Blockade of the Epidermal Growth Factor Receptor Signaling Inhibits Angiogenesis Leading to Regression of Human Renal Cell Carcinoma Growing Orthotopically in Nude Mice

Daniel Kedar, Cheryl H. Baker, Jerald J. Killion, Colin P. N. Dinney, and Isaiah J. Fidler

Departments of Cancer Biology [D. K., C. H. B., J. J. K., C. P. N. D., I. J. F.] and Urology [D. K., C. P. N. D.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

We determined whether blockade of the epidermal growth factor-receptor (EGF-R) signaling pathway by oral administration of the EGF-R tyrosine kinase inhibitor PKI166 can inhibit angiogenesis and growth of SN12PM6 human renal cell carcinoma (HRCC) in the kidney of nude mice and whether gemcitabine can potentiate these effects. In vitro treatment of HRCC cells with PKI166 inhibited EGF-R autophosphorylation, which correlated with a decrease in expression of Bcl-xl protein and phosphorylation of signal transducers and activators of transcription, particularly signal transducers and activators of transcription 3. PKI166 also decreased expression of vascular endothelial growth factor and basic fibroblast growth factor in a dose-dependent manner. Oral administration of PKI166 or PKI166 and injected gemcitabine or gemcitabine alone beginning 7 days after implantation of SN12PM6 cells into the kidney of athymic nude mice reduced the volume of tumors by 26, 61, and 23%, respectively. In another experiment 28 days after the orthotopic implantation of SN12PM6 cells, nephrectomy was performed followed by 4 weeks of treatment. Treatment with PKI166 and, more so, PKI166 plus gemcitabine significantly inhibited lung metastasis, corresponding to a significant increase in overall length of survival. EGF-R activation was significantly blocked by therapy with PKI166 and was associated with a significant reduction in expression of vascular endothelial growth factor and interleukin-8, decreased microvessel density, decreased staining of proliferating cell nuclear antigen, and increased tumor cell apoptosis. Collectively, the data indicate that targeting activation of EGF-R on HRCC produces significant therapeutic benefits.

INTRODUCTION

RCC occurs nearly twice as often in men as in women with median age in the mid-60s (1–3). At the time of diagnosis, the majority of patients with RCC present with metastases (1–3). Radical nephrectomy is the only curative therapy for localized RCC, and in only a few cases is surgical resection of locally recurrent RCC or of disease at a solitary metastatic site associated with long-term survival (1–3). Metastatic RCC is highly resistant to chemotherapy and radiotherapy. A significant response to IFN, IL-2, or both (4) can occur, but it is rarely durable. In short, the identification of new therapeutic agents for this fatal cancer is urgently needed.

One candidate family of agents is the PTKs, which play a key role in the control of cell proliferation (5). A significant number of oncogenes and proto-oncogenes, including the EGF-R, are PTKs (5–7). Under physiological conditions, the binding of EGF to its receptor (EGF-R) leads to receptor tyrosine kinase activity and subsequently to a complex cascade of events culminating in cell proliferation, which is enhanced by antiapoptotic effects (8). PKI166, a novel EGF-R tyrosine kinase inhibitor of the pyrrolo-pyrimidine class (9), reversibly inhibits the intracellular domain of the EGF-R kinase, resulting in inhibition of cell proliferation and stimulation of apoptotic events (10, 11). Because increased expression of EGF, tumor growth factor α and EGF-R in surgical specimens of RCC correlates with rapidly progressive disease (12–16), we determined whether administration of PKI166 to nude mice implanted orthotopically with HRCC would down-regulate the EGF-R signaling pathway and, hence, inhibit tumor growth and metastasis.

MATERIALS AND METHODS

HRCC Line and Culture Conditions. For these studies, we used the highly metastatic SN12C-PM6 HRCC cell line (17) maintained in EMEM (M. A. Bioproducts, Walkersville, MD) supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, l-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin.
mixtures (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: reovirus type 3, pneumonia virus, K virus, Thielier’s encephalitis virus, Sendai virus, min virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Reagents. PKI166 [4-((R)-phenethylamino)-6-(hydroxyl)phenyl-7H-pyrrolo[2,3-d]-pyrimidine], a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). For in vivo administration, PKI166 was dissolved in DMSO/0.5% Tween 80 and then diluted 1:20 in water (9). All of the antibodies were purchased as listed: rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal rabbit antihuman IL-8 (Biosource International, Camarillo CA); rabbit anti-bFGF (Sigma Chemical Co., St. Louis, MO); rat antimouse CD31/PECAM-1 and peroxidase-conjugated rat antimonoe IgG1 (PharMingen, San Diego, CA); mouse anti-PCNA clone PC 10 (DAKO A/S, Copenhagen, Denmark); monoclonal mouse antihuman EGF-R (activated form) IgG1 (Chemicon, Temecula, CA); polyclonal rabbit antihuman EGF-R (Santa Cruz Biotechnology); peroxidase-conjugated F(ab')₂ goat antirabbit IgG F(ab')₂, peroxidase-conjugated rat antimouse IgG F(ab')₂ fragment, AffiniPure Fab' fragment goat antimouse IgG, peroxidase-conjugated goat antirat IgG, and Texas Red-conjugated goat antirat IgG (Jackson Research Laboratories, West Grove, CA); Alexa Fluor 594-conjugated goat antimouse IgG and Alexa Fluor 594-conjugated goat antirabbit IgG (Molecular Probes, Eugene, OR); peroxidase-conjugated rat antimouse IgG2a (Sercotec; Harlan Bioproducts for Science, Inc., Indianapolis, IN); anti-Bcl-xl (Santa Cruz Biotechnology); monoclonal antiphosphotyrosine MAb 4G10, anti-STAT3, and anti-phos-STAT3 (Upstate Biotechnology, Lake Placid, NY); horseradish peroxidase-conjugated donkey antirabbit IgG (Amersham Life Science, Inc., Arlington Heights, IL); and sheep antimouse and human IgG (Sigma Immunochemicals, St. Louis, MO). Other reagents were Hoechst Dye 3342 MW 615.9 (Hoechst, Warrington, PA), stable 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL), 3-amino-9-ethycarbazole (Biogenex Laboratories, San Ramon, CA), and Gill’s hematoxylin (Sigma Chemical Co.). Prolong solution was purchased from Molecular Probes, and pepsin was purchased from Biomedica (Foster City, CA).

MTT (M2128) was purchased from Sigma Chemical Co., and a stock solution was prepared by dissolving 5 mg of MTT in 1 ml of PBS and filtering the solution to remove particulates. The solution was protected from light, stored at 4°C, and used within a month. The ECL detection system was purchased from Amersham, Inc., and the VEGF and bFGF ELISA kits from R&D Systems (Minneapolis, MN). The TUNEL assay was performed using a commercial Apoptosis Detection kit (Promega, Madison, WI) with modifications.

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-Fredrick 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, SN12C-PM6 cells were harvested from confluent 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 >90% viability were used for the injections. Injection of cells into the renal subcapsule was performed as described previously (17). The mice were killed when the controls became moribund (<60 days). The size and weight of the tumors in the kidney were recorded. Histopathology confirmed the nature of the disease. For IHC and histology staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin. The other part was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C.

Therapy of Established HRC Tumors Growing in the Kidney of Athymic Nude Mice. Seven days after the implantation of tumor cells into the renal subcapsule, the mice were randomized into four treatment groups as follows (n = 10): (a) oral vehicle solution for PKI166 (DMSO/0.5% Tween 80 diluted 1:20 in water) and i.p. HBSS (control group); (b) twice weekly i.p. injections of 125 mg/kg gemcitabine alone; (c) thrice weekly oral administration of 100 mg/kg PKI166 alone; and (d) thrice weekly oral administration of 100 mg/kg PKI166 combined with twice weekly i.p. injections of 125 mg/kg gemcitabine.

To determine overall survival of mice and prevention of lung metastasis, we carried out another study. Mice were implanted with SN12C-PM6 cells in the RCC, and 28 days later nephrectomy was performed. The mice were then divided into four treatment groups (n = 10) as described above. The mice were killed when moribund or at the end of 28 days of treatment. The lungs were harvested and fixed in Bouin’s solution, and the number of peripheral metastases was determined under a dissecting microscope. All of the metastases were confirmed by histological examination of fixed tissue sections stained with H&E.

Necropsy Procedures and Histological Studies. Mice were killed by nembutal overdose and weighed. Kidneys with tumors were excised and weighed. For IHC and H&E staining procedures, one part of the tumor tissue was formalin-fixed and paraffin-embedded, and another part was embedded in OCT compound (Miles, Inc.), rapidly frozen in liquid nitrogen, and stored at −70°C.

IHC Determination of VEGF, IL-8, bFGF, PCNA, CD31/PECAM-1, EGF-R, and Activated EGF-R. Paraffin-embedded tissues were used for identification of VEGF, IL-8, bFGF, and PCNA. Sections (4–6 μm thick) 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%, 80% ethanol/ddH2O v/v) and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA were microwaved 5 min for “antigen retrieval” (18). All of the other paraffin-embedded tissues were treated with pepsin (Biomedra) for 15 min at 37°C and washed with PBS (19). Frozen tissues used for identification of CD31/PECAM-1, EGF-R, and activated EGF-R 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), 1:1 acetone/chloroform (v/v; 5 min), and acetone (5 min), and washed with PBS. Immuno-histochemical procedures were performed as described previously (20). 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). When staining for EGF-R or activated EGF-R, no counterstaining was used. Sections analyzed for activated EGF-R were pretreated with goat antimouse IgG1 F(ab′)2 fragment (1:10 dilution in PBS) for 8–12 h before incubation with a primary antibody. The samples were then incubated at 4°C for 18 h with a 1:200 dilution (v/v) of monoclonal mouse antihuman antibody for activated EGF-R. A positive reaction for EGF-R or activated EGF-R was visualized by incubating the slides for 1 h with a 1:200 dilution of Alexa Fluor 594 conjugated goat antirabbit or Alexa Fluor 594 conjugated goat antimouse, respectively, at room temperature for 1 h in the dark. Fluorescent bleaching was minimized by covering the slides with 90% glycerol and 10% PBS. Control samples exposed to secondary antibody alone showed no specific staining.

**IHC Staining for Apoptotic Tumor Cells and Endothelial Cells.** The TUNEL assay was performed using a commercially available apoptosis detection kit with modifications as described previously (10, 11). Immunofluorescence microscopy was performed using an epifluorescence microscope equipped with narrow band pass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to select for red fluorescence. Images were captured using a cooled 3CCD camera (Photometrics, Tucson, AZ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas Image Analysis software (Bioscan, Edmond, WA) installed on a Compaq computer with Pentium chip, a frame grabber, an optical disk storage system, and a Sony Mavigraph UP-D7000 digital color printer (Tokyo, Japan). Images were additionally processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to total number of endothelial cells in 5–10 random 0.011-mm² fields at ×400 magnification. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random 0.159-mm² fields at ×100 magnification.

**Quantification of MVD, PCNA, TUNEL, and Absorbance.** For the quantification of MVD, 10 random 0.159 mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified according to the method described previously (21, 22). For the quantification of IHC reaction intensity, the absorbance of 100 VEGF-, IL-8-, and bFGF-positive cells in 10 random 0.039-mm² fields at ×200 magnification taken from treated tumor tissues was measured using the Optimas Image Analysis software (23–25). The samples were not counterstained so that the absorbance would be attributable solely to the product of the IHC reaction. VEGF, IL-8, and bFGF cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and expressed as a ratio of tumor cell expression to normal kidney gland expression multiplied by 100 (21, 22). The number of PCNA+ and TUNEL+ cells was quantified in 10 random 0.159-mm² fields at ×100 magnification.

**In Vitro Cytotoxicity Assay.** In all of the assays, 1000 tumor cells were seeded into 38-mm² wells of flat-bottomed 96-well plates in triplicate and allowed to adhere overnight. The cultures were then washed and refed with medium (negative control) or medium containing PKI166 with or without gemcitabine. After 5 days (control cultures did not reach confluence), the number of metabolically active cells was determined by MTT assay (26). After 2–4 h of incubation in medium containing 0.42 mg/ml of MTT, the cells were lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells was monitored by an MR-5000 96-well microtiter plate reader at 570 nm (Dynatech, Inc., Chantilly, VA). Growth inhibition was calculated from the formula: cytostasis (%) = [1 – (AB)] × 100, where A is the absorbance of treated cells and B is the absorbance of the control cells.

**Western Blot Analysis of EGF-R Autophosphorylation after Treatment with PKI166.** Serum-starved SN12C-PM6 cells were treated with PKI166 (0.01–1.0 μM) for 1 h and then incubated with or without 40 ng/ml recombinant human EGF for 15 min, washed, and 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. Protein lysates were analyzed on 7.5% SDS-PAGE and transferred onto 0.45-μm nitrocellulose membranes. The filters were blocked with 3% BSA in Tris-buffered saline [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl], probed with either polyclonal rabbit anti-human EGF-R (1:1000) or monoclonal antiphosphotyrosine (MAb 4G10; 1:2000) in TTBS, and incubated with horseradish peroxidase-conjugated donkey antirabbit IgG (1:2000; Sigma Immunochemicals) or sheep antimouse IgG (1:2000), respectively, in TTBS. Protein bands were visualized by the ECL detection system.

**Western Blot Analysis of Bcl-xl and STAT3 Phosphorylation after Treatment with PKI166.** EGF-R signaling has been linked recently to apoptosis through a STAT3 pathway (27–29), and expression of the activated form of STAT3 induces transcription of reporter constructs containing promoter sequences derived from the bcl-x gene (30). Because a strong correlation exists between elevated levels of members of the Bcl-2 family of antiapoptotic regulatory proteins, including Bcl-xl protein and human cancer (31), we assessed STAT3 and Bcl-xl in the observed apoptotic events. Activated STAT3 (phosphorylated STAT3) has been shown to protect cells from apoptosis (32, 33). Serum-starved SN12C-PM6 cells were treated with PKI166 (2.5 μM) for 1 h, and then incubated with or without 40 ng/ml recombinant human EGF for 15 min, washed, scraped, and prepared as described above. Western blot
analysis was performed using anti-BCL-xl, anti-STAT3, and anti-phos-STAT3. Protein lysates were analyzed on 7.5% SDS-PAGE and transferred onto 0.45-μm nitrocellulose membranes. The filters were blocked with 3% BSA in Tris-buffered saline [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl], probed with polyclonal rabbit antihuman Bcl-xl (1:1000), monoclonal antiphospho-STAT3 (1:2000), or monoclonal rabbit antihuman STAT3 in TTBS, and incubated with horseradish peroxidase-conjugated donkey antirabbit IgG (1:2000; Sigma Immunochemicals) in TTBS. Protein bands were visualized by the ECL detection system.

**Methods**. The immunoreactive proteins were detected by incubating the blot with the corresponding peroxidase-conjugated IgG and visualized using the ECL system.

**RESULTS**

Inhibition of EGF-R Autophosphorylation, Bcl-xl Expression, and STAT3 Phosphorylation in HRCCs by PKI166. In the first set of experiments, SN12C-PM6 cells incubated for 15 min with serum-free medium containing 40 ng/ml EGF exhibited high levels of autophosphorylated EGF-R (M, 170,000 band) detected by Western blots using antiphosphotyrosine sera (Fig. 1A). Pretreatment of the cells with PKI166 for 1 h followed by a 15-min treatment with EGF (0.01–1.0 μM) inhibited the autophosphorylation in a dose-dependent manner. The M, 170,000 band confirmed as EGF-R by Western blot analysis using anti-EGF-R antisera did not decrease in response to increasing concentrations of PKI166 (Fig. 1A).

Treatment with PKI166 (2.5 μM) in the absence or presence of EGF (40 ng/ml) reduced concentration of phosphorylated STAT3 and Bcl-xl (Fig. 1B), suggesting a link between the effects of PKI166 at the membrane level and phosphorylated STAT3-induced control of apoptosis. The protein level of STAT3 did not vary in response to treatment.

**In Vitro Cytotoxicity Mediated by Gemcitabine and PKI166.** SN12C-PM6 cells were incubated for 5 days in medium containing increasing concentrations (0–5.0 μM) of gemcitabine in the absence or presence of a noncytostatic concentration (0.05 μM) of PKI166. The cytotoxicity mediated by gemcitabine was enhanced by PKI166, and the IC50 of 0.312 μM for gemcitabine decreased to 0.078 μM (P < 0.05) when the cells were exposed to both gemcitabine and PKI166.

**Inhibition of RCC Growth and Metastasis.** The kidneys of athymic nude mice were injected with SN12C-PM6 cells. Seven days later, the mice were randomized into four treatment groups of 10 mice each. The first control group received oral vehicle solution for PKI166 (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 three weekly oral administrations of 100 mg/kg PKI166 alone, and the fourth group received daily oral administrations of 100 mg/kg PKI166 plus twice weekly i.p. injections of 125 mg/kg gemcitabine. All of the mice were killed on day 35. The data summarized in Table 1 show that thrice weekly oral administrations of PKI166 alone or twice weekly i.p. injections of gemcitabine alone decreased median kidney tumor volume (258 and 270 mm3, respectively, versus 349 mm3 in controls). The combination of gemcitabine and PKI166 produced a significant decrease in median volume of kidney tumors (136 mm3; P = 0.034). Oral treatments with PKI166 alone or in combination with gemcitabine were well tolerated, as shown by the maintenance of body weight in all of the treatment groups (Table 1).

The effect of the treatments on overall survival and incidence of lung metastasis after nephrectomy were determined by...
a separate study in mice injected in the kidney with SN121C-PM6 human renal cancer cells (1 × 10⁶) were injected into the kidney of nude mice. Seven days later, groups of mice (n = 10) were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, thrice weekly oral feedings of PKI166 (100 mg/kg) alone, PKI166 in combination with gemcitabine, or saline (control). All mice were killed on day 35.

### Table 1: Therapy of HRCC growing in the kidney of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence</th>
<th>Tumor volume (mm³)</th>
<th>Body weight (g)</th>
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<tr>
<td>Saline control</td>
<td>10/10°</td>
<td>349 ± 260–968</td>
<td>26 ± 23–31</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10/10°</td>
<td>270 ± 199–683</td>
<td>27 ± 20–32</td>
</tr>
<tr>
<td>PKI166</td>
<td>10/10°</td>
<td>258 ± 245–717</td>
<td>26 ± 20–31</td>
</tr>
<tr>
<td>PKI166 + gemcitabine</td>
<td>9/10°</td>
<td>136 ± 208–489</td>
<td>27 ± 21–32</td>
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</table>

° SN12P6M6 HRCCs (1 × 10⁶) were injected into the kidney of nude mice. Seven days later, groups of mice (n = 10) were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, thrice weekly oral feedings of PKI166 (100 mg/kg) alone, PKI166 in combination with gemcitabine, or saline (control). All mice were killed on day 35.

° Number of positive mice/number of mice injected.

° P < 0.001 as compared with controls.

Production of VEGF, IL-8, and bFGF by RCC cells was significantly reduced (P < 0.001) 35 days after initiation of treatment with PKI166 or PKI166 and gemcitabine as compared with control or gemcitabine alone (Fig. 3; Table 3). Control cells produced 146 pg/ml VEGF and 563 pg/ml bFGF. Cells treated with 0.5 μM PKI166 produced 137 pg/ml VEGF and 345 pg/ml bFGF (15% and 45% reduction as compared with control, respectively). Cells treated with 2.5 μM PKI166 produced 76 pg/ml VEGF and 222 pg/ml bFGF (48% and 61% reduction as compared with control cells, respectively).

MVD (measured by staining with antibodies against CD31 correlated with expression of VEGF, IL-8, and bFGF. Treatment with PKI166 or PKI166 and gemcitabine significantly reduced tumor MVD from 72 ± 8 in control tumors to 47 ± 8, 59 ± 11, 33 ± 3, respectively (P < 0.001; Fig. 3; Table 3). The CD31/TUNEL fluorescence double-labeling technique (10, 11) revealed that at 28 days of treatment, endothelial cells were not undergoing apoptosis. The absence of endothelial cell apoptosis in tumors that did not express EGF suggests that the decrease in MVD was attributable to a decrease in VEGF, IL-8, and bFGF production.

### DISCUSSION

At the time of diagnosis, 20–30% of patients present with metastasis, and 50–60% of patients develop metachronous metastatic disease (34, 35). Blockade of the EGF-R signaling pathway by oral administration of the EGF-R tyrosine kinase inhibitor PKI166 combined with i.p. injections of gemcitabine significantly inhibited the growth of HRCC implanted into the kidney of nude mice. Whereas treatment with PKI166 alone or gemcitabine alone reduced the growth of the primary neoplasms by 24%, the combination of PKI166 and gemcitabine decreased the growth of primary kidney cancer by 61%, leading to significant prolongation of survival after nephrectomy. IHC analysis of the kidney cancers demonstrated down-regulation of activated EGF-R in lesions from mice treated with PKI166 alone or PKI166 combined with gemcitabine. This effect was accompanied by down-regulation of proangiogenic molecules VEGF, IL-8, and bFGF. Treatment of mice with PKI166 alone or in combination with gemcitabine was also associated with a decrease in tumor cell proliferation (PCNA⁺) and an increase in...
apoptosis (TUNEL+) of tumor cells that correlated with a significant decrease in MVD (CD31+). Interestingly, double staining of endothelial cells with antibodies against CD31 and TUNEL (10, 11) did not identify apoptotic endothelial cells, suggesting that the observed decrease in MVD was because of a decrease in VEGF, IL-8, and bFGF.

Gemcitabine (2’, 2’-difluoro-2’-deoxycytidine), a nucleoside analogue of cytidine, is phosphorylated inside cells, targeting DNA and RNA (36). Data obtained with ovarian carcinoma cells indicate that the drugs block the G1 and G2 M checkpoints of the cell cycle and, hence, enhance apoptosis (36).

Previous data (37) demonstrated the activity of gemcitabine in the treatment of HRCC xenografts in nude mice. Multiple studies investigating chemotherapy agents have clearly demonstrated that RCC is a chemotherapy-resistant tumor (34, 38). The results of 155 clinical trials using 80 single agents demonstrate an overall median response rate of 4% (39). Treatment with gemcitabine produced a response rate of 8% (40). Combined therapy with IFN-α increased the efficacy of gemcitabine in terms of tumor response in immunodeficient nude mice (37).

Overexpression of EGF-R is a frequent event in HRCC and has been proposed as a prognostic parameter (12–16, 41, 42). Binding of EGF or tumor growth factor α activates the EGF-R tyrosine kinase, which in turn transduces the mitogenic signal by specific phosphorylation of intracellular substrates (6). Depending on the nature of signals that initiate the apoptotic process, active and passive modes of cell death may be distinguished. Apoptosis induced by the engagement of the cell death receptor Fas (43) and radiation-induced, p53-dependent cell suicide (44) provide examples for active modes of cell death. By contrast, apoptosis in the absence of extracellular signals may be viewed as a passive or default process. Extracellular signals preventing death by exogenous growth factors/cytokines, of which the EGF-R signaling pathway is but one example, have recently received widespread attention. Activation of EGF-R has been shown to affect the functional state and/or expression patterns of members of the antiapoptotic Bcl-2 family (45–48), possibly via the STAT3 pathway (29, 49, 50), providing direct links between extracellular survival signals and intracellular mechanisms of survival regulation. Our present data on EGF-R-dependent ex-
pression of the phosphorylated (activated) form of STAT3 and expression of Bcl-xl (Bcl-2 family member) in HRCC are consistent with recent published results (31, 45–50). We provide evidence that inhibition of the EGF-R pathway by PKI166 decreased phosphorylation of STAT3, which was associated with a down-regulation in Bcl-xl expression, suggesting that EGF-R-mediated Bcl-xl expression is dependent on the tyrosine kinase activity of the receptor. Taken together, these findings suggest that receptor tyrosine kinases can inhibit cell death by two independent mechanisms. The first rests on phosphorylation cascades and thus represents a comparatively rapid response triggered by ligand binding. The second mechanism leads to

Table 3  IHC analysis of HRCC in the kidney of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumor cells</th>
<th>Endothelial cells</th>
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<tr>
<td></td>
<td>PCNA⁺ (%)</td>
<td>TUNEL⁺ (%)</td>
</tr>
<tr>
<td>Control</td>
<td>68 ± 6⁺</td>
<td>5 ± 0.4⁺</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>54 ± 2⁺</td>
<td>8 ± 1⁺</td>
</tr>
<tr>
<td>PKI166</td>
<td>47 ± 6⁺</td>
<td>9 ± 3⁺</td>
</tr>
<tr>
<td>PKI166 + gemcitabine</td>
<td>35 ± 6⁺</td>
<td>12 ± 3⁺</td>
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⁺ Mean ± SD PCNA⁺ cells/field determined by measuring 10 random 0.159-mm² fields at ×100 magnification.
⁻ Mean ± SD TUNEL⁺ cells/field determined by measuring 10 random 0.159-mm² fields at ×100 magnification.
⁻ Mean ± SD optical density.
⁻ Mean ± SD CD31/TUNEL⁺ cells/field, determined by measuring 10 random 0.011-mm² fields at ×400 magnification. Fluorescence double labeling was performed on frozen tissue sections.
⁻⁺ P < 0.05 as compared with controls.
⁻⁻ P < 0.001 as compared with controls.

Fig. 3  Immunohistochemical analysis. Tumors were harvested from control mice and mice treated with gemcitabine, PKI166, or both gemcitabine and PKI166. The sections were immunostained for expression of PCNA (to show cell proliferation), TUNEL (to show cell death), VEGF, IL-8, and bFGF. Tumors from mice treated with PKI166 plus gemcitabine had decreased PCNA⁺ and increased TUNEL⁺ cells. Tumors from mice treated by PKI166 or by the combination of gemcitabine and PKI166 expressed lower levels of VEGF, IL-8, and bFGF.
up-regulation of expression of antiapoptotic proteins of the Bcl-2 family by activation of STAT3 (45–50).

In summary, we show that blockade of the EGF-R signaling pathway by the PTK inhibitor PKI1166 in combination with gemcitabine significantly inhibits HRCC in nude mice and prolongs survival. The inhibition of both primary tumor growth and lung metastasis production after nephrectomy is mediated by direct antitumor effects of PKI1166, resulting in a decrease in the phosphorylation of EGF-R and/or expression of proteins important for tumor cell survival. Therefore, this combination may be useful for the treatment of HRCC, a devastating disease.

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REFERENCES


