

Inhibition of the Phosphatidylinositol 3'-Kinase-AKT Pathway Induces Apoptosis in Pancreatic Carcinoma Cells *in Vitro* and *in Vivo*

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Abstract

The phosphatidylinositol 3'-kinase (PI3k)-AKT survival pathway is activated in many malignancies. We observed constitutive AKT phosphorylation (on S473) consistent with pathway activation in seven of nine human pancreatic carcinoma cell lines *in vitro*. Exposure of the cells to two structurally distinct inhibitors of PI3k (wortmannin and LY294002) resulted in a dose-dependent induction of apoptosis in six of seven of the cell lines that displayed constitutive AKT phosphorylation but not in either of the cell lines that did not. The mitogen-activated protein/extracellular signal-regulated kinase kinase-mitogen-activated protein kinase inhibitor PD98059 also induced apoptosis in two of the cell lines, including one of the LY294002-insensitive lines (AsPC-1). Exposure of orthotopic L3.6pl pancreatic tumors to LY294002 resulted in dose-dependent inhibition of tumor growth, and decreased peritoneal and liver metastases, effects that were associated with an inhibition of AKT phosphorylation and increased terminal deoxynucleotidyl transferase-mediated nick end labeling staining characteristic of apoptosis. Furthermore, a suboptimal dose of LY294002 (25 mg/kg) produced additive inhibition of tumor growth when combined with a suboptimal dose of gemcitabine (62 mg/kg). Together, our results establish that the PI3k/AKT pathway is constitutively activated in a majority of human pancreatic cancer cell lines and establish that the pathway is a promising target for therapeutic intervention.

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer death in both men and women of the United States, although it represents only 2.3% of newly diagnosed cancer cases a year (1). At the time of diagnosis, >80% of patients

present with either locally advanced or metastatic disease, and this fact coupled with the inherently aggressive nature of the disease and lack of effective therapies all contribute to an extremely poor prognosis for the vast majority of patients. Therefore, understanding of the molecular mechanisms of pancreatic cancer progression remains a high priority in efforts to define better targets for therapeutic intervention.

Studies using archival pancreatic tumor tissues and pancreatic carcinoma cell lines have identified a number of genetic and biochemical abnormalities that are common in human pancreatic cancer. Mutations in the *K-ras* oncogene that lock the protein in a constitutively active (GTP-bound) state accumulate early in disease progression and occur in 75–90% of pancreatic adenocarcinomas (2). *K-ras* signaling probably promotes the neoplastic phenotype via activation of a downstream protein kinase cascade that controls a wide variety of cellular processes, including membrane trafficking, cellular proliferation, differentiation, and cytoskeletal organization (reviewed in Ref. 3).

One of the key downstream targets of the Ras family is class I PI3k² (4). PI3k phosphorylates membrane phosphatidylinositol on the 3' hydroxyl of the inositol ring to create polyphosphoinositides (phosphatidylinositol -di and -triphosphates) that recruit a number of PH domain-containing proteins to the cell membrane. The PDK1 and its target, the protein serine/threonine kinase AKT/PKB, are recruited to the cell membrane by their PH domains, which results in AKT activation and the subsequent propagation of several different downstream survival signals (reviewed in Ref. 5). The AKT survival pathway is regulated negatively by the MMAC/PTEN (mutated in multiple advanced cancers/phosphatase on chromosome ten) lipid phosphatase, which selectively dephosphorylates the 3' site on polyphosphoinositides produced by PI3k. Importantly, the gene for MMAC/PTEN is inactivated or deleted in a many solid tumors, including some pancreatic adenocarcinomas (6). In addition to mutations in *K-ras* and MMAC, many receptor tyrosine kinases (EGF receptor, platelet-derived growth factor receptor, c-erb2, insulin-like growth factor-1 receptor, and fibroblast growth factor receptor) are overexpressed in human pancreatic cancer (7–11), and all of them can activate Ras and AKT. Finally, the *AKT2* gene is located within a genomic locus (19q13) that is amplified in a subset of pancreatic tumor specimens and cell lines (12, 13). Together, these observations suggest that the

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² The abbreviations used are: PI3k, phosphatidylinositol 3'-kinase; PH, pleckstrin homology; PDK1, phosphatidylinositol 3'-kinase-dependent kinase-1; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor.

PI3k-AKT pathway is a point of convergence for survival signaling in pancreatic cancer.

Chemical inhibitors of PI3k (wortmannin and LY294002) have been used extensively to study the role of the PI3k/AKT pathway in normal and transformed cells (14, 15). The inhibitors also have antitumor activity *in vitro* and *in vivo* in a variety of tumor types (16, 17; reviewed in Ref. 18), and it is possible that cells expressing constitutively active AKT become dependent on its survival-promoting effects. Here we assessed the prevalence of AKT pathway activation in a panel of human pancreatic carcinoma cell lines and tested the effects of PI3k inhibitors on apoptosis *in vitro* and *in vivo*.

Materials and Methods

Materials. All of the antibodies were purchased from commercial sources as listed: rabbit polyclonal anti-AKT, antiphosphorylated (serine 473)-AKT, anti-ERK, and antiphosphorylated ERK (T202/Y204) antibodies, from Cell Signaling Technology (Beverly, MA); rabbit polyclonal antiactin, from Sigma (St. Louis, MO); and rabbit polyclonal anti-Ki-67 antibodies from DAKO (Carpinteria, CA). Gemcitabine (HCl, 29,29-difluorodeoxycytidine) and LY294002 were purchased from Eli Lilly (Indianapolis, IN). Wortmannin and PD98059 were purchased from Calbiochem (San Diego, CA).

Cells and *in Vitro* Culture Conditions. L3.6pl human pancreatic cancer cells were established from Colo-357 fast-growing cells by orthotopic "recycling" in nude mice (described in Ref. 19). Other cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained on plastic in MEM supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamin solution (Life Technologies, Inc., Grand Island, NY), incubated in 5% CO₂-95% air at 37°C. The cultures were free of *Mycoplasma* (as assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Immunoblotting. Cells were grown to 75–80% confluence in complete medium and then for 24–36 h in serum-free complete MEM. Cells were then incubated with or without 1 μM wortmannin or 10 μM PD98059 for 1 h and lysed in a buffer containing 1% Triton, 150 mM NaCl, and 25 mM Tris-acetate (pH 7.4) supplemented with protease inhibitors (Complete Mini; Boehringer-Mannheim, Indianapolis, IN), sodium orthovanadate, glycerophosphate, and sodium fluoride. Lysates were mixed with Laemmli's reducing sample buffer, resolved by 10% SDS-PAGE, and electrophoretically transferred onto 0.2-μm nitrocellulose membranes. Blots were blocked in 5% nonfat dry milk and incubated overnight (4°C) with primary antibodies. The blots were washed, probed with species-specific secondary antibodies coupled to horseradish peroxidase (Amersham, Arlington Heights, IL), and antibody-antigen complexes were detected by enhanced chemiluminescence (NEN, Boston, MA). All of the experiments were performed in triplicate with lysates prepared from independent experiments.

Quantification of Apoptosis *in Vitro*. Cells maintained in MEM medium with 5% FBS or serum-free MEM at 70–75% confluence were exposed to the indicated concentrations of

wortmannin, LY294002, or PD98059 for 48 h. The cells were collected with 0.25% trypsin/0.02% EDTA. Apoptosis was measured by propidium iodide staining and flow cytometry (20). All of the LY294002 experiments were performed in triplicate. Results from the PD98059 experiments are representative of two independent experiments. In all of the cases but one levels of apoptosis were comparable in cells maintained in 5% serum *versus* serum-free medium, and the results from the former experiments are shown. The only exception to this was the case of L3.6pl cells exposed to PD98059, where significant levels of apoptosis were only observed when cells were maintained in serum-free medium, and these results are presented.

Tumor Xenograft Studies. Male athymic nude mice (NCr-nu) were obtained from the animal production area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and NIH, and their use in these experiments was approved by the Institutional Animals Care and Use Committee. The mice were used when they were 8–10 weeks of age. Colo-357-L3.6pl cells were harvested from subconfluent cultures by treatment with 0.25% trypsin and 0.02% EDTA.

Trypsinization was stopped with medium containing 10% FBS, and cells were washed once in serum-free medium and resuspended in HBSS. Only single-cell suspensions displaying >90% viability were used for injections. Orthotopic tumor cell injections of 1 × 10⁶ cells were performed as described elsewhere (19, 21). Seven days later, the mice were randomly assigned to four treatment groups of 10 mice each. The first group received twice-weekly i.p. injections of 5% DMSO in HBSS as control. The second, third, and fourth groups received twice-weekly i.p. injections of LY294002 at 10, 25, and 100 mg/kg. For s.c. tumors, 1 × 10⁶ cells were mixed with Matrigel (Collaborative Research, Bedford, MA; 3.5 mg of Matrigel in 0.5 ml of HBSS). Two bilateral flank tumors were generated in each mouse. Seven days later, the mice were randomly assigned to four treatment groups of 5 mice each. The first group received twice-weekly i.p. injections of LY294002 (25 mg/kg), the second group received gemcitabine (62 mg/kg), the third group received a combination of 25 mg/kg LY294002 and 62 mg/kg gemcitabine, and the last group received 5% DMSO in HBSS as control. The experiment was repeated using different doses of gemcitabine (125 mg/kg, 62 mg/kg, and 31 mg/kg; data not shown).

Treated mice were closely monitored for any signs of progressive disease and sacrificed when moribund (at 3–4 weeks). Body weight, size and weight of the primary tumors, the incidence of regional (celiac and para-aortal) lymph node metastasis, and the number of liver and peritoneal nodules were all determined. Tumor volume was measured along the longest orthogonal axis and calculated using the formula: volume = length × width²/2, where width was the shortest measurement. Histopathology confirmed the nature of the

disease. For immunohistochemistry and histology staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, and another part was embedded in OCT compound (Miles, Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -70°C .

Immunohistochemistry. Paraffin-embedded tissues were used for identification of AKT, pAKT, Ki-67, and for analysis of DNA fragmentation using the TUNEL assay. Four to 6- μm -thick sections were mounted on positively charged Superfrost slides (Fisher Scientific, Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, followed by treatment with a graded series of alcohol [100, 95, and 80% ethanol/double-distilled H_2O (v/v)] and rehydrated in PBS (pH 7.5). Sections were incubated with Tris-citrate target retrieval solution from DAKO at 95°C for 20 min. Slides were then allowed to cool for another 20 min, followed by sequential rinsing in Tris-buffered saline-Tween 20 [Tris HCl 0.5 M, NCl 0.3 M, and Tween 20 0.15% (pH 7.6)]. All of the samples were incubated with 3% hydrogen peroxide in methanol (v/v) for 12 min to block endogenous peroxidase, washed with PBS (pH 7.5), and incubated in protein blocking solution [5% normal human serum/0.5% normal goat serum in PBS (v/v)] for 20 min. For TUNEL analyses we used an *in situ* cell death detection kit obtained from Boehringer-Mannheim. Briefly, the samples were incubated with equilibration buffer (30 mM Trizma, 140 mM Na cacodylate, and 1 mM CoCl_2) containing nucleotide mix and terminal deoxynucleotidyl transferase in a humidified chamber for 1 h at 37°C in the dark. The reaction was terminated by immersing the samples in TB buffer [300 mM NaCl and 30 mM sodium citrate (pH 7.2)] for 15 min, followed by three washes to remove unincorporated dUTP. For AKT, pAKT, and Ki-67 staining we used a catalyzed signal amplification system obtained from DAKO. Briefly, slides were sequentially incubated with intervening washes for 15 min in the following: primary antibodies diluted 1:200, biotinylated goat antirabbit antibodies, streptavidin-biotin complex, amplification reagent, and peroxidase-labeled streptavidin. Positive reactions for AKT, pAKT, Ki-67, and TUNEL were visualized by incubating the slides with diaminobenzidine/hydrogen peroxide (DAKO) as a substrate. The sections were then rinsed with distilled water, counterstained with Gill's hematoxylin (Sigma), and mounted with Universal Mount (Research Genetics). Background reactivity for TUNEL was determined by processing slides in the absence of terminal deoxynucleotidyl transferase (negative control). Maximum reactivity was observed by preincubating the tissue sections with DNase I and served to confirm the quality of the specimens. Control samples for AKT, pAKT, and Ki-67 staining exposed to secondary antibody alone showed no nonspecific staining. The staining experiments were performed in duplicate.

Quantification of Immunohistochemical Tissue Staining. Microscopy was performed using a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) equipped with Sony three-chip camera (Sony Corporation of America, Montvale, NJ) and Optimas Image Analysis software (Bioscan, Edmond, WA) installed in a Compaq computer with Pentium chip, a frame grabber, an optical disk storage system, and a Sony color printer. For the quantification of TUNEL and Ki-67 expression, the number of positive tumor cells was counted in 10 random 0.159- mm^2 fields at $\times 100$ and divided by the

total number of cells per field. The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the immunohistochemical reaction.

Statistical Analyses. Data were expressed as mean \pm SD of mean and compared by unpaired Student's *t* test. Differences between the groups were considered statistically significant at $P < 0.025$ for a one-tailed test.

Results

Constitutive AKT Phosphorylation Correlates with Sensitivity to PI3k Inhibitor-induced Apoptosis *in Vitro*. As a first step toward characterizing the role of the PI3k-AKT pathway in human pancreatic cancer, we measured the levels of AKT phosphorylation in a panel of human pancreatic cell lines by Western blotting. Seven of nine cell lines demonstrated activated AKT at baseline, which was inhibited by treatment with PI3k inhibitor wortmannin (Fig. 1A). Because recent studies have implicated the PI3k/AKT pathway in cell survival, we also characterized the effects of wortmannin and another PI3k inhibitor (LY294002) on DNA fragmentation characteristic of apoptosis. LY294002 is a synthetic flavinoid that acts as a potent, competitive, reversible inhibitor of the ATP-binding site of class I PI3k (22). Treatment with either inhibitor resulted in a dose-dependent induction of apoptosis in the Colo-357-L3.6pl and Panc-1 cell lines expressing high baseline levels of total and activated AKT (Fig. 2A). The PI3k inhibitors were less effective in the CFPAC-1, HPAF, CAPAN-2, and MiaPaCa-2 cells expressing moderate baseline levels of activated AKT, and they failed to induce apoptosis in the Hs766T, BxPc-3, and AsPc-1 cells (Fig. 2B). Among the three insensitive cell lines, only the AsPC-1 cells displayed significant basal AKT phosphorylation (Fig. 1A). Direct sequencing of exons 2–7 of PTEN in L3.6pl, Hs766T, and Panc-1 cells revealed that they contained wild-type copies of the gene (data not shown), and immunoblot analysis confirmed that all three expressed detectable levels of PTEN protein (Fig. 1B). Therefore, the constitutive AKT phosphorylation observed in L3.6pl and Panc-1 cells did not appear to have been caused by loss of wild-type PTEN, although the results do not rule out the possibility that the lipid phosphatase activity of PTEN was decreased in the cells. Irrespective of the mechanism(s) underlying AKT activation, the results demonstrated that the levels of apoptosis induced by PI3k inhibition correlated closely with baseline levels of activated AKT in all but one of the cell lines.

Other studies have implicated ERK/MAPK in cell survival, and ERK activation is also common in cancer. Therefore, we assessed ERK activation status in four of our cell lines by immunoblotting with an anti-ERK phosphospecific antibody. Levels of phosphorylation were compared in cells maintained in 5% serum, cells preincubated for 24 h in serum-free medium, and serum-starved cells exposed to EGF (100 ng/ml). Basal phosphorylation was determined by incubating the cells for 1 h with the synthetic MAP/ERK kinase-MAPK inhibitor, PD98059 (10 μM). ERK phosphorylation was constitutive (*i.e.*, not modulated by serum or EGF) in AsPC-1, L3.6pl, and Mia PaCa-2 but was induced by serum and EGF in the BxPC-3 cells (Fig. 3). PD98059 induced concentration-dependent DNA fragmentation characteristic of apoptosis in

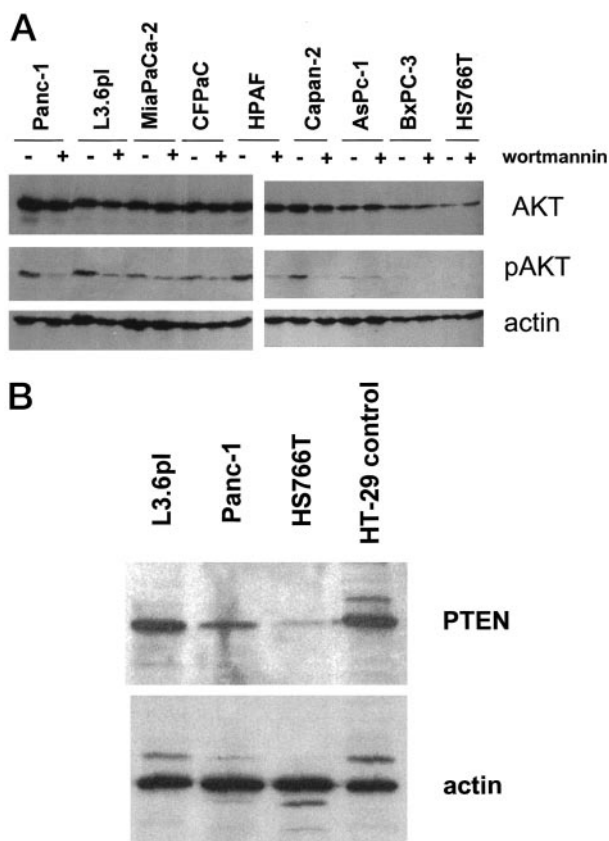


Fig. 1. Total and activated (phosphorylated on serine 473) AKT protein levels in human pancreatic cancer cells. **A**, paired samples of serum-starved cells at 75–80% confluence were incubated with or without 1 μ M wortmannin for 1 h and lysed in a buffer containing 1% Triton, 150 mM NaCl, and 25 mM Tris-acetate (pH 7.4) supplemented with protease and phosphatase inhibitors. Proteins were resolved by 10% SDS-PAGE and detected with specific primary antibodies. Results are representative of three independent experiments. **B**, analysis of PTEN protein expression. Lysates were resolved by 10% SDS-PAGE, and PTEN was detected by immunoblotting. Blots were then stripped and reprobed with a rabbit polyclonal antiactin antibody (from Sigma) to confirm equal protein loading. The human HT-29 colon adenocarcinoma line contains wild-type PTEN and served as a positive control. Although PTEN protein was detected in all of the cell lines, note that the lowest levels were observed in the line (Hs766T) that demonstrated low constitutive AKT phosphorylation.

AsPC-1 cells maintained in complete medium and in L3.6pl cells exposed to the drug in serum-free medium, but not in BxPC-3, Mia PaCa-2, HS766T, or Panc-1 (Fig. 4; data not shown). Importantly, PD98059 had no effect on AKT phosphorylation in the L3.6pl cells (Fig. 3), demonstrating that its cytotoxicity was not because of indirect effects of the inhibitor on the AKT pathway.

Effects of PI3K Inhibition on Tumor Growth in Vivo.

Tumors generated by orthotopic implantation of the metastatic L3.6pl cell line were used to evaluate the effects of LY294002 on growth and metastasis in an orthotopic xenograft model. The L3.6pl model was selected because of its well-characterized growth properties (19, 21) and the high sensitivity of L3.6pl cells to drug-induced apoptosis *in vitro*. Therapy with LY294002 (10, 25, and 100 mg/kg) was initiated 7 days after tumor implantation. One mouse died before the

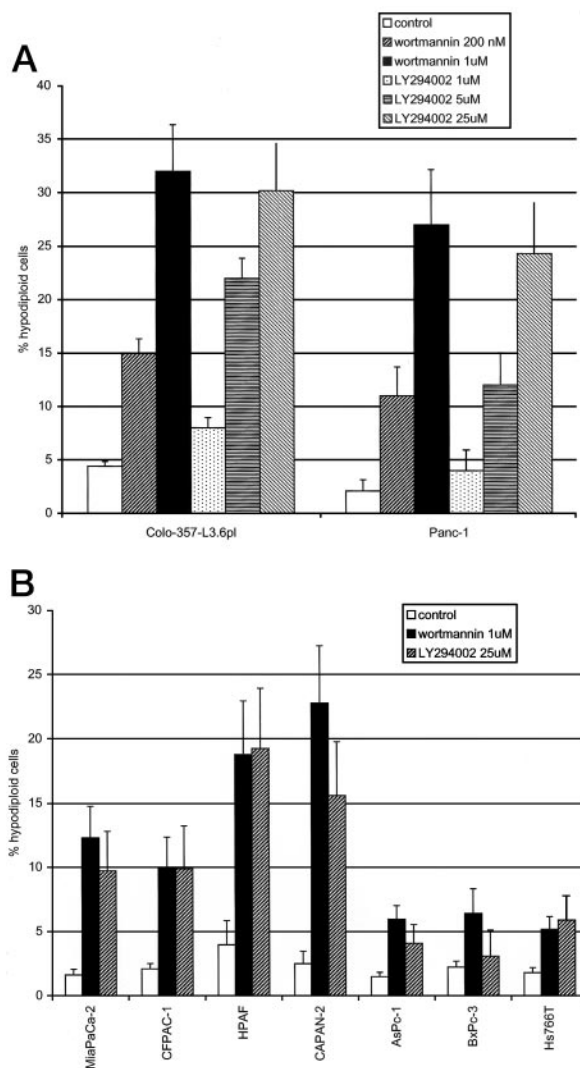


Fig. 2. Sensitivity of human pancreatic carcinoma cells to the treatment with PI3K inhibitors. Cells maintained in MEM with 5% FBS were incubated with or without wortmannin and LY294002 for 48 h. The cells were collected with 0.25% trypsin/0.02% EDTA, and apoptosis was measured by propidium iodide staining and flow cytometry. All experiments were performed in triplicate. Data were expressed as mean; bars, \pm SD. **A**, treatment with wortmannin and LY294002 resulted in a dose-dependent apoptotic cell death in the Colo-357-L3.6pl and Panc-1 cell lines. **B**, PI3K inhibitors were less effective in the treatment of the CFPAC-1, HPAF, CAPAN-2, and MiaPaCa-2 cells, and failed to induce apoptosis in the AsPc-1, Hs766T, and BxPc-3 pancreatic carcinoma cells.

onset of treatment. All of the mice were sacrificed after 4 weeks of treatment. Detailed necropsy revealed that all of the mice had pancreatic tumors (Table 1). Treatment with 100 mg/kg LY294002 significantly reduced mean pancreatic tumor burden as compared with the control mice (control versus LY294002 100 mg/kg; $P < 0.005$). In addition, the incidence of liver and peritoneal metastases was also reduced by this dose of the drug. Treatment with 10 mg/kg and 25 mg/kg of LY294002 were less effective in decreasing tumor burden, and number of liver and peritoneal metastases than treatment with 100 mg/kg of LY294002 (control versus

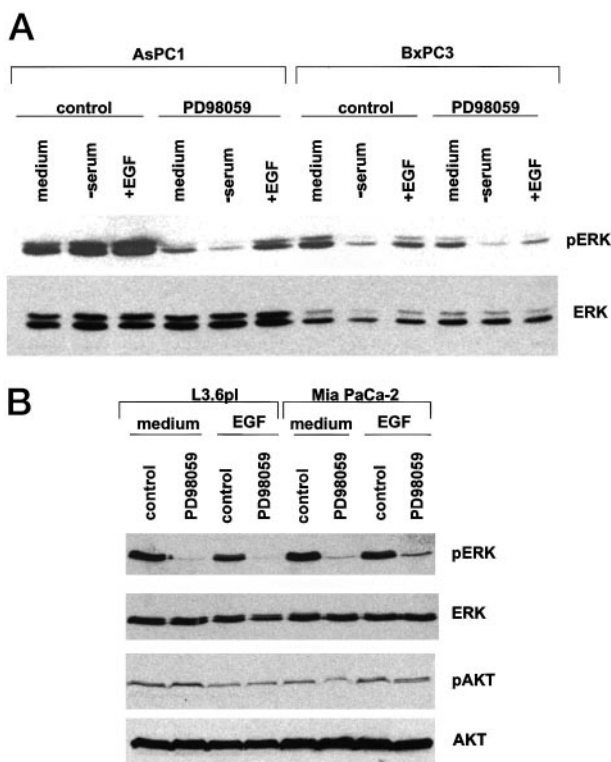


Fig. 3. Analysis of ERK phosphorylation in pancreatic cancer cell lines. **A**, ERK phosphorylation in AsPC-1 and BxPC-3. Cells were either maintained in complete MEM or preincubated in serum-free MEM for 24 h. Cells were then incubated with or without 10 mM PD98059 for 1 h. Finally, one pair of serum-starved plates was incubated with 100 ng/ml EGF for 10 min. Cells were harvested and lysed, and levels of total and phosphorylated ERKs were measured by immunoblotting as described in "Materials and Methods." **B**, effects of PD98059 on ERK and AKT phosphorylation in L3.6pl and Mia PaCa-2. Cells were preincubated in serum-free MEM before incubation with PD98059 and/or EGF as described above. Cells were harvested and lysed, and levels of total and phosphorylated ERKs and AKT were measured by immunoblotting as described in "Materials and Methods."

LY294002 10 mg/kg and control versus LY294002 25 mg/kg; $P > 0.025$, not significant). All of the mice tolerated LY294002 at the dose levels selected and maintained or gained body weight (Table 1).

Tumors harvested from the different groups were processed for routine histology and immunohistochemical analyses. Microscopically, all of the tumors displayed areas of necrosis. Cell proliferation was evaluated by immunohistochemistry using an anti-Ki-67 antibody, and apoptosis was quantified using the TUNEL method (Fig. 5). Tumors in the high dose group (100 mg/kg LY294002) displayed lower Ki-67 staining and higher TUNEL staining than controls (Fig. 5). In control tumors, the mean number of Ki-67- and TUNEL-positive cells was 228 ± 65 and 128 ± 37 , respectively; whereas Ki-67 staining dropped to 169 ± 53 and TUNEL staining increased to 266 ± 72 in tumors obtained from animals treated with 100 mg/kg LY294002 (Ki-67, control versus LY294002 100 mg/kg, $P < 0.025$; TUNEL, control versus LY294002 100 mg/kg, $P < 0.0005$). To confirm that LY294002 blocked the PI3k-AKT pathway *in vivo*, AKT phosphorylation status was measured by immunohistochemistry

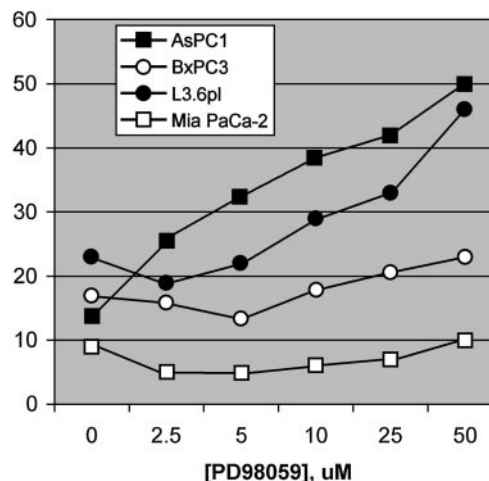


Fig. 4. Effects of PD98059 on DNA fragmentation associated with apoptosis. AsPC-1, BxPC-3, and Mia PaCa-2 cells (75–80% confluent) were exposed to the indicated concentrations of PD98059 for 48 h, and DNA fragmentation was measured by propidium iodide staining and FACS analysis as described in "Materials and Methods." Similar results were obtained when these cell lines were exposed to drug in serum-free medium. L3.6pl cells were grown to 80% confluence in complete medium. The medium was then replaced with serum-free complete MEM, and cells were exposed to the indicated concentrations of PD98059 in this medium for 48 h before analysis of DNA fragmentation by propidium iodide staining and FACS analysis. Similar results were obtained in two independent experiments.

with a phosphospecific anti-AKT (serine 473) antibody. The results revealed reductions in both total and phosphorylated AKT levels in tumors treated with 100 mg/kg of LY294002 compared with controls (Fig. 6), confirming appropriate drug targeting *in vivo*.

PI3k Inhibition Promotes the Antitumoral Effects of Gemcitabine. Gemcitabine is a nucleoside analogue that is currently considered one of the most active conventional agents in pancreatic cancer. In previous work we have shown that inhibitors of the EGF receptor (23, 24) and other biologically active agents^{3,4} promote the antitumoral effects of gemcitabine in pancreatic tumor xenografts *in vivo*. Therefore, we characterized the effects of LY294002 plus gemcitabine on the growth of L3.6pl tumors. s.c. tumors were used in these studies to better monitor tumor volumes throughout the course of therapy. Doses of LY294002 (25 mg/kg) and gemcitabine (62 mg/kg) were selected that produced partial effects on tumor growth in pilot studies (Table 1; data not shown). All of the mice were sacrificed after 3 weeks of the treatment because of high tumor burden in control animals. The results (summarized in Table 2) revealed that treatment with LY294002 alone produced a 25% reduction in tumor volume (control versus LY294002 group; $P > 0.025$, not significant), and gemcitabine alone produced a 31% reduction in tumor volume (control versus gemcitabine group;

³ S. Nawrocki *et al.* Effects of the proteasome inhibitor PS-341 on apoptosis and angiogenesis in orthotopic human pancreatic tumor xenografts, submitted for publication.

⁴ M. Jean *et al.* The erbB antagonist CI-1033 inhibits growth of orthotopic pancreatic tumors by direct induction of apoptosis and inhibition of angiogenesis, submitted for publication.

Table 1 Therapy of human pancreatic carcinoma growing in the pancreas of nude mice

One million Colo-357-L3.6pl cells were injected into the pancreas of nude mice. Seven days later, the mice were randomly assigned to four treatment groups of 10 mice each. The first group received twice-weekly i.p. injections of 5% DMSO in HBSS as control. The second, third, and fourth groups received twice-weekly i.p. injections of LY294002 at 10, 25, and 100 mg/kg. All mice were sacrificed after 4 weeks of treatment.

Treatment groups	Tumor weight in milligrams Mean \pm SD	Lymph nodes metastases	Liver metastases	Peritoneal carcinomatosis	Body weight in grams Mean \pm SD	
					Before	After
Control	921.7 \pm 266.8	10/10	9/10	4/10	26.54 \pm 2.62	26.45 \pm 2.36
LY294002 10 mg/kg	826.1 \pm 221.5	9/9	8/9	3/9	26.13 \pm 2.45	27.98 \pm 2.54
LY294002 25 mg/kg	740.8 \pm 214.6	9/10	8/10	3/10	26.87 \pm 2.98	29.08 \pm 3.04
LY294002 100 mg/kg	615.3 \pm 184.5 ^a	8/10	5/10	1/10	26.64 \pm 2.43	29.04 \pm 2.89

^a $P < 0.005$ versus control.

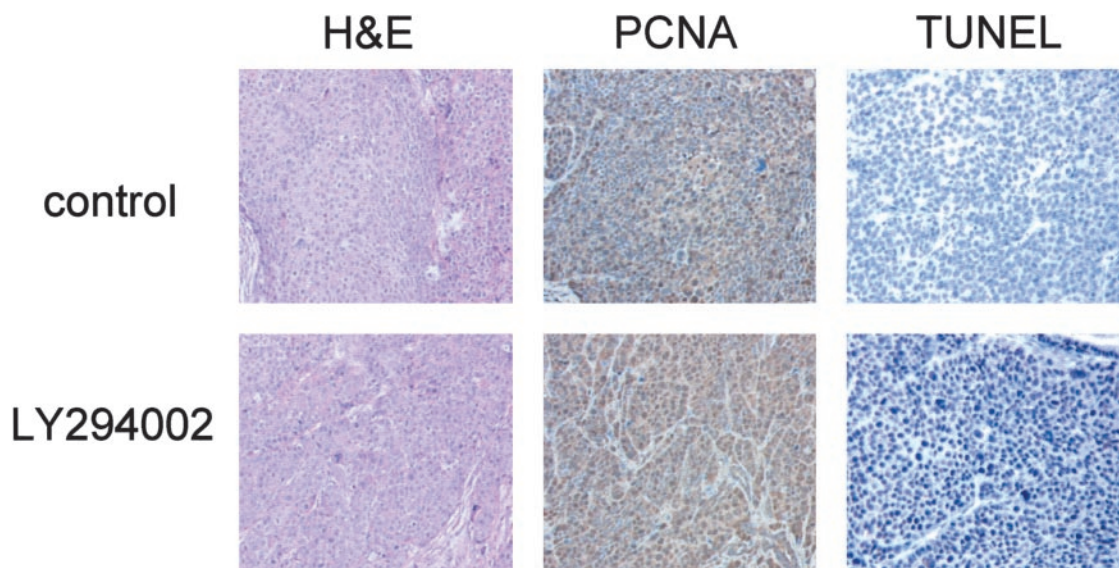


Fig. 5. Proliferation and apoptosis in orthotopic tumor xenografts. Mice bearing established (7-day) orthotopic L3.6pl tumors were treated biweekly with 100 mg/kg LY294002 via i.p. injection. After 4 weeks of therapy, animals were sacrificed, and excised tumors were analyzed by H&E staining, expression of Ki-67 antigen, or apoptosis as described in "Materials and Methods."

$P < 0.025$). Combined therapy with LY294002 and gemcitabine produced a 54% reduction in tumor volume compared with control tumors, and the combination was superior to the use of gemcitabine or LY294002 alone (LY294002 + gemcitabine group versus control, gemcitabine group, or LY294002 group; $P < 0.0005$). The magnitude of tumor growth inhibition in animals treated with the LY294002+gemcitabine combination suggests that the drugs interact in an additive fashion.

Discussion

Recent studies have implicated the PI-3 kinase/AKT pathway in the suppression of apoptosis in a variety of different model systems. We found that seven of nine arbitrarily selected human pancreatic adenocarcinoma cell lines displayed elevated baseline levels of activated AKT. Of interest, all seven of the lines have mutations in codon 12 of *K-ras* gene (25),⁵ whereas the two cell lines that did not display constitutive AKT phosphory-

lation express either wild-type *K-ras* (BxPC-3; Ref. 25) or a form of *K-ras* that is mutated at codon 61 (Hs766T; Ref. 26). Although our observations do not establish a causal relationship between codon 12 mutant (active) *K-ras* isoforms and PI3k-AKT activation, they strongly suggest that a relationship might exist (4, 27, 28). In ongoing studies we are characterizing the effects of enforced expression of mutant active *K-ras* codon 12 versus codon 61 in the BxPC-3 cells that still possess endogenous wild-type protein.

We also found that wortmannin and LY294002 induced apoptosis in six of seven of the cell lines that displayed elevated baseline AKT phosphorylation, whereas the cells that did not express constitutively active AKT (BxPC-3 and HS766T) were resistant. The only exception to this rule was AsPc-1, which displayed constitutive (albeit low level) AKT phosphorylation but did not undergo apoptosis after exposure to either inhibitor. Previous work has demonstrated that the *AKT2* gene is amplified in AsPc-1 cells, and it is possible that this contributes to their resistance (12, 13). More importantly, wortmannin only marginally down-regulated phos-

⁵ V. Bondar, unpublished observations.

Fig. 6. Determination of AKT and activated AKT in pancreatic cancer xenografts. Mice bearing s.c. L3.6pl tumors were treated with 100 mg/kg LY294002 via biweekly i.p. injection. Mice were sacrificed after 4 weeks, and levels of total and phosphorylated (S473) AKT were measured by immunohistochemistry. Note that LY294002 therapy reduced levels of phosphorylated AKT to background levels, whereas the effects of therapy on total AKT levels were much less pronounced, confirming drug targeting *in vivo*.

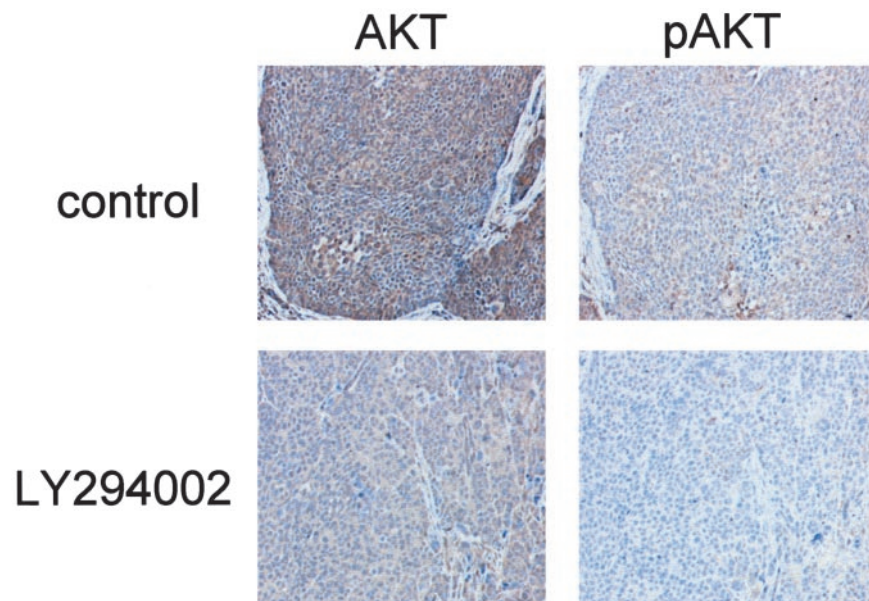


Table 2 Therapy of human pancreatic carcinoma growing subcutaneously in nude mice

One million of Colo-357-L3.6pl cells were mixed with Matrigel (3.5 mg of Matrigel in 0.5 ml of HBSS). Each mouse underwent two s.c. injections in bilateral flank regions. Seven days later, the mice were randomly assigned to four treatment groups of 5 mice each. The first group received twice-weekly i.p. injections of LY294002 at 25 mg/kg, the second group received gemcitabine at 62 mg/kg, the third group received combination of 25 mg/kg LY294002 and 62 mg/kg gemcitabine, and the last group received 5% DMSO in HBSS as control. All mice were sacrificed after 3 weeks of treatment.

Treatment groups	Tumor volume in mm ³ Mean ± SD		Body weight in grams Mean ± SD	
	Before	After	Before	After
Control	191.8 ± 76.9	2314.1 ± 874.8	23.12 ± 3.18	27.52 ± 3.26
Gemcitabine 62 mg/kg	183.9 ± 67.6	1596 ± 350.1 ^a	23.66 ± 2.87	28.18 ± 2.54
LY294002 25 mg/kg	175 ± 41.1	1738.1 ± 387.4	23.09 ± 3.08	28.27 ± 3.34
LY294002 25 mg/kg + Gemcitabine 62 mg/kg	181.3 ± 51.3	1071.4 ± 224.3 ^b	22.92 ± 2.78	29.3 ± 3.01

^a $P < 0.025$ versus control.

^b $P < 0.0005$ versus all other groups.

pho-AKT levels in the cells, which contrasted sharply with the effects of wortmannin in the cells that underwent apoptosis, where complete down-regulation of phosphorylation was observed (Fig. 1). It is possible that PI3k is insensitive to these inhibitors in AsPc-1 cells and/or that they possess an alternative pathway for AKT phosphorylation not shared by the other cell lines. Another major pathway activated in human tumors is mediated by the ERK subfamily of MAPKs (29, 30). We found the pathway to be constitutively active in three of four of the human pancreatic cancer cell lines interrogated. More importantly, the MAP/ERK kinase-MAPK inhibitor PD98059 induced apoptosis in two of them, including the LY294002-resistant AsPC-1 cells. Together, these results confirm that AKT and the ERKs regulate independent survival pathways in pancreatic cancer cells. They also suggest that the *in vivo* effects of PD98059 and other inhibitors of the ERK pathway on the growth of human pancreatic tumors should be evaluated in future studies.

Previous reports have demonstrated that PI3k inhibitors produce antiproliferative effects on BxPC-3 cells in culture

(15) and in xenograft tumors *in vivo* (31). Although these results would appear to be at odds with data presented in this paper, we suspect that the growth inhibition observed in the previous studies was not because of apoptosis. The PI3k inhibitors used in this study and the previous ones are not completely specific for the type-I PI3ks (see below), and it is possible that the growth inhibition was caused by interference with one of these other important cellular targets. Our inability to detect constitutive AKT phosphorylation in the BxPC-3 cells is consistent with results obtained by independently by another laboratory (32).

It is also possible that cytotoxic effects of wortmannin and LY294002 were because of PI3k-AKT-independent effects. Both wortmannin and LY294002 inhibit the class II and III PI3ks, the ataxia telangiectasia mutated protein kinase and the related ATR kinase, the PAF400 and telomeric repeat amplification protocol components of the histone acetylase complex, and DNA-dependent protein kinase (reviewed in Ref. (18)). Furthermore, LY294002 inhibits the unrelated casein kinase-2 with potency similar to its effects on PI3k (33).

The tight correlation between constitutive AKT phosphorylation and drug sensitivity in our panel strongly supports a central role for AKT in drug-induced cell death. However, additional studies are required to directly determine the potential role(s) of these other drug targets in inhibitor-induced apoptosis.

The PI3k-AKT survival pathway plays a critical role in many aspects of cellular homeostasis (4, 5). As a result, we were concerned that PI3k inhibitors would interfere with the survival and/or proliferation of critical populations of normal cells and would display unacceptable toxicity. However, bi-weekly i.p. administration of up to 100 mg/kg LY294002 was exceptionally well tolerated, and this dose of drug produced significant inhibition of AKT phosphorylation, reduced tumor cell proliferation, and increased apoptosis in orthotopic L3.6pl pancreatic tumor xenografts. Furthermore, combined therapy with intermediate doses of LY294002 and gemcitabine was also well-tolerated, and produced additive inhibition of tumor growth in the s.c. L3.6pl tumors. It is possible that we could have increased the dose of LY294002 used in the combination therapy experiments to 100 mg/kg without producing significant toxicity and that this would have produced stronger effects on tumor growth. Depending on drug availability, we hope to be able to conduct more extensive combination therapy studies in the future. The decrease in total AKT levels observed in LY294002-treated tumors was unexpected, and we do not have an explanation for the observation at present, although it is conceivable that AKT itself or some other PH domain-containing protein regulates AKT expression in pancreatic tumor cells. Daily i.p. administration of LY294002 caused dry and scaly skin in mice in a previous study (34), presumably as the result of increased epidermal cell apoptosis that resulted in hyperkeratosis. We suspect that the increased toxicity observed in the study by Hu *et al.* (34) was related to the more aggressive regimen used.

The molecular basis of the broad-spectrum drug resistance inherent to pancreatic cancer is largely unknown. The "classical" mechanisms of drug resistance (*i.e.*, overexpression of drug efflux pumps) have been documented in pancreatic cancer, but they cannot explain the modest activities of MDR-independent agents like gemcitabine and 5-fluorouracil. Activation of the PI3k pathway in pancreatic cancer cells might be one of the drug resistance mechanisms responsible for these failures. Indeed, the PI3k-AKT survival pathway can mediate resistance to proapoptotic stimuli in fibroblasts and epithelial cells (27, 35). Ng *et al.* (14) demonstrated that wortmannin and LY294002 enhanced apoptosis induced by gemcitabine in otherwise drug-resistant pancreatic cancer cells. Additional studies are required to address this possibility and determine the extent to which inhibition of the PI3k/AKT pathway restores drug sensitivity.

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