those from OCIP#7, it is possible that ERK1/2 activation in the latter is less dependent on EGFR, and therefore, less sensitive to receptor blockade by OSI-774.

In accordance with our previous findings (8), wortmannin monotherapy was able to enhance gemcitabine-induced apoptosis in OCIP#2 xenografts. This effect was additionally potentiated by the inclusion of OSI-774 in the treatment regimen. ERK1/2 has been shown to inhibit apoptosis in hematopoietic cells by blocking caspase activation after cytochrome *c* release from the mitochondria and by phosphorylating BAD (12). The mechanisms by which PKB/Akt me-

diates cell survival include regulation of glycogen synthase kinase-3$\beta$ (7), BAD (13), nuclear factor -$\kappa$B, and Bcl-X$_L$ (14). Not surprisingly, the reduction of both phosphoERK1/2 and phosphoPKB/Akt levels by OSI-774 and wortmannin caused a greater increase in apoptosis than the reduction of each alone. However, gemcitabine-induced apoptosis in OCIP#7 xenografts was only evident with the combined use of OSI-774 and wortmannin but not with wortmannin alone. The status and/or expression levels of other signaling molecules such as p53, Ki-Ras, and other receptor tyrosine kinases may contribute to the variable responses to EGFR blockade and PI3k inhibition in the two patient xenografts.

Recently, there is increasing evidence demonstrating the therapeutic potential of EGFR blockade in the management of pancreatic cancer and other malignancies. For instance, the EGFR inhibitor PKI116 and the anti-EGFR antibody C225 have been reported to block EGFR phosphorylation in L3.6pl human pancreatic carcinoma xenografts leading to decreased production of vascular epidermal growth factor, apoptosis of endothelial cells, and, therefore, inhibition of tumor-induced angiogenesis (15). Furthermore, the combination of PKI116 or C225 and gemcitabine caused more prominent tumor shrinkage than either agent used alone (15).
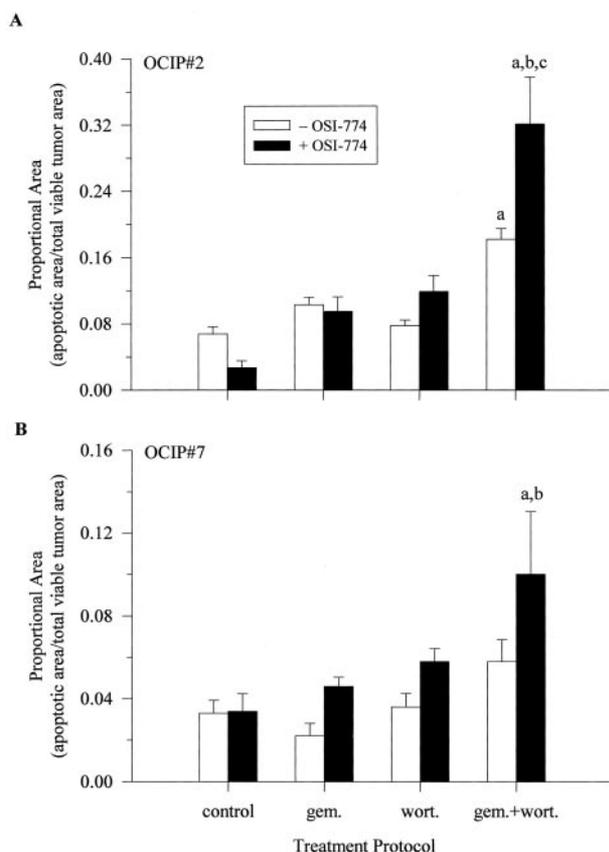
Fig. 5. Proportional area of tumors undergoing apoptosis in response to various treatment protocols in the absence (□) or presence (■) of OSI-774 in OCIP#2 (A) and OCIP#7 (B) xenografts (n = 4/group). gem., gemcitabine; wort., wortmannin. a, significantly different from vehicle control; b, significantly different from OSI-774 alone; c, significantly different from similar treatment without OSI-774; bars, ±SE.

Sirotnak et al. (16) showed that chemosensitization by yet another EGFR inhibitor ZD1839 ("Iressa"; Astra-Zeneca) does not require high levels of EGFR expression in the target tumors. Induction of apoptosis by cytotoxic drugs appears to up-regulate EGFR (17) possibly as a survival mechanism, which also makes cells more susceptible to the effect of subsequent EGFR inhibition. This may partially explain why EGFR inhibitors and cytotoxic drugs in combination are more efficacious than either class of agents used alone. It should be noted that the above effects are not exclusive to EGFR blockade. Herceptin, a selective anti-HER-2/neu antibody, has also been shown to induce greater inhibition of growth of human breast cancer xenografts when used in combination with a variety of chemotherapeutic agents (e.g., paclitaxel, doxorubicin) than when used alone (18). Taken together, these observations indicate the importance of characterizing the tumor of a patient from biopsies in the design of treatment protocols involving receptor tyrosine kinase or other signal transduction inhibitors.

It is apparent that the use of a single signal transduction inhibitor cannot antagonize all of the potentially relevant survival pathways in pancreatic cancers. For instance, OSI-774 would not overcome the downstream effects of mutant Ki-Ras. Wortmannin can reverse the effects of the two afferent pathways by directly inhibiting PI3k. However, mutant Ki-Ras or activated EGFR or other receptor tyrosine kinases can also suppress apoptosis and promote survival via PI3k-independent pathways (19). Therefore, combinations of signal transduction inhibitors will likely be required for efficacious reversal of drug resistance in pancreatic cancer patients. The ability of signal transduction inhibitors to enhance the antitumor activity of cytotoxic drugs additionally supports their introduction into the clinic.

In conclusion, it was demonstrated that OSI-774 suppresses EGFR and ERK1/2 phosphorylation, and promotes gemcitabine-induced apoptosis in combination with wortmannin. The variability of responses observed in the two primary pancreatic cancer xenografts under study suggests that characterization of patient tumors should be performed to optimize the efficacy of treatment regimens involving signal transduction inhibitors.

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