Targeted Molecular Therapy for Oral Cancer With Epidermal Growth Factor Receptor Blockade

A Preliminary Report

Jeffrey N. Myers, MD, PhD; F. Christopher Holsinger, MD; B. Nebiyou Bekele, PhD; Emily Li, DDS; Samar A. Jasser, BA; Jerald J. Killion, PhD; Isaiah J. Fidler, DVM, PhD

Background: Overexpression of epidermal growth factor receptor (EGF-R) is associated with increased malignant potential and correlates with poor clinical outcome in head and neck cancer. Therefore, inhibition of the EGF-R pathway provides an ideal target for molecular therapy. We examined in vitro and in vivo effects of PKI166, an orally administered EGF-R inhibitor, on 2 human squamous cell carcinoma of the oral cavity cell lines, Tu159 and MDA1986.

Study Design: Basic science, laboratory investigation.

Results: For Western blotting, Tu159 and MDA1986 cells were pretreated for 1 hour and then stimulated with EGF. The EGF-R–specific tyrosine kinase autophosphorylation was inhibited completely by PKI166 at all doses tested (1-10 µg/mL). By means of a tetrazolium-based viable cell assay, PKI166 was shown to arrest the growth of Tu159 and MDA1986 cells. The inhibitory concentration (50%), calculated from regression lines on the linear portion of the growth inhibition graphs, was 0.18µM (R=0.98) for Tu159 cells and 0.23µM (R=0.97) for MDA1986 cells. Nude mice were inoculated subcutaneously with 1 x 10⁶ Tu159 tumor cells and observed for 7 days. Next, daily doses of PKI166 (0, 10, or 50 mg/kg) were delivered by orogastric lavage for 28 days and the animals were observed for tumor growth. PKI166 significantly reduced tumor growth in mice treated for 1 month with oral PKI166 in a dose-dependent fashion.

Conclusions: Targeted molecular therapy with EGF-R blockade arrests the growth of oral cancer in vitro and reduces its proliferation in an experimental xenograft animal model.


IN 2002, SQUAMOUS cell carcinoma of the head and neck (HNSCC) is predicted to account for nearly 40000 new cancers in the United States, equal in incidence to leukemia and greater than all endocrine tumors.¹ Worldwide, cancers of the oral cavity and pharynx represent an even greater public health problem, responsible for almost 200000 deaths annually.² Squamous cell carcinoma of the oral cavity (SCCCO) accounts for nearly 50% of all newly diagnosed cancers in India and is a leading cause of cancer death in France.² Despite improvements in locoregional control, morbidity and mortality rates have improved little during the past 30 years.³ Targeted molecular therapy offers an exciting new approach to treat human malignancy.⁴ The tyrosine kinase inhibitor STI-571 has shown promise in early clinical trials for the treatment of chronic myeloid leukemia.⁵ Its success highlights the potential for anticancer drugs based on the specific molecular abnormality present in a human cancer.

The epidermal growth factor receptor (EGF-R) pathway provides an attractive target for molecular therapy for HNSCC. Overexpression of the EGF-R correlates with a poor outcome in patients with HNSCC⁶ and other human epithelial tumors.⁷ The EGF-R is a 170-kd transmembrane glycoprotein consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with intrinsic tyrosine kinase activity.⁸ Once activated, the EGF-R intracellular domain phosphorylates both the receptor itself and several crucial second messenger effector molecules.⁹ Activation of this signaling pathway triggers DNA synthesis and a mitogenic cascade, resulting in cell proliferation.¹⁰¹¹ Therefore, inhibition of the EGF-R pathway and its tyrosine kinase signaling activity may provide an ideal target for the molecular treatment of HNSCC.

Several strategies have been developed to block the EGF-R. These include antisense technology, ligand-linked toxins, monoclonal antibodies, and small-
MATERIALS AND METHODS

ANIMALS

Male athymic nude mice (NCR-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 in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with Animal Care and Use Guidelines of The University of Texas M. D. Anderson Cancer Center, Houston. They were 8 to 12 weeks old when they were used for this study.

CELL LINES AND CULTURE CONDITIONS

Tu159 and MDA1986 are human SCCOC cell lines derived from individual patients undergoing surgery, primarily at M. D. Anderson Cancer Center.18,19 The cells were grown in vitro in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 1-glutamine. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were free of Mycoplasma species and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler encephalitis virus, Sendai virus, minute virus, ectromelia virus, and lactate dehydrogenase virus (assayed by MA Bioproducts, Walkersville, Md). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

INHIBITION OF EGF-R AUTOPHOSPHORYLATION IN HUMAN SCCOC

In our first experiment, we determined whether treatment of Tu159 and MDA1986 cells with PKI166 could inhibit EGF-stimulated tyrosine phosphorylation of the EGF-R. Tu159 and MDA1986 cells, incubated 15 minutes with serum-free medium but containing EGF, exhibited high levels of autophosphorylated EGF-R as detected by antiphosphotyrosine antiserum on Western blots of anti–EGF-R–immunoprecipitated cell lysates. Next, pretreatment of cells with PKI166 for 60 minutes, followed by a 15-minute treatment with EGF, inhibited the autophosphorylation in a dose-dependent manner (0-10 µg/mL). Expression of the 170-kd EGF-R protein was found to be down-modulated by the addition of EGF in the absence of PKI166. Receptor autophosphorylation was found to be maximal under these conditions, and the EGF-R–specific tyrosine autophosphorylation was inhibited completely by PKI166 at all doses tested (1-10 µg/mL) in both cell lines (Figure 1 and Figure 2).

RESULTS

MEDIATION OF IN VITRO CYTOTOXICITY OF SCCOC

Tu159 and MDA1986 cells were incubated for 5 days in medium, either with or without PKI166. As seen in molecule tyrosine kinase inhibitors. A phase 1 clinical trial recently demonstrated the safety and preliminary efficacy of the monoclonal antibody preparation C225.13 PKI166 is a newly identified, low-molecular-weight EGF-R tyrosine kinase inhibitor that is administered orally.14 PKI166 not only inhibited tumor growth in an orthotopic model of human pancreatic adenocarcinoma but also induced apoptosis in tumor-associated endothelial cells.15 Although a handful of in vitro studies have examined the role of EGF-R tyrosine kinase inhibitors in HNSCC,16,17 to our knowledge, no confirmation in an experimental animal model has been reported.

We present a preclinical evaluation of an EGF-R tyrosine kinase inhibitor and its antitumoral effects on in vitro and in vivo proliferation of human SCCOC.

REAGENTS

PKI166 (4-[R]-phenethylamino-6-[hydroxyl]phenyl-7H-pyrrolo[2,3-d]-pyrimidine) was synthesized and obtained (Novartis International AG, Basel, Switzerland). For in vivo administration, PKI166 was dissolved in dimethyl sulfoxide (0.5%) and then diluted 1:20 in Hanks balanced saline solution.14 Tetrazolium (MTT) was purchased (Sigma-Aldrich Corp, St Louis, Mo), and a stock solution was prepared by dissolving 5 mg of MTT in 1 mL of phosphate-buffered saline and filtering the solution to remove particles. The solution was protected from light, stored at 4°C, and used within 1 month.

WESTERN BLOTTING

The Tu159 and MDA1986 cells were plated into 6-well (38 mm²) plates at a concentration of 4 × 10⁵ cells per well and then incubated in serum-free medium for 24 hours. Treated cells were preincubated with inhibitor (1-10 µg/mL) (controls were preincubated with dimethyl sulfoxide alone) for 1 hour, then epidermal growth factor (EGF) (40 ng/mL) was added for 15 minutes; the cells were then washed with phosphate-buffered saline containing 5 mM edetic acid and 1 mM sodium orthovanadate. Cells were scraped into lysis buffer (1% Triton X-100, 20 mM Tris pH 8.0, 137 mM sodium chloride, 10% glycerol (vol/vol), 2 mM edetic acid, 1 mM phenylmethylsulfonyl fluoride, 20 µg/mL aprotinin, leupeptin and trypsin inhibitors, 2 mM sodium orthovanadate) and centrifuged to remove insoluble protein. Samples were diluted in sample buffer (0.5 mM Tris hydrochloride, pH 6.8, 10% sodium dodecyl sulfate, 1 M dithiothreitol, 10% (vol/vol) glycerol, and 1% bromphenol blue) and boiled. The proteins (30 µg/mL) were resolved on 7.5% polyacrylamide gel electrophoresis and transferred onto 0.45-µg nitrocellulose membranes. The 7.3% gels were used to probe with antiphosphotyrosine antiserum on Western blots of anti–EGF-R–immunoprecipitated cell lysates. We present a preclinical evaluation of an EGF-R tyrosine kinase inhibitor and its antitumoral effects on in vitro and in vivo proliferation of human SCCOC.

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20 (vol/vol) in Tris-buffered saline, probes with mouse monoclonal antiphosphotyrosine (IgG2bκ) (1:5000) (Upstate Biotechnology, Inc, Lake Placid, NY) in 5% nonfat milk, and incubated with horseradish peroxidase–conjugated sheep anti–mouse immunoglobulin (1:2000) (Amersham Life Science Inc, Arlington Heights, Ill) in 5% nonfat milk. The blots were also probed with sheep anti–EGF-R (UBI Inc), diluted 1:1000, in 5% nonfat milk and incubated with peroxidase–conjugated donkey anti–sheep IgG (1:3000) (Sigma Immunocoumialchemicals, St Louis, Mo) in 5% nonfat milk. Finally, all blots were probed with antiactin (1:1000) in 5% nonfat milk (Sigma Immunocoumialchemicals), followed by horseradish peroxidase–conjugated donkey anti–rabbit immuno- 

globulin (1:2000) (Amersham Inc) in 5% nonfat milk. Protein bands were visualized by the Enhanced Chemilumi-

nescence detection system (Amersham Inc).

MTT CELL PROLIFERATION ASSAY

PKI166 was tested against the Tu159 and MDA1986 cell lines by means of an MTT-based assay. The MTT assay measures cell proliferation, based on the ability of live cells to use MTT and convert it into dark-blue formazan.50 One thousand cells were plated into 38-mm² wells of 96-well tissue culture plates. The cells were grown in Dulbecco modified Eagle medium supplemented with sodium pyruvate, essential amino acids, and 10% fetal bovine serum. After a 24-hour attachment period, the cells were refed with medium (negative control with dimethyl sulfoxide alone) or medium containing PKI166. After a 5-day incubation, the number of metabolically active cells was determined by MTT assay. The conversion of MTT to formazan by metabolically active cells was measured by a 96-well microtiter plate reader at an optical density at 570 nm (MR-5000; Dynatech Laboratories Inc, Chantilly, Va). Growth inhi-

bition was calculated from the following formula: cytostasis (%) = [(1−(A/B))] × 100, where Δ is the absorbance of treated cells and B is the absorbance of control cells.

REDUCTION OF IN VIVO GROWTH OF Tu159 SCCOC XENOGRAFTS

On the basis of these in vitro data, a pilot study was performed to determine the effect of PKI166 on the growth of SCCOC in nude mice. Groups of 5 mice were inoculated subcutaneously with 1 × 10⁶ Tu159 cells each and observed for 7 days. Then, the animals were treated daily with oral PKI166 (0, 10, or 50 mg/kg) for 28 days. As shown in Figure 5, the growth of Tu159 xenografts was reduced in a dose-dependent manner. With the repeated-measures analysis (log-transformed data), differences in the growth in tumor size over time (as measured by the time × dose interaction) were statistically significant (P = .008). Differences between the control animals and the 50-mg dose group (P = .004) and between the 10-mg and 50-mg groups (P = .02) were statistically significant. Differences between control animals and the 10-mg dose group were not statistically significant. Inferences drawn from the untransformed tumor volume data and the square root–transformed data were similar to those observed for the log-transformed data.

COMMENT

Blockade of the EGF-R signaling pathway by the novel tyrosine kinase inhibitor PKI166 suppressed the growth of human SCCOC. PKI166 specifically inhibited the autophosphorylation mediated by the EGF-R tyrosine kinase pathway, as measured by Western blotting. Furthermore, the in vitro cytotoxicity of PKI166 on 2 SCCOC cell lines was confirmed by MTT assay. Finally, we present the first report, to our knowledge, of in vivo suppression of SCCOC tumor growth in an experimental animal model using oral tyrosine kinase inhibitor–EGF-R blockade.

The proliferation of HNSCC has been correlated with increased expression of EGF-R and its ligands, EGF and
transforming growth factor α (TGF-α). The HNSCCs express higher levels of EGF-R and TGF-α than corresponding normal tissues.21 Furthermore, the concomitant expression of both EGF-R and its ligand TGF-α suggests that an autocrine control mechanism may be important in the development of these tumors.22,23 In head and neck cancer, overexpression of EGF-R and TGF-α has been shown to correlate with aggressive malignant progression and poor clinical outcome.6,24

Targeted molecular therapy for HNSCC has focused on the use of anti–EGF-R antibody preparations. Tumor proliferation in cell culture and tumor xenografts in athymic mice have been inhibited by these antibodies, which block EGF binding to EGF-R.25,26 When injected into mice bearing tumor xenografts, mouse anti–EGF-R antibody preparations can cause partial tumor regression. The addition of concomitant chemotherapeutic agents (cisplatin or doxorubicin) is necessary for a more complete tumor response.27 A chimeric version of the 225 monoclonal antibody (C225) in which the mouse antibody variable regions are linked to human constant regions exhibited an improved in vivo therapeutic effect at high doses. These promising results with C225 led to phase 1 and 2 clinical trials that are now under way.13

The use of an orally administered compound that inhibits the proliferation of HNSCC has several advantages over treatment with receptor-specific antibodies. These advantages include drug availability, no immunologic reactivity, and direct intracellular effects on EGF-R. Phase 1 trials are currently under way to assess the pharmacokinetic bioavailability and toxicity of PKI166.28 Early reports14,28,29 suggest that this class of compounds has low levels of systemic toxic effects, limited to fatigue, nausea, and rash.30 The crucial biological activity of EGF-R pathway inhibition may be derived from its ability to suspend cell proliferation. Blockade of the EGF-R signaling pathway...
results in cellular arrest at the G1 restriction point, which has been shown to increase sensitivity to cytotoxicity mediated by radiation or chemotherapeutic agents. While halted by EGF-R inhibition, cancer cells may be more susceptible to concomitant cytotoxic agents (paclitaxel or cisplatin) and adjuvant radiotherapy. Studies are under way in our laboratory to evaluate the efficacy of PKI166 when given in combination with paclitaxel, with the use of an orthotopic nude mouse model of oral cancer.

In summary, the blockade of the EGF-R signaling pathway with a tyrosine kinase inhibitor arrests the growth of oral cancer in vitro and reduces its proliferation in an experimental xenograft animal model. These preliminary results require further confirmation with an orthotopic model of oral cancer as well as preclinical studies to determine the safety of tyrosine kinase inhibitors in humans. These studies are now under way in our laboratory.

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Corresponding author and reprints: Jeffrey N. Myers, MD, PhD, Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 441, Houston, TX 77030-4009 (e-mail: jmyers@mdanderson.org).

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