

Optimization for the Blockade of Epidermal Growth Factor Receptor Signaling for Therapy of Human Pancreatic Carcinoma¹

Carmen C. Solorzano, Cheryl H. Baker, Rachel Tsan, Peter Traxler, Pamela Cohen, Elisabeth Buchdunger, Jerry J. Killion, and Isaiah J. Fidler²

Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [C. C. S., C. H. B., R. T., J. J. K., I. J. F.], and Novartis Pharma, CH-4002 Basel, Switzerland [P. T., P. C., E. B.]

ABSTRACT

We determined the optimal administration schedule of a novel epidermal growth factor receptor (EGFR) protein tyrosine kinase inhibitor (PKI), PKI 166 (4-(*R*)-phenethylamino-6-(hydroxyl)phenyl-7H-pyrrolo[2,3-*d*]-pyrimidine), alone or in combination with gemcitabine (administered *i.p.*) for therapy of L3.6pl human pancreatic carcinoma growing in the pancreas of nude mice. Seven days after orthotopic implantation of L3.6pl cells, the mice received daily oral doses of PKI 166. PKI 166 therapy significantly inhibited phosphorylation of the EGFR without affecting EGFR expression. EGFR phosphorylation was restored 72 h after cessation of therapy. Seven days after orthotopic injection of L3.6pl cells, groups of mice received daily or thrice weekly oral doses of PKI 166 alone or in combination with gemcitabine. Treatment with PKI 166 (daily), PKI 166 (3 times/week), or gemcitabine alone produced a 72%, 69%, or 70% reduction in the volume of pancreatic tumors in mice, respectively. Daily oral PKI 166 or thrice weekly oral PKI 166 in combination with injected gemcitabine produced 97% and 95% decreases in volume of pancreatic cancers and significant inhibition of lymph node and liver metastasis. Daily oral PKI 166 produced a 20% decrease in body weight, whereas treatment 3 times/week did not. Decreased microvessel density, decreased proliferating cell nuclear antigen staining, and increased tumor cell and endothelial cell apoptosis correlated with therapeutic success. Collectively, our results demonstrate that three weekly oral administrations of an EGFR tyrosine kinase inhibitor in combination with

gemcitabine are sufficient to significantly inhibit primary and metastatic human pancreatic carcinoma.

INTRODUCTION

The progressive growth and metastasis of neoplasms, including pancreatic cancers, depend in part on angiogenesis (1), the extent of which is determined by the balance between proangiogenic and antiangiogenic molecules released by tumor cells and normal host cells (2, 3). Cancer of the exocrine pancreas is characterized by extensive local invasion and early lymphatic and hematogenous metastasis (4, 5). At the time of diagnosis, more than 80% of patients present with either locally advanced or metastatic disease (6). The inability to detect pancreatic cancer at an early stage, its aggressiveness, and the lack of effective systemic therapy are responsible for rapid death from this disease. Indeed, only 1–4% of all patients with adenocarcinoma of the pancreas survive 5 years after diagnosis (7, 8). For patients with advanced pancreatic cancer, even the recent introduction of the deoxycytidine analogue gemcitabine does not extend median survival beyond 6 months (9). Clearly, novel approaches to therapy of human pancreatic carcinoma are urgently needed.

Candidates include the various growth factors that mediate tumor progression. Human pancreatic cancer cells growing in culture express high levels of EGFR³ and produce TGF- α (10), and *in vivo*, human pancreatic cancers overexpress EGFR and all five known ligands (10–13). Moreover, receptor protein tyrosine kinases such as EGFR (11), c-erbB2 (14), insulin-like growth factor I (15), and fibroblast growth factor receptor (16) are highly expressed in human pancreatic cancer tissues and pancreatic cancer cell lines. The overexpression of EGFR, TGF- α , and EGF in human pancreatic tumors correlates with rapidly progressive disease (17), and expression of a truncated EGFR was associated with inhibition of pancreatic cancer cell growth and enhanced sensitivity to cisplatin (18).

Human pancreatic cancer cells secrete the proangiogenic molecules VEGF, IL-8, and basic fibroblast growth factor (19, 20). VEGF, currently regarded as the major proangiogenic factor for most types of human cancer (21), is strongly induced by EGF and TGF- α (22, 23). Thus, both EGFR-mediated proliferation and angiogenesis can contribute to the progressive growth

Received 2/28/01; revised 5/22/01; accepted 5/30/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported in part by Cancer Center Support Core Grant CA16672 and Grant R35-CA42107 (to I. J. F.) from the National Cancer Institute, NIH.

² To whom requests for reprints should be addressed, at Department of Cancer Biology-173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-8577; Fax: (713) 792-8747; E-mail: ifidler@mdanderson.org.

³ The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; IL-8, interleukin 8; PCNA, proliferating cell nuclear antigen; IHC, immunohistochemistry; MVD, microvessel density; PKI, protein tyrosine kinase inhibitor; PECAM, platelet/endothelial cell adhesion molecule; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; FBS, fetal bovine serum; MAb, monoclonal antibody.

of human pancreatic carcinoma and have been independently evaluated as targets for therapy (1, 3, 14, 16, 18–26).

Recent work from our laboratory demonstrated that daily oral administration of an EGFR tyrosine kinase inhibitor, PKI 166(4-(*R*)-phenethylamino-6-(hydroxyl)phenyl-7H-pyrrolo[2.3-*d*]pyrimidine), in combination with gemcitabine inhibited progressive growth and metastasis of human pancreatic cancer implanted orthotopically in nude mice. Combination therapy with PKI 166 plus gemcitabine produced significant therapeutic effects mediated in part by induction of apoptosis in tumor-associated endothelial cells (27). Whether daily doses of PKI 166 were essential or optimal for therapy remained unclear. The purpose of the present study was to determine the optimal administration schedule of PKI 166 for therapy of human pancreatic carcinoma growing in the pancreas of nude mice. We show that thrice weekly oral administration of PKI 166 combined with twice weekly gemcitabine did not have toxic effects and produced significant therapeutic effects by inducing apoptosis in tumor cells and tumor-associated endothelial cells.

MATERIALS AND METHODS

Pancreatic Cancer Cell Lines and Culture Conditions.

The highly metastatic human pancreatic cancer cell line L3.6pl was maintained in DMEM supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, *L*-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and a penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were free of *Mycoplasma* and the following pathogenic murine viruses: (a) reovirus type 3; (b) pneumonia virus; (c) K virus; (d) Theiler's encephalitis virus; (e) Sendai virus; (f) minute virus; (g) mouse adenovirus; (h) mouse hepatitis virus; (i) lymphocytic choriomeningitis virus; (j) ectromelia virus; and (k) lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 week after recovery from frozen stocks.

Reagents. PKI 166, a novel EGFR tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). For *in vivo* administration, PKI 166 was dissolved in DMSO/0.5% Tween 80 and then diluted 1:20 (v/v) in water (28). All antibodies were purchased from the manufacturers indicated below: (a) rabbit anti-VEGF/vascular permeability factor, Santa Cruz Biotechnology (Santa Cruz, CA); (b) polyclonal rabbit antihuman IL-8, Biosource International (Camarillo CA); (c) rat antimouse CD31/PECAM-1 and peroxidase-conjugated rat antimouse IgG1, PharMingen (San Diego, CA); (d) mouse anti-PCNA clone PC 10, DAKO A/S (Copenhagen, Denmark); (e) mouse antihuman EGFR (activated form) IgG1, Chemicon (Temecula, CA); (f) monoclonal mouse antihuman IgG1 EGFR clone 30, Biogenex (San Ramon, CA); (g) peroxidase-conjugated F(ab')₂ goat antirabbit IgG F(ab')₂, peroxidase-conjugated goat antimouse IgG F(ab)₂ fragment, aff-nipure Fab fragment goat antimouse IgG, and peroxidase-conjugated goat antirat IgG, Serotec, Harlan Bioproducts for Science, Inc. (Indianapolis, IN); (h) Texas Red-conjugated goat antirat IgG, Jackson Research Laboratories (West Grove, CA); (i) peroxidase-conjugated rat antimouse IgG2a, Serotec, Harlan Bioproducts for Science, Inc.; (j) monoclonal antiphosphoty-

rosine MAb 4G10 and polyclonal sheep antihuman EGFR, Upstate Biotechnology (Lake Placid, NY); and (k) MAb anti-EGFR (clone EGFRI; Amersham, Arlington Heights, IL). Other reagents were human IgG (Sigma Chemical Co., St. Louis, MO), Hoechst dye 3342 molecular weight 615.9 (Hoechst, Warrington, PA), stable 3,3'-diaminobenzidine (Research Genetics, Huntsville, AL), 3-amino-9-ethylcarbazole (Biogenex), and Gill's hematoxylin (Sigma Chemical Co.). Prolong solution was purchased from Molecular Probes (Eugene, OR). Pepsin was purchased from Biomed (Foster City, CA). The enhanced chemiluminescence detection system was purchased from Amersham. TUNEL was performed using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

Western Blot Analysis of EGFR Autophosphorylation after Treatment and Removal of PKI 166. Serum-starved L3.6pl cells were treated with PKI 166 (0.5 μM) for 60 min and then incubated with or without 40 ng/ml recombinant human EGF for 15 min, washed, scraped into PBS containing 5 mM EDTA and 1 mM sodium orthovanadate, and centrifuged. The pellet was resuspended in lysis buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 0.15 unit/ml aprotinin], sonicated, and centrifuged to recover insoluble protein. To determine how long the effects of the inhibitor would last after its removal, L3.6pl cells were serum-starved, treated with 0.5 μM PKI 166 for 60 min, and washed with serum-free medium for 24, 48, or 72 h. Cells were then challenged with or without 40 ng/ml recombinant human EGF for 15 min, washed, and treated as described above. Immunoprecipitation was performed using MAb anti-EGFR (clone EGFRI) as described previously (26). Immunoprecipitates were analyzed on 7.5% SDS-PAGE and transferred onto 0.45-μm nitrocellulose membranes. The filters were blocked with 3% BSA in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl, probed with either polyclonal sheep antihuman EGFR (1:1000) or monoclonal antiphosphotyrosine (MAb 4G10; 1:2000) in TTBS (0.1% Tween 20 in Tris-buffered saline), and incubated with horseradish peroxidase-conjugated donkey antisheep IgG (1:2000; Sigma Chemical Co.) or sheep antimouse IgG (1:2000), respectively, in TTBS. Protein bands were visualized by enhanced chemiluminescence detection system.

Animals and Orthotopic Implantation of Tumor Cells.

Male athymic nude mice (NCI-nu) were purchased 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. The facilities were approved by the American Association for Accreditation of Laboratory Animal Care and were in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8–12 weeks old.

To produce tumors, L3.6pl cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with greater than 90% viability were used for the

injections. Injection of cells into the pancreas was performed as described previously (19). The mice were killed when the controls became moribund (5–6 weeks) or at predetermined times for pharmacokinetics determinations. The size and weight of the primary pancreatic tumors, the incidence of regional (celiac and para-aortal) lymph node metastasis, and the presence or absence of liver metastases were recorded. Histopathology confirmed the identity of the disease. For IHC 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, Elkhart, IN), frozen rapidly in liquid nitrogen, and stored at -70°C .

Therapy of Established Human Pancreatic Carcinoma Tumors Growing in the Pancreas of Athymic Nude Mice. Seven days after implantation of tumor cells into the pancreas, five mice were killed, and the presence of tumor lesions was determined. At that time, the median tumor volume was 18 mm^3 . Histological examination confirmed the lesions to be actively growing pancreatic cancer. The mice were randomized into six groups as follows ($n = 10$): (a) group 1, oral vehicle solution for PKI 166 (DMSO/0.5% Tween 80 diluted 1:20 in water) and i.p. HBSS (control group); (b) group 2, twice weekly i.p. injections of 125 mg/kg gemcitabine alone; (c) group 3, daily oral administration of 50 mg/kg PKI 166 alone; (d) group 4, thrice weekly oral administration of 50 mg/kg PKI 166 alone; (e) group 5, daily oral administration of 50 mg/kg PKI 166 combined with twice weekly i.p. injections of 125 mg/kg gemcitabine; and (f) group 6, thrice weekly oral administration of 50 mg/kg PKI 166 combined with twice weekly i.p. injections of 125 mg/kg gemcitabine.

Necropsy Procedures and Histological Studies. Mice were euthanized and weighed. Primary tumors in the pancreas were excised and weighed. For IHC and H&E staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, and the part was embedded in OCT compound (Miles), frozen rapidly in liquid nitrogen, and stored at -70°C . Visible liver metastases were counted with the aid of a dissecting microscope and processed for H&E staining. All macroscopically enlarged regional (celiac and para-aortal) lymph nodes were harvested, and the presence of metastatic disease was confirmed by histology.

Pharmacodynamic End Point of Target Inhibition. Seven days after implantation of tumor cells into the pancreas, mice were randomized into two treatment groups ($n = 12$): (a) group a, daily oral administration of 50 mg/kg PKI 166; or (b) group b, daily oral vehicle solution for PKI 166 (DMSO/0.5% Tween 80 diluted 1:20 in water). Mice from group a were treated for 7 days, at which time treatment was stopped, and three mice were killed. The remaining mice in this group were killed (three mice at a time) 24, 48, and 72 h after cessation of treatment. All tumors were processed for IHC as described previously (25). The mice from group b were killed ($n = 3$) at the same time as the mice from group a and served as controls.

Immunohistochemical Determination of VEGF, IL-8, PCNA, CD31/PECAM-1, and EGFR. Paraffin-embedded tissues were used for identification of VEGF, IL-8, and PCNA. Sections ($4\text{--}6\text{-}\mu\text{m}$ thick) were mounted on positively charged Superfrost slides (Fisher Scientific, Houston, TX) and dried overnight. Sections were deparaffinized in xylene followed by

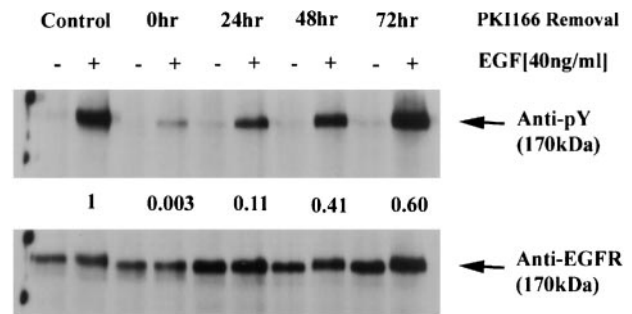


Fig. 1 Inhibition of EGF-induced autophosphorylation of EGFR. L3.6pl human pancreatic carcinoma cells were incubated for 60 min in serum-free medium (control) or medium containing $0.5\text{ }\mu\text{M}$ PKI 166. All cultures were then stimulated for 15 min with 40 ng/ml EGF. The cultures were washed and refed with serum-free medium. The levels of EGFR protein (M_r 170,000) and phosphorylated EGFR (M_r 170,000) were determined by Western blotting. Expression of EGFR did not vary among the groups. PKI 166 inhibited phosphorylation of the EGFR.

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 analyzed for PCNA were microwaved for 5 min for antigen retrieval (29). All other paraffin-embedded tissues were treated with pepsin (Biomed) for 15 min at 37°C and washed with PBS (30). Frozen tissues used for identification of CD31/PECAM-1, EGFR, and activated EGFR were sectioned ($8\text{--}10\text{ }\mu\text{m}$), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), acetone/chloroform (v/v; 5 min), and acetone (5 min) and washed with PBS. Immunohistochemical procedures were performed as described previously (25). A positive reaction was visualized by incubating the slides with stable 3,3'-diaminobenzidine for 10–20 min. The sections were rinsed with distilled water, counterstained with Gill's hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics). No counterstaining was used when staining for EGFR or activated EGFR. Sections analyzed for activated EGFR were pretreated with goat antimouse IgG F(ab)₂ fragment (1:10 dilution in PBS) for 4–6 h before incubation with the primary antibody. Control samples exposed to secondary antibody alone showed no specific staining.

Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells). Frozen tissues were sectioned ($8\text{--}10\text{ }\mu\text{m}$), mounted on positively charged slides, air-dried for 30 min, and fixed in cold acetone for 5 min, in 1:1 acetone:chloroform (v/v) for 5 min, and in acetone for 5 min. Samples were washed three times with PBS, incubated with protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and then incubated with the appropriate dilution (1:400; v/v) of rat monoclonal antimouse CD31 antibody (human cross-reactive) for 18 h at 4°C . After the samples were rinsed four times for 3 min each with PBS, they were incubated with the appropriate dilution (1:200) of Texas Red-conjugated secondary goat antirat antibody for 1 h at room temperature in the dark. Samples were washed twice with PBS containing 0.1% Brij and washed with PBS for 5 min. TUNEL was performed using a commercially available apoptosis detec-

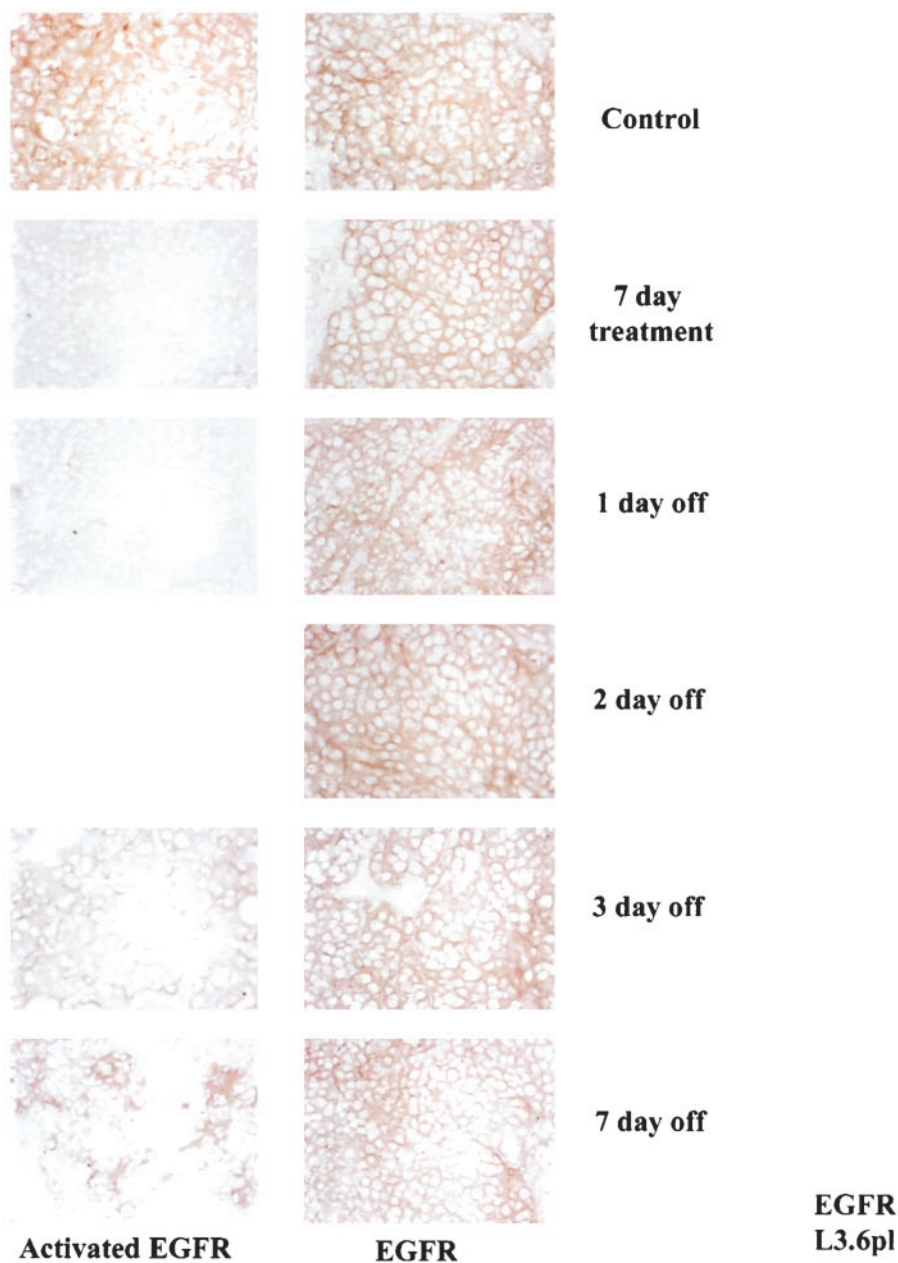


Fig. 2 IHC determination of EGFR and activated EGFR in pancreatic cancers after withdrawal of PKI 166. L3.6pl human pancreatic cancer cells were implanted into the pancreas of nude mice. Daily oral doses of 50 mg/kg PKI 166 were given for 7 days. Groups of mice ($n = 3$) were killed on day 7 (the last day of treatment). Other groups of mice ($n = 3$) were killed 1, 2, 3, or 7 days after cessation of therapy. The pancreatic lesions were harvested and processed for IHC to demonstrate expression of EGFR and phosphorylated activated EGFR. The expression of EGFR did not vary among the treatment groups. In contrast, seven daily oral doses of PKI 166 significantly inhibited expression of activated EGFR. Activated EGFR was reexpressed in lesions harvested from mice no earlier than 3 days after cessation of therapy.

tion kit with the following modifications: samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After being washed twice for 5 min each with PBS, the samples were incubated with equilibration buffer (from kit) for 10 min at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mixture, and terminal deoxynucleotidyl transferase enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h in the dark. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were washed three times for 5 min to remove unincorporated fluo-

rescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 $\mu\text{g/ml}$ Hoechst stain for 10 min at room temperature. Fluorescent bleaching was minimized by treating slides with an enhancing reagent (Prolong solution). Immunofluorescence microscopy was performed using a $\times 40$ objective and an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a Sony 3-chip camera (Sony Corp. of America, Montvale, NJ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas image analysis software (BioScan, Edmond, WA) installed on a Compaq computer with a

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

Treatment group ^a	Pancreatic tumors						
	Incidence	Tumor volume (mm ³)		Metastasis		Body weight (g)	
		Median	Range	Liver	Lymph	Median	Range
Saline control	10/10 ^b	574	445–770	4/10 ^b	9/10 ^b	22	18–30
Gemcitabine	10/10	230 ^c	64–445	3/10	5/10	24	17–26
PKI 166, 7 days/wk	10/10	162 ^d	16–274	2/10	8/10	24	21–31
PKI 166, 7 days/wk + gemcitabine	10/10	18 ^d	6–80	0/10	1/10	18 ^e	15–24
PKI 166, 3 days/wk	10/10	174 ^c	88–607	1/10	7/10	25	21–31
PKI 166, 3 days/wk + gemcitabine	10/10	27 ^d	6–72	1/10	3/10	22	15–25

^a L3.6pl human pancreatic cancer cells (1×10^6) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, daily or triweekly oral feedings of PKI 166 (50 mg/kg) alone, gemcitabine and PKI 166, or saline (control). All mice were killed on day 35.

^b Number of positive mice/number of mice injected.

^c $P < 0.001$ versus control.

^d $P < 0.0001$ versus control.

^e $P < 0.01$ versus control.

Pentium chip, a frame grabber, an optical disc storage system, and a Sony Mavigraph UP-D7000 Digital color printer (Tokyo, Japan). Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm² fields at $\times 400$ magnification. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random 0.159-mm² fields at $\times 100$ magnification.

Quantification of MVD, PCNA, and Absorbance. For the quantification of MVD, 10 random 0.159-mm² fields at $\times 100$ magnification were captured for each tumor, and microvessels were quantified according to the method described previously (31, 32). For the quantification of the immunohistochemical reaction intensity, the absorbance of 100 VEGF- and IL-8-positive cells in 10 random 0.039-mm² fields at $\times 100$ magnification of tumor tissues was measured using Optimas image analysis software (19, 20, 26). The samples were not counterstained, so the absorbance was due solely to the product of the immunohistochemical reaction. VEGF and IL-8 cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and expressed as the ratio of tumor cell expression to normal pancreatic gland expression multiplied by 100 (31, 32). For the quantification of PCNA expression, the number of positive cells was quantified in 10 random 0.159-mm² fields at $\times 100$ magnification.

Statistical Analysis. Pancreatic tumor volume; expression of VEGF and IL-8; quantification of PCNA, TUNEL, and CD31; and the percentage of apoptotic endothelial cells were compared by unpaired Student's *t* test. The incidence of metastasis between groups was compared using Fisher's exact test.

RESULTS

Duration of *in Vitro* Inhibition of EGFR Autophosphorylation. We have reported that pretreatment of L3.6pl cells with PKI 166 for 60 min followed by a 15-min treatment with

EGF inhibited EGF-stimulated tyrosine phosphorylation of the EGFR in a dose-dependent manner (0.01–0.5 μ M; Ref. 27). In the present study, we incubated L3.6pl cells with serum-free medium containing 40 ng/ml EGF for 15 min. The cells exhibited high levels of autophosphorylated EGFR (M_r 170,000 band) detected by antiphosphotyrosine antisera. To determine how long the inhibitory effect of PKI 166 lasted after its removal, cells pretreated with 0.5 μ M PKI 166 for 60 min were washed and incubated with serum-free medium for 24, 48, or 72 h and then reexposed to 40 ng/ml EGF. A progressive increase in autophosphorylation of EGFR reached the maximum by 72 h after PKI 166 was removed. The M_r 170,000 band was confirmed to be EGFR by Western blot analysis using anti-EGFR antisera (Fig. 1).

***In Vivo* Pharmacokinetics of PKI 166.** Mice given pancreatic implants of L3.6pl cells were treated with daily oral doses of 50 mg/kg PKI 166 for 7 consecutive days. Three mice were killed within 1 h after the last treatment, and the pancreatic tumors were harvested. IHC using specific anti-EGFR antibodies and antibodies specific against tyrosine-phosphorylated (activated) EGFR demonstrated that tumors from mice treated for 7 days with oral PKI 166 and tumors from control mice (receiving vehicle solution) expressed similar levels of EGFR, whereas only tumors from control mice were positive for activated EGFR (Fig. 2). Tumors were harvested 24 h, 48 h, 72 h, or 7 days after cessation of PKI 166 treatment. At all time points, the tumors expressed similar levels of EGFR protein. In contrast, treatment with oral PKI 166 (50 mg/kg/dose) inhibited phosphorylation of the EGFR for up to 3 days after cessation of therapy (Fig. 2), indicating that the inhibition of EGFR phosphorylation by PKI 166 does not require daily oral administration.

Effectiveness of Triweekly versus Daily Oral PKI 166 Administration in Suppressing Growth and Metastasis of Human L3.6pl Pancreatic Cancer in Nude Mice. Athymic nude mice were given injections of 1×10^6 viable L3.6pl cells in the pancreas. Seven days later, the mice were randomized into six treatment groups of 10 mice each. The first control group received oral vehicle solution for PKI 166 (DMSO/0.5% Tween 80 diluted 1:20 in water) and i.p. HBSS, the second group received twice weekly i.p. injections of 125 mg/kg gemcitabine alone, the third

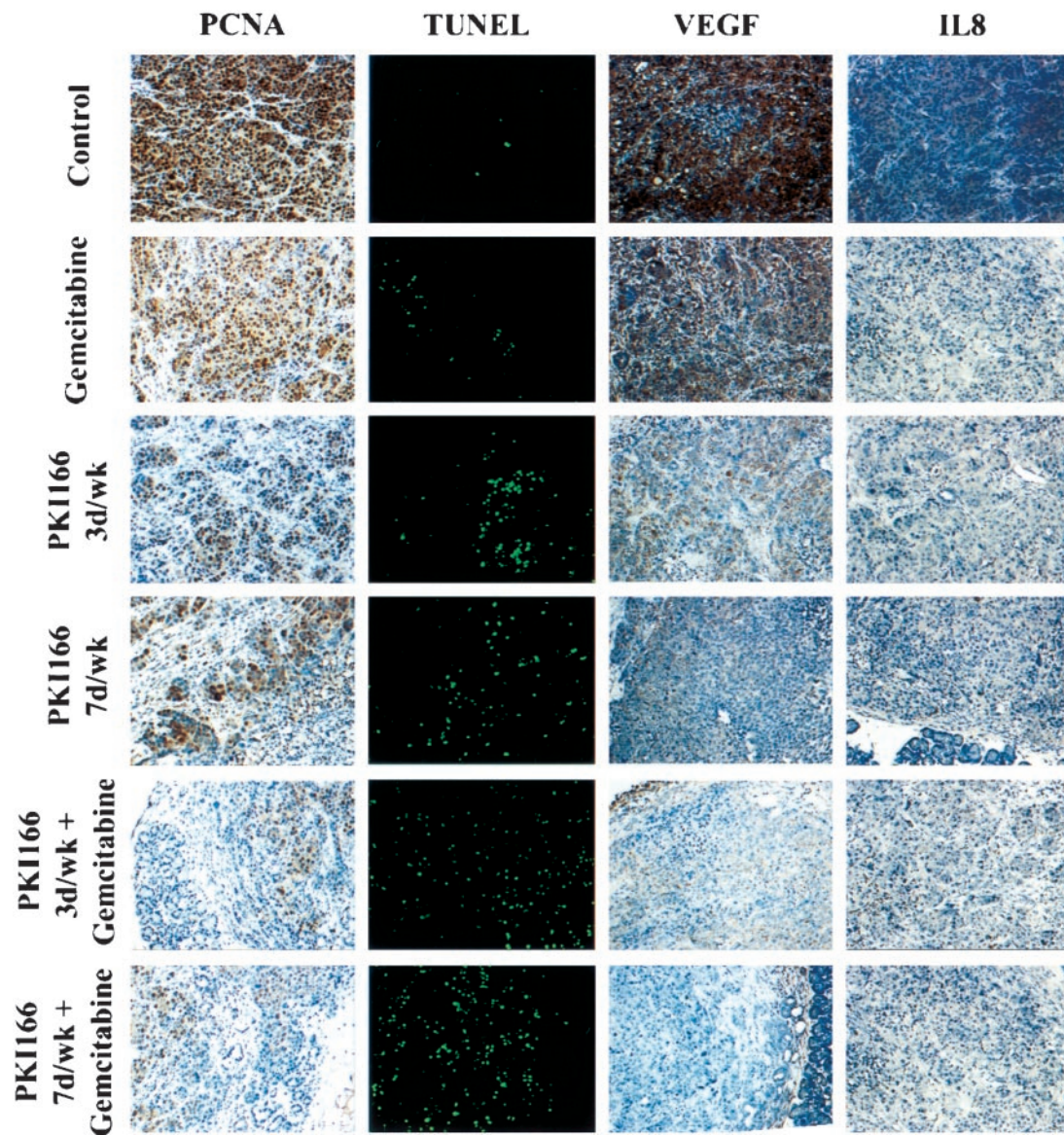


Fig. 3 Immunohistochemical analyses of tumors from control or PKI 166-treated mice. Tumors were harvested from control mice, mice treated with gemcitabine, mice treated 3 times/week with PKI 166, mice treated daily with PKI 166, mice treated 3 times/week with PKI 166 + gemcitabine, or mice treated daily with PKI 166 + gemcitabine. The sections were immunostained for expression of PCNA (to show cell proliferation), TUNEL (FITC; to show apoptosis), VEGF, and IL-8. Tumors from mice treated with PKI 166 alone or with PKI 166 + gemcitabine exhibited marked decreases in the number of PCNA-positive cells and increases in the number of TUNEL-positive cells. Tumors from mice treated with both compounds (regardless of the scheduling of oral PKI 166) had decreased immunoreactivity for VEGF and IL-8.

group received daily oral administrations of 50 mg/kg PKI 166 alone, the fourth group received thrice weekly oral administration of 50 mg/kg PKI 166 alone, the fifth group received daily oral administrations of 50 mg/kg PKI 166 and twice weekly i.p. injections of 125 mg/kg gemcitabine, and the sixth group received thrice weekly oral administration of 50 mg/kg PKI 166 and twice weekly i.p. injections of 125 mg/kg gemcitabine. All mice were killed on day 35 because the control mice were moribund.

Detailed necropsy revealed that all of the mice had tumors in the pancreas. The data summarized in Table 1 show that daily oral PKI 166 alone, thrice weekly oral PKI 166 alone, or twice weekly

i.p. injections of gemcitabine alone significantly decreased median tumor volume as compared with that seen in control mice (162, 174, 230, and 574 mm³, respectively; $P < 0.001$). The combination of gemcitabine and PKI 166 given daily or thrice weekly produced a greater decrease in median volume of pancreatic tumors (to 18 and 27 mm³, respectively; $P < 0.0001$). Daily oral administration of PKI 166 combined with gemcitabine decreased body weight 20% ($P < 0.01$), whereas thrice weekly administration of PKI 166 and twice weekly administration of gemcitabine did not decrease body weight.

Visible liver metastases (enumerated with the aid of a

Table 2 Immunohistochemical analysis of human pancreatic carcinoma in the pancreas of control and treated nude mice

Treatment group ^a	Tumor cells				Endothelial cells	
	PCNA ⁺	TUNEL ⁺	VEGF	IL-8	CD31 ⁺	% TUNEL ⁺
Control	594 ± 47 ^b	13 ± 6 ^b	265 ± 21 ^c	235 ± 7 ^c	82 ± 25 ^b	1.6 ^d
Gemcitabine	353 ± 18	47 ± 13 ^e	245 ± 35	165 ± 7 ^e	65 ± 1	2.6
PKI 166, 3 days/wk	165 ± 4 ^e	71 ± 8 ^f	180 ± 10 ^e	175 ± 7 ^e	37 ± 4 ^e	20 ^e
PKI 166, 7 days/wk	95 ± 25 ^e	100 ± 20 ^f	145 ± 7 ^e	170 ± 14 ^e	29 ± 6 ^e	22 ^e
PKI 166, 3 days/wk + gemcitabine	84 ± 14 ^e	85 ± 13 ^f	143 ± 15 ^e	160 ± 14 ^e	9 ± 3 ^f	27 ^f
PKI 166, 7 days/wk + gemcitabine	85 ± 13 ^e	127 ± 17 ^f	124 ± 28 ^e	155 ± 7 ^e	8 ± 1 ^f	30 ^f

^a L3.6pl human pancreatic cancer cells (1×10^6) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, daily (3 or 7 days a week) oral feedings of PKI 166 (50 mg/kg) alone, PKI 166 in combination with gemcitabine, or saline (control). All mice were killed on day 35.

^b The number (mean ± SD) of positive cells/field determined from measurement of 10 random 0.159-mm² fields at $\times 100$ magnification.

^c Mean ± SD absorbance, determined as described in "Materials and Methods."

^d The number (mean ± SD) of CD31/TUNEL-positive cells in 10 random 0.011-mm² fields at $\times 400$ magnification. Fluorescent double labeling was performed on frozen tissue sections.

^e $P < 0.05$ as compared with controls.

^f $P < 0.001$ as compared with controls.

dissecting microscope) were present in 40% of control mice and 0–10% of the PKI 166 and gemcitabine combination-treated groups (Table 1). Histologically positive regional lymph node metastases were found in 90% of control animals, in 50% of gemcitabine-treated animals, and in 70–80% of PKI 166-treated animals. However, only 10–30% of animals receiving both gemcitabine and PKI 166 had histologically positive regional lymph node metastases (Table 1).

Histology and IHC Analyses. Tumors harvested from the different treatment groups were processed for routine histological and IHC analyses. Tumors from mice treated with gemcitabine and PKI 166 exhibited necrotic zones and contained a large number of infiltrating cells. Cell proliferation and apoptosis were analyzed by using anti-PCNA antibodies and the TUNEL method, respectively (Fig. 3). The mean number of PCNA-positive tumor cells in control tumors was 594 ± 47 . After therapy with gemcitabine, PKI 166 thrice weekly, or PKI 166 daily, it was 353 ± 18 , 165 ± 4 , and 95 ± 25 , respectively (Table 2). The lowest number of PCNA-positive cells was found in tumors of mice treated with both gemcitabine and PKI 166, regardless of the schedule of oral PKI 166 administration (84 ± 14 and 85 ± 13 ; $P < 0.05$ versus control).

The mean number of TUNEL-positive cells was inversely correlated with PCNA positivity. In control tumors, the mean number of TUNEL-positive cells was 13 ± 6 ; in gemcitabine-treated tumors, it was 47 ± 13 ; in daily PKI 166-treated tumors, it was 100 ± 20 ; and in tumors treated thrice weekly with PKI 166, it was 71 ± 8 . In combination-treated tumors, it was 127 ± 17 for daily treatments and 85 ± 13 for triweekly treatments (control versus gemcitabine, $P < 0.05$; all others versus control, $P < 0.001$). There was no significant difference in the number of TUNEL-positive tumor cells in tumors treated with gemcitabine or PKI 166 alone as compared with tumors treated with the combination therapy. The calculated ratio of PCNA-positive cells:TUNEL-positive cells was 1.1, 0.64, 0.43, and 3.61 in mice treated with gemcitabine alone, PKI 166 alone, combination therapy, and control mice, respectively (Fig. 3; Table 2).

Production of VEGF and IL-8 by the pancreatic cancer cells was significantly reduced ($P < 0.001$) 35 days after initi-

ation of treatment with PKI 166 or PKI 166 and gemcitabine as compared with control or gemcitabine treatment alone (Fig. 3, Table 2). To determine whether blockade of the EGFR signaling pathway by PKI 166 down-regulated expression of VEGF and IL-8 in the L3.6pl cells, we incubated the cells for 72 h in medium supplemented with 5% FBS with or without $1 \mu\text{M}$ PKI 166 or 10 nM gemcitabine. The culture supernatants were harvested, and the levels of VEGF and IL-8 proteins were determined by ELISA. Control cells produced 1400 pg/ml VEGF and 1440 pg/ml IL-8. Gemcitabine-treated cells produced 1280 pg/ml VEGF and 2000 pg/ml IL-8. Cells treated with PKI 166 produced 180 pg/ml VEGF ($P < 0.001$) and 640 pg/ml IL-8 ($P < 0.01$), suggesting that blockade of the EGFR signaling pathway decreased the production of two important proangiogenic molecules. Basic fibroblast growth factor protein was unaffected.

MVD (measured by staining with antibodies against CD31) was directly proportional to expression of VEGF and IL-8; *i.e.*, we found a significant reduction in tumor MVD per field after treatment with PKI 166 (24 ± 18) or combination therapy (24 ± 13) as compared with control tumors (60 ± 23) or gemcitabine-treated tumors (52 ± 18 ; control versus PKI 166, $P < 0.0005$; control versus PKI 166 and gemcitabine, $P < 0.0002$; Fig. 4; Table 2).

Finally, the CD31/TUNEL fluorescent double-labeling technique (27) revealed that many endothelial cells in tumors treated with PKI 166 or PKI 166 and gemcitabine underwent apoptosis (yellow reaction). A significant increase in the percentage of apoptotic endothelial cells over total endothelial cells was found in pancreatic tumors harvested 28 days after the initiation of treatment with PKI 166 (20 ± 15) or PKI 166 + gemcitabine (30 ± 20) as compared with control tumors or gemcitabine-treated tumors ($P < 0.001$; Fig. 4; Table 2).

DISCUSSION

Our data confirm that blockade of the EGFR signaling pathway by oral administration of the novel EGFR tyrosine kinase inhibitor PKI 166 combined with i.p. injection of gemcitabine significantly inhibits the growth and metastasis of hu-

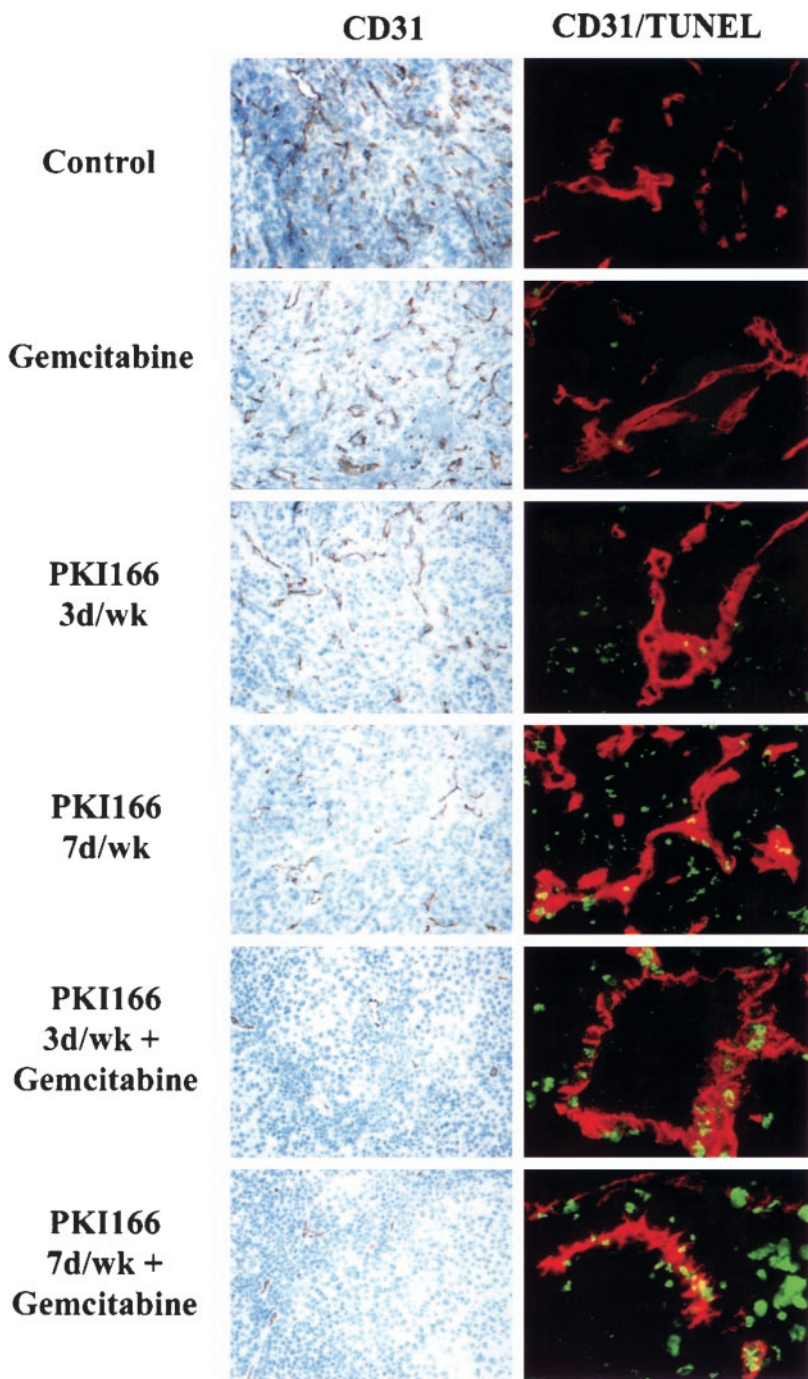


Fig. 4 Vascular density and endothelial cell apoptosis. Pancreatic tumors (L3.6pl cells) from mice treated with vehicle (control), mice treated with gemcitabine, mice treated 3 times/week with PKI 166, mice treated daily with PKI 166, mice treated 3 times/week with PKI 166 + gemcitabine, or mice treated daily with PKI 166 + gemcitabine were harvested on day 28 of the treatment. Sections were immunostained for expression of CD31/PECAM-1 (MVD) or anti-CD31 antibodies (Texas Red) and TUNEL (FITC-green). Multiple sections were examined, and a representative sample (×400) is shown. Treatment with PKI 166 or gemcitabine decreased mean vessel density. Treatment with both PKI 166 and gemcitabine significantly decreased the number of CD31-positive endothelial cells. This decrease was directly correlated with the induction of apoptosis. *Fluorescent red*, CD31-positive endothelial cells; *fluorescent green*, TUNEL-positive cells; *yellow*, TUNEL-positive, CD31-positive cells.

man pancreatic carcinoma cells implanted into the pancreas of nude mice. Under both *in vitro* and *in vivo* conditions, control L3.6pl cells and cells treated with PKI 166 expressed similar levels of nonactivated EGFR. Treatment with PKI 166 inhibited phosphorylation of the EGFR. The receptor was rephosphorylated 48–72 h after cessation of PKI 166 administration.

PKI 166 is a reversible inhibitor of the EGFR (and c-erbB2) tyrosine kinase (28). The compound inhibits the intracellular domain of the EGFR tyrosine with an IC₅₀ value of 0.7

nM (27). Enzyme kinetic studies are in accordance with ATP-competitive inhibitor of the EGFR kinase,⁴ consistent with the interaction of the compound with the ATP-binding pocket of the kinase.

Administration of a single 100 mg/kg oral dose of PKI 166

⁴ P. Traxler, P. Cohen, and E. Buchdunger, unpublished data.

to A431 tumor-bearing athymic nude mice produced high concentrations of the test compounds in plasma [maximum concentration (C_{max}), 9.6 μM] as well as in tumor (C_{max} , 21.9 μM). Twenty-four h after dosing, substantial concentrations of the drug could still be detected in tumors ($C_{24\text{h}}$, 1.5 μM), exceeding by a factor of 150 the IC_{50} for inhibition of autophosphorylation in A431 cells (measured by Western blotting). Elimination of PKI 166 from the tumor was slower than that from plasma. No accumulation of the drug was observed after multiple dosing to mice.⁴

In *in vivo* experiments with A431 tumor-bearing mice, daily oral doses of 100 mg/kg or doses of 200 mg/kg every second day were well tolerated without body weight loss. Higher doses (<150 mg/kg/day) produced increased toxic effects (body weight loss).⁴

Human pancreatic carcinoma L3.6pl cells implanted into the pancreas of nude mice express high levels of activated EGFR. Oral administration of PKI 166 on the schedule of three times per week in combination with gemcitabine produced therapeutic effects equivalent to that of daily oral administration of PKI 166. However, daily oral administration produced a 20% loss in body weight, whereas the 3 times/week schedule did not produce any weight loss or other toxic reactions. Immunohistochemical analyses of the pancreatic cancers demonstrated down-regulation of activated EGFR in lesions from mice treated with PKI 166 (with or without gemcitabine). In agreement with our previous report (27), the decrease in activated EGFR was associated with down-regulation in expression of VEGF and IL-8 and a decrease in MVD due to increased apoptosis of tumor-associated endothelial cells.

The coexpression of EGFR with at least one of its ligands correlates with rapid progression of pancreatic cancer (14). Subsequent to ligand binding, the EGFR dimerizes to become activated through autophosphorylation and transphosphorylation (33). Activated EGFR can also regulate apoptosis (34, 35), and inactivation of activated EGFR inhibits EGF-induced receptor autophosphorylation, entry of cells into S phase, and cyclin E-associated kinase activity, consequently inhibiting accumulation of cells in the G_1 phase of the cell cycle (36). EGFR is also expressed on some dividing endothelial cells (22, 23). Our data show that in mice given daily or thrice weekly oral doses of PKI 166, the human pancreatic tumors expressed the EGFR but not the phosphorylated activated EGFR. The treatment decreased cell proliferation (PCNA positive) and increased the number of apoptotic tumor cells (TUNEL positive). Immunohistochemical analyses of the tumor specimens led us to conclude that treatment with PKI 166 (alone or in combination with gemcitabine) also produced apoptosis in tumor-associated endothelial cells and hence decreased mean vessel density within the tumors. The concomitant decrease in expression of VEGF and IL-8, which serve as survival factors for endothelial cells (36–45), could also have contributed to the decrease in vessel density.

In summary, we show that three weekly oral administrations (plus gemcitabine) of PKI 166 produce as significant therapy of human pancreatic carcinoma in nude mice as daily oral administrations, but without any measurable toxicity. This combination therapy therefore offers a superior approach to the treatment of metastatic pancreatic cancer.

REFERENCES

- Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nat. Med.*, *1*: 27–31, 1995.
- Folkman, J., and Klagsbrun, M. Angiogenic factors. *Science (Wash. DC)*, *253*: 442–447, 1987.
- Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, *86*: 353–364, 1996.
- Warshaw, A. L., and Fernandez-del Castillo, C. Pancreatic carcinoma. *N. Engl. J. Med.*, *326*: 455–465, 1992.
- Evans, D. B., Abbruzzese, J. L., and Rich, T. R. Cancer of the pancreas. *In*: V. T. deVita, S. Hellman, and S. A. Rosenberg (eds.), *Cancer: Principles and Practice of Oncology*, 5th ed., pp. 1054–1087. Philadelphia: J. B. Lippincott, 1997.
- Wanebo, H. J., and Vezeridis, M. P. Pancreatic carcinoma in perspective: a continuing challenge. *Cancer (Phila.)*, *78*: 580–591, 1996.
- Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1999. *CA Cancer J. Clin.*, *49*: 8–31, 1999.
- Fernandez, E., La Vecchia, C., Porta, M., Negri, E., Lucchini, F., and Levi, F. Trends in pancreatic cancer mortality in Europe, 1955–1989. *Int. J. Cancer*, *57*: 786–792, 1994.
- Burriss, H. A., III, Moore, M. J., Andersen, J., Green, M. R., and Rothenberg, M. L. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J. Clin. Oncol.*, *15*: 2403–2413, 1997.
- Smith, J. J., Derynck, R., and Korc, M. Production of transforming growth factor α in human pancreatic cancer cells: evidence for a superagonist autocrine cycle. *Proc. Natl. Acad. Sci. USA*, *84*: 7567–7570, 1987.
- Korc, M., Chandrasekar, B., Yamanaka, Y., Friess, H., Buchler, M., and Beger, H. G. Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor α . *J. Clin. Investig.*, *90*: 1352–1360, 1993.
- Ebert, M., Yokoyama, M., Kobrin, M. S., Friess, H., Lopez, M. E., Buchler, M. W., Johnson, G. R., and Korc, M. Induction and expression of amphiregulin in human pancreatic cancer. *Cancer Res.*, *54*: 3959–3962, 1994.
- Kobrin, M. S., Funatomi, H., Friess, H., Buchler, M. W., Stathis, P., and Korc, M. Induction and expression of heparin-binding EGF-like growth factor in human pancreatic cancer. *Biochem. Biophys. Res. Commun.*, *202*: 1705–1709, 1994.
- Yamanaka, Y., Friess, H., Kobrin, M. S., Buchler, M., Kunz, J., Beger, H. G., and Korc, M. Overexpression of HER2/neu oncogene in human pancreatic carcinoma. *Hum. Pathol.*, *24*: 1127–1134, 1993.
- Bergmann, U., Funatomi, H., Yokoyama, M., Beger, H. G., and Korc, M. Insulin-like growth factor I overexpression in human pancreatic cancer: evidence for autocrine and paracrine roles. *Cancer Res.*, *55*: 2007–2011, 1995.
- Wagner, M., Lopez, M. E., Cahn, M., and Korc, M. Suppression of fibroblast growth factor receptor signaling inhibits pancreatic cancer growth *in vitro* and *in vivo*. *Gastroenterology*, *114*: 798–807, 1998.
- Yamanaka, Y., Friess, H., Kobrin, M. S., Buchler, M., Beger, H. G., and Korc, M. Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. *Anticancer Res.*, *13*: 565–569, 1993.
- Wagner, M., Cao, T., Lopez, M. E., Hope, C., van Nostrand, K., Kobrin, M. S., Fan, H. U., Buchler, M. W., and Korc, M. Expression of a truncated EGF receptor is associated with inhibition of pancreatic cancer cell growth and enhanced sensitivity to cisplatin. *Int. J. Cancer*, *68*: 782–787, 1996.
- Bruns, C. J., Harbison, M. T., Kuniyasu, H., Eue, I., and Fidler, I. J. *In vivo* selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia*, *1*: 50–62, 1999.
- Kuniyasu, H., Ellis, L. M., Evans, D. B., Abbruzzese, J. L., Fenoglio, C. J., Bucana, C. D., Cleary, K. R., Tahara, E., and Fidler, I. J. Relative expression of E-cadherin and type IV collagenase genes pre-

- dicts disease outcome in patients with resectable pancreatic carcinoma. *Clin. Cancer Res.*, 5: 25–33, 1999.
21. Ferrara, N. The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Res. Treat.*, 36: 127–137, 1995.
22. Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T., and Gillespie, G. Y. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol. Biol. Cell*, 4: 121–133, 1993.
23. Schreiber, A. B., Winkler, M. E., and Derynck, R. Transforming growth factor- α : a more potent angiogenic mediator than epidermal growth factor. *Science (Wash. DC)*, 232: 1250–1253, 1986.
24. Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am. J. Pathol.*, 151: 1523–1530, 1997.
25. Rak, J., Filmus, J., and Kerbel, R. S. Reciprocal paracrine interactions between tumour cells and endothelial cells: the “angiogenesis progression” hypothesis. *Eur. J. Cancer*, 32A: 2438–2450, 1996.
26. Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R., and Dinney, C. P. Antiepidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin. Cancer Res.*, 5: 257–265, 1999.
27. Bruns, C. J., Solorzano, C. C., Harbison, M. T., Ozawa, S., Tsan, R., Fan, D., Abbruzzese, J., Traxler, P., Buchdunger, E., Radinsky, R., and Fidler, I. J. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res.*, 60: 2926–2935, 2000.
28. Traxler, P., Buchdunger, E., Furet, P., Gschwind, H.-P., Ho, P., Mett, H., O’Reilly, T., Pfaar, U., and Thomas, H. Preclinical profile of PKI 166, a novel and potent EGF-R tyrosine kinase inhibitor for clinical development. *Clin. Cancer Res.*, 5: 3750s, 1999.
29. Shi, S. R., Key, M. E., and Kalra, K. L. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.*, 39: 741–748, 1991.
30. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis: correlation in invasive breast carcinoma. *N. Engl. J. Med.*, 324: 1–8, 1991.
31. Yoneda, J., Kuniyasu, H., Crispens, M. A., Price, J. E., Bucana, C. D., and Fidler, I. J. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J. Natl. Cancer Inst. (Bethesda)*, 90: 447–454, 1998.
32. Radinsky, R., Risin, S., Fan, D., Dong, Z., Bielenberg, D., Bucana, C. D., and Fidler, I. J. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin. Cancer Res.*, 1: 19–31, 1995.
33. Pazin, M. J., and Williams, L. T. Triggering signaling cascades by receptor tyrosine kinases. *Trends Biochem. Sci.*, 17: 374–378, 1992.
34. Uckun, F. M., Narla, R. K., Jun, X., Zeren, T., Venkatachalam, T., Waddick, K. G., Rostostev, A., and Myers, D. E. Cytotoxic activity of epidermal growth factor-genistein against breast cancer cells. *Clin. Cancer Res.*, 4: 901–912, 1998.
35. Uckun, F. M., Narla, R. K., Zeren, T., Yanishevski, Y., Myers, D. E., Waurzyniak, B., Ek, O., Schneider, E., Messinger, Y., Chelstrom, L. M., Gunther, R., and Evans, W. *In vivo* toxicity, pharmacokinetics, and anticancer activity of genistein linked to recombinant human epidermal growth factor. *Clin. Cancer Res.*, 4: 1125–1134, 1998.
36. Mendelsohn, J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin. Cancer Res.*, 3: 2703–2707, 1997.
37. Benjamin, L. E., Golijanin, D., Itin, A., Pode, D., and Keshet, E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J. Clin. Invest.*, 103: 159–165, 1999.
38. Benjamin, L. E., and Keshet, E. Conditioned switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc. Natl. Acad. Sci. USA*, 94: 8761–8766, 1997.
39. Kumar, R., Yoneda, J., Bucana, C. D., and Fidler, I. J. Regulation of distinct steps of angiogenesis by different angiogenic molecules. *Int. J. Oncol.*, 12: 749–757, 1998.
40. Kitadai, Y., Haruma, K., Sumii, K., Yamamoto, S., Ue, T., Yokozaki, H., Yasui, W., Ohmoto, Y., Fidler, I. J., Tahara, E., and Kajiyama, G. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am. J. Pathol.*, 152: 93–100, 1998.
41. Xu, L., Xie, K., Mukaida, N., Matsushima, K., and Fidler, I. J. Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Res.*, 59: 5822–5829, 1999.
42. Syridopoulos, I., Brogi, E., Kearney, M., Sullivan, A. B., Cetrulo, C., Isner, M., and Losordo, D. W. Vascular endothelial growth factor inhibits endothelial cell apoptosis induced by tumor necrosis factor- α : balance between growth and death signals. *J. Mol. Cell. Cardiol.*, 29: 1321–1330, 1997.
43. Gerber, H. P., Dixit, V., and Ferrara, N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J. Biol. Chem.*, 273: 13313–13316, 1998.
44. Nor, J. E., Christensen, J., Mooney, D. J., and Polverini, P. J. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am. J. Pathol.*, 154: 375–384, 1999.
45. Watanabe, Y., and Dvorak, H. V. Vascular permeability factor/vascular endothelial growth factor inhibits anchorage-disruption-induced apoptosis in microvessel endothelial cells by inducing scaffold formation. *Exp. Cell Res.*, 233: 340–349, 1997.