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

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

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-(hydroxy)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 EGF 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 tyrrosine 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 EGFR 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 EGFR 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 EGF-mediated proliferation and angiogenesis can contribute to the progressive growth
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, 1-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 anti-human IL-8, Biosource International (Camarillo CA); (c) rat antimmouse CD31/PECAM-1 and peroxidase-conjugated rat antianimal 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 antianimal IgG F(ab)2 fragment, affini- nure Fab fragment goat antianimal IgG, and peroxidase-conjugated goat antianimal IgG, Serotec, Harlan Bioproducts for Science, Inc. (Indianapolis, IN); (h) Texas Red-conjugated goat antianimal IgG, Jackson Research Laboratories (West Grove, CA); (i) peroxidase-conjugated rat antianimal IgG2a, Serotec, Harlan Bioproducts for Science, Inc.; (j) monoclonal antiphospho-rosine MAb 4G10 and polyclonal sheep antianimal EGFR, Upstate Biotechnology (Lake Placid, NY); and (k) MAb anti-EGFR (clone EGFR; 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-ethylocarbazole (Biogenex), and Gill’s hematoxylin (Sigma Chemical Co.). Prolong solution was purchased from Molecular Probes (Eugene, OR). Pepsin was purchased from Biomedia (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 EGFR for 15 min, washed, resuspended 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 EGFR for 15 min, washed, and treated as described above. Immunoprecipitation was performed using MAb anti-EGFR (clone EGFR) 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 antianimal 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 antialpse IgG (1:2000; Sigma Chemical Co.) or sheep antitmouse 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 mm³. 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 (4–6 μm thick) were mounted on positively charged Superfrost slides (Fisher Scientific, 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 H₂O (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 (Biomeda) 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 (8–10 μm), 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 (8–10 μm), 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 sections 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 antirabbit 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. 1](image-url)
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 fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 μg/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 ×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 (Bio-scan, Edmond, WA) installed on a Compaq computer with a

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

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence</th>
<th>Tumor volume (mm³)</th>
<th>Metastasis</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
<td>Liver</td>
</tr>
<tr>
<td>Saline control</td>
<td>10/10^a</td>
<td>574</td>
<td>445–770</td>
<td>4/10^b</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10/10</td>
<td>230</td>
<td>64–445</td>
<td>3/10</td>
</tr>
<tr>
<td>PKI 166, 7 days/wk</td>
<td>10/10</td>
<td>162^c</td>
<td>16–274</td>
<td>2/10</td>
</tr>
<tr>
<td>PKI 166, 7 days/wk + gemcitabine</td>
<td>10/10</td>
<td>18^d</td>
<td>6–80</td>
<td>0/10</td>
</tr>
<tr>
<td>PKI 166, 3 days/wk</td>
<td>10/10</td>
<td>174^f</td>
<td>88–607</td>
<td>1/10</td>
</tr>
<tr>
<td>PKI 166, 3 days/wk + gemcitabine</td>
<td>10/10</td>
<td>27^g</td>
<td>6–72</td>
<td>1/10</td>
</tr>
</tbody>
</table>

* L.36pl 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.

a Number of positive mice/number of mice injected.

b P < 0.001 versus control.

c P < 0.0001 versus control.

EGF inhibited EGF-stimulated tyrosine phosphorylation of the EGFR in a dose-dependent manner (0.01–0.5 μM; Ref. 27). In the present study, we incubated L.36pl cells with serum-free medium containing 40 ng/ml EGF for 15 min. The cells exhibited high levels of autophosphorylated EGFR (M, 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 μM 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, 170,000 band was confirmed by 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 L.36pl Pancreatic Cancer in Nude Mice.** Athymic nude mice were given injections of 1 × 10⁶ viable L.36pl 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
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. 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.

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 mm$^3$, 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 mm$^3$, 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
combination with gemcitabine, or saline (control). All mice were killed on day 35. 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 gemcitabine-treated animals, and in 70 groups (Table 1). Histologically positive regional lymph node metastases (Table 1).

Histological and IHC analyses. Tumors from mice treated with 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).

**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-
human 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 IC$_{50}$ value of 0.7 nM (27). Enzyme kinetic studies are in accordance with ATP-competitive inhibitor of the EGFR kinase, 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 ($C_{\text{max}}$), 9.6 $\mu$M) as well as in tumor ($C_{\text{max}}$, 21.9 $\mu$M). Twenty-four h after dosing, substantial concentrations of the drug could still be detected in tumors ($C_{\text{24h}}$, 1.5 $\mu$M), 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 downregulation 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 EFG-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


Clinical Cancer Research

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


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/8/2563

Cited articles  This article cites 42 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/8/2563.full.html#ref-list-1

Citing articles  This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/7/8/2563.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.