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

A Preliminary Report

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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 × 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 200 000 deaths annually.² Squamous cell carcinoma of the oral cavity (SCCOC) 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.⁸,⁹ Once activated, the EGF-R intracellular domain phosphorylates both the receptor itself and several crucial second messenger effector molecules.¹⁰ Activation of this signaling pathway triggers DNA synthesis and a mitogenic cascade, resulting in cell proliferation.¹¹,¹² 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-
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).

MEDICATION 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
20 (vol/vol) in Tris-buffered saline, probed with mouse monoclonal antiphosphotyrosine (IgG2bK) (1:5000) (Up-state 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 horseradish peroxidase–conjugated donkey anti–sheep IgG (1:3000) (Sigma Immunchemicaless, St Louis, Mo) in 5% nonfat milk. Finally, all blots were probed with antiactin (1:1000) in 5% nonfat milk (Sigma Immunchemicaless), followed with horseradish peroxidase–conjugated donkey anti–rabbit immunoglobulin (1:2000) (Amersham Inc) in 5% nonfat milk. Protein bands were visualized by the Enhanced Chemiluminescence detection system (Amersham Inc).

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.30 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 inhibition was calculated from the following formula: cytostasis (%)={(1− (A/B)) × 100}, where A is the absorbance of treated cells and B is the absorbance of control cells.

**Figure 3** and **Figure 4**, a dose-dependent cytotoxicity was seen with the addition of PKI166. Both cell lines had similar patterns of growth inhibition by PKI166. The IC₅₀ was calculated from regression lines on the linear portion of the growth inhibition graphs. The IC₅₀ was 0.18µM (R=0.98) for Tu159 cells and 0.23µM (R=0.97) for MDA1986 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.22 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 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

![Image](image1.png)

**Figure 1.** Effect of PKI166 on tyrosine phosphorylation in Tu159 cells of squamous cell carcinoma of the oral cavity by Western blotting. EGF indicates epithelial growth factor; EGF-R, EGF receptor; and P, phosphorylated.

![Image](image2.png)

**Figure 2.** Effect of PKI166 on tyrosine phosphorylation in MDA1986 cells of squamous cell carcinoma of the oral cavity by Western blotting. EGF indicates epithelial growth factor; EGF-R, EGF receptor; and P, phosphorylated.

![Image](image3.png)

**Figure 3.** PKI166-mediated cytotoxicity of Tu159 oral cancer cells as measured by tetrazolium-based viable cell assay. Tu159 cells were sensitive to the effects of PKI166 in a dose-dependent manner. The inhibitory concentration (50%) was 0.18µM (R=0.98) for Tu159 cells.

![Image](image4.png)

**Figure 4.** PKI166-mediated cytotoxicity of MDA1986 oral cancer cells as measured by tetrazolium-based viable cell assay. The MDA1096 cells were sensitive to the effects of PKI166 in a dose-dependent manner. The inhibitory concentration (50%) was 0.23µM (R=0.97) for MDA1986 cells.

![Image](image5.png)

**Figure 5.** Therapeutic effect of epidermal growth factor receptor blockade by means of systemic treatment with PKI166 on the growth of Tu159 xenograft tumors in nude mice. The growth of Tu159 tumor xenografts was inhibited in a dose-dependent manner in animals treated for 1 month with oral PKI166. Differences in the growth of tumors 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.
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.

Accepted for publication January 18, 2002.

This study was presented at the Fifth International Conference on Head and Neck Cancer, San Francisco, Calif, July 31, 2000.

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REFERENCES