0026-895X/08/7303-627–638$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2008 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 73:627–638, 2008

Vol. 73, No. 3
41160/3301056
Printed in U.S.A.

Antitumor Mechanisms of Systemically Administered Epidermal Growth Factor Receptor Antisense Oligonucleotides in Combination with Docetaxel in Squamous Cell Carcinoma of the Head and Neck

Sufi Mary Thomas, Michelene Jeter Ogagan, Maria L. Freilino, Sandy Strychor, Dustin R. Walsh, William E. Gooding, Jennifer Rubin Grandis, and William C. Zamboni

Departments of Otolaryngology (S.M.T., M.J.O., M.L.F., J.R.G.), Pharmaceutical Sciences (S.S., D.R.W., W.C.Z.), Biostatistics (W.E.G.), Pharmacology (J.R.G.), and Medicine (W.C.Z.), University of Pittsburgh and the University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Received September 6, 2007; accepted November 19, 2007

ABSTRACT

Squamous cell carcinoma of the head and neck (SCCHN) is one of the most common malignancies worldwide, with low 5-year survival rates. Current strategies that block epidermal growth factor receptor (EGFR) have limited effects when administered as single agents. Targeting EGFR via intratumoral administration of phosphorothioate-modified antisense oligonucleotides has antitumor efficacy in xenograft models of SCCHN. Because intratumoral delivery of therapeutic agents has limited clinical application, the present study was undertaken to examine the therapeutic mechanisms of systemically delivered phosphorothioate-modified EGFR antisense oligonucleotides alone, or in combination with docetaxel, in a SCCHN xenograft model. EGFR antisense oligonucleotides were administered at 5 mg/kg i.p. daily in athymic mice bearing 1483 human SCCHN xenografts alone or in combination with docetaxel at 2.5 mg/kg i.p. once a week for 4 weeks. Administration of EGFR antisense oligonucleotides in combination with docetaxel improved antitumor efficacy and resulted in lower expression levels of EGFR, fewer proliferating cells, and more apoptotic cells in the tumors compared with controls. Systemic administration of phosphorothioated EGFR antisense oligonucleotides for 30 days increased the retention of docetaxel in the tumor by approximately 4-fold compared with tumors treated with docetaxel alone or docetaxel and EGFR sense oligonucleotides ($P < 0.05$). Combination of EGFR antisense oligonucleotides with low doses of docetaxel has antitumor efficacy, and it may be an effective treatment strategy for SCCHN.

Standard therapeutic regimens for squamous cell carcinoma of the head and neck (SCCHN) patients include surgery, radiation, and chemotherapy. Surgical extirpation often leads to functional impairment and the toxic effects of radiation and chemotherapy can be debilitating. Recent evidence suggests that combining standard therapy with agents that inhibit specific molecular targets may increase patient survival (Bonner et al., 2006). The epidermal growth factor receptor (EGFR) is overexpressed in 90% of SCCHN tumors. Activated EGFR triggers increased cell proliferation, angiogenesis, and metastasis, contributing to tumor progression and decreased survival and indicating that EGFR may serve as a therapeutic target for SCCHN. Various strategies targeting EGFR have demonstrated antitumor efficacy in preclinical models. Recently the Food and Drug Administration approved the use of the anti-EGFR antibody cetuximab as a monotherapy or in combination with radiotherapy for SCCHN patients. In addition to blocking EGFR activation, down-modulating EGFR protein levels via an antisense DNA approach has been reported to be an effective way of inhibiting EGFR-mediated growth. A direct comparison between EGFR antisense gene targeting and an EGFR tyrosine kinase-specific inhibitor or EGFR monoclonal antibodies demonstrated increased growth inhibition with antisense gene treatment compared with either of the other EGFR-targeting modalities (Grandis et al., 1997). Intratumoral injections of lipid formulations with antisense EGFR gene therapy and

This work was supported by National Institutes of Health grants R01-CA77308 and P50-CA097190 (to J.R.G.) and a research grant from sanofi-aventis (to J.R.G. and W.C.Z.).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.041160.

ABBREVIATIONS: SCCHN, squamous cell carcinoma of the head and neck; EGFR, epidermal growth factor receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; STAT, signal transducer and activator of transcription.

antisense EGFR oligonucleotides reduced SCCHN tumor growth in vivo without evidence of toxicity (He et al., 1998; Niwa et al., 2003). However, given the obstacles associated with intratumoral administration, the concerns regarding gene therapy raised by the death of the patient at the University of Pennsylvania in 1999, and the potential toxicity of liposomes, we initiated studies to examine therapeutic targeting of EGFR using systemically administered phosphorothioated antisense oligonucleotides. Phosphorothioate modifications improve the stability of oligonucleotides in the plasma by increasing their resistance to nuclease degradation. Preclinical studies with antisense phosphorothioate-modified oligonucleotides demonstrated successful down-regulation of the antiapoptotic proteins, X-linked inhibitor of apoptosis and Bcl-2, via systemic delivery in mice (Hu et al., 2003). However, emerging evidence suggests that no single treatment regimen results in sustained antitumor efficacy. In the last decade, the field of oncology has witnessed the development of combined chemotherapeutic agents, with therapies targeting signal transduction molecules in cancer cells (Wang et al., 2001).

In this study, we tested the antitumor efficacy of systemically administered EGFR antisense oligonucleotides alone and combined with a low dose of the chemotherapeutic agent docetaxel. Docetaxel is a taxane analog that is active both as monotherapy and in combination with molecular targeting agents, including bevacizumab in patients with solid tumors (Dreyfuss et al., 1996; Schöffski et al., 1999; Herbst et al., 2007). Combining EGFR inhibitors with chemotherapy has been reported to result in added tumor growth inhibition in several cancer cell lines, including ovary, breast, and colon (Ciardiello et al., 2000). The plasma, tumor, and tissue disposition of taxane analogs has been previously evaluated in preclinical models (Bissery et al., 1995; Sparreboom et al., 1998; Strychor et al., 2005). However, the plasma and tumor disposition of docetaxel has not been evaluated in combination with agents that target EGFR. Here, we report the feasibility and explore the antitumor mechanisms of a novel therapeutic strategy combining low-dose docetaxel with systemically delivered EGFR antisense oligonucleotides for the treatment of SCCHN.

Materials and Methods

Cells and Reagents. Human SCCHN cells 1483 and PCI-15b used in this study are previously described, well characterized cell lines (Sacks et al., 1988; Heo et al., 1989). Cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in presence of 5% CO_2. Docetaxel (Taxotere) was obtained from sanofi-aventis (Bridgewater, NJ) as a solution of 10 mg/ml in 13% ethanol (Clarke and Rivory, 1999). Docetaxel was further diluted to a concentration of 0.5 mg/ml in saline. Phosphorothioate-modified antisense and sense oligonucleotides targeting EGFR mRNA were obtained from the University of Pittsburgh DNA Synthesis Facility (Pittsburgh, PA) as described previously (Niwa et al., 2003). The oligonucleotides were dissolved in water and further diluted in saline. Antibodies used for immunoblotting and immunohistochemistry included anti-EGFR monoclonal antibody (BD Transduction Laboratories, Lexington, KY); β-actin monoclonal mouse IgM (Calbiochem, San Diego, CA); phospho-AKT (Ser473; Cell Signaling Technology Inc., Danvers, MA); phospho-Stat3 (Tyr705; Cell Signaling Technology Inc.), and Ki67 (Dako Denmark A/S, Glostrup, Denmark). Apoptosis was measured by TUNEL (Roche Applied Science, Mannheim, Germany).

In Vivo Studies. Animal use and care was in strict compliance with institutional guidelines established by the University of Pittsburgh, Institutional Animal Care and Use Committee. Female athymic nude mice (Harlan, Indianapolis, IN) between the age of 5 and 6 weeks were injected with 1×10^6 1483 cells in 100 μl of Hanks' balanced salt solution subcutaneously on the left flank. Treatment was initiated 8 days after inoculation when tumors reached an average volume of 15 mm^3. Tumor volumes were calculated using the formula: length × $(\text{width})^2/2$, where length is the largest diameter and width is the smallest diameter perpendicular to the length. Mice were randomized into treatment groups based on the tumor volumes. Five mice were randomly assigned to vehicle control (saline), EGFR sense oligonucleotide alone, EGFR antisense oligonucleotide alone, or docetaxel alone. Ten mice were assigned to the docetaxel plus EGFR sense oligonucleotide and docetaxel plus EGFR antisense oligonucleotide groups. EGFR antisense and sense oligonucleotides were administered at 5 mg/kg i.p. once daily for 30 days alone or in combination with docetaxel administered i.p. at 2.5 mg/kg once a week alone. Tumors were measured twice a week in two dimensions, with a vernier caliper. At the end of the study, tumors were excised, and they were divided into three sections. One section was snap frozen for molecular analyses by immunoblotting. The second section was fixed in 10% buffered formalin for immunohistochemical analyses. Blood was collected from anesthetized mice via cardiac puncture using heparinized syringes. Part of the blood was analyzed for hematological and serum chemistry parameters by Antech Diagnostics (Farmingdale, NY).

Pharmacokinetic studies were carried out on docetaxel concentrations in plasma and tumors of mice administered a single dose of docetaxel (2.5 mg/kg i.p.) alone or in combination with EGFR antisense oligonucleotides (5 mg/kg i.p.). Plasma and tumor tissue were harvested at 0.25, 1, 3, 6, and 24 h after administration of docetaxel alone or docetaxel injected 30 min after EGFR antisense oligonucleotide administration. In a separate study, the concentration of docetaxel in the tumor was assessed in mice administered multiple doses of both agents over 30 days. Specifically mice ($n = 5$ per treatment arm) were treated for 30 days (720 h) with EGFR antisense or sense oligonucleotides administered at 5 mg/kg i.p. once daily for 30 days alone or in combination with docetaxel administered i.p. at 2.5 mg/kg once a week. At the end of the study mice were anesthetized with isofluorane, and approximately 0.8 to 1 ml of blood was collected, by cardiac puncture using heparinized syringes. Blood was transferred to microcentrifuge tubes, and the tubes were immediately placed on ice. Plasma was harvested from the blood samples after centrifugation at 12,000*g* for 4 min at 4°C, and it was snap frozen in a dry ice-ethanol bath. Mice were euthanized by cervical dislocation, and the tumor tissue was harvested, weighed, and snap frozen in a dry ice-ethanol bath. Plasma samples were processed using solid phase extraction, whereas tumor samples were processed using acetonitrile precipitation followed by solid phase extraction (Strychor et al., 2005). Docetaxel concentrations in plasma and tumors were determined by a liquid chromatography/mass spectrometry assay as described previously (Parise et al., 2003).

Immunoblotting. Xenograft tumors were minced on dry ice and suspended in 500 μl of lysis buffer (10 mM Tris HCl, pH 7.6, 50 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM NaV_3O_4, 1% Triton X-100, and 1× protease inhibitor cocktail tablet that included a broad-spectrum potent inhibitor of protein tyrosine phosphatases (Roche Applied Science). The lysates were sonicated, and the supernatant was collected after centrifugation at 13,000 rpm for 5 min. Forty micrograms of protein was fractionated through 8% SDS polyacrylamide gels, and it was analyzed via immunoblotting for biomarker modulation. Autoradiograms were scanned, and the bands were quantified using the DigiDoc 1000 digital imaging system (Alpha Innotech, San Leandro, CA). Values obtained were normalized to β-actin levels and positive control lysates run on every gel. Cell lysates obtained from a well characterized HNSCC cell line, PCI-15b, were used as the positive controls. Relative intensities were averaged across tumors

from the same group, and the cumulative results were graphed using Prism version 6 (GraphPad Software Inc., San Diego, CA).

Immunohistochemistry. Four-micrometer-thick sections of paraffin-fixed tumor samples from the EGFR antisense oligonucleotide alone and EGFR sense oligonucleotide alone treatment groups were removed, and they were adhered to slides by heating overnight at 57°C in a dry slide incubator. The specimens were deparaffinized, rehydrated, and placed in a 1:10 diluted mixture of methanol/H_2O_2 for 15 min at room temperature, and then they were rinsed two times with distilled water. Slides were placed in boiling citrate buffer for 10 min to enable antigen retrieval, and then they were examined for Ki67 expression. Sections from paraffin-embedded tissues were also examined for apoptosis by TUNEL (Roche Applied Science) according to the manufacturer's instructions. The average number of positive cells was determined from five separate fields under 400× magnification. Cumulative findings from blinded assessments of sections by two independent investigators were graphed using GraphPad Prism version 6.

Pharmacokinetic Analysis. The compartmental pharmacokinetic analysis of docetaxel in plasma and tumors was performed using maximum likelihood estimation in the ADAPT II modeling program as described previously (Zamboni et al., 1999). The estimation procedure and variance model used in the compartmental pharmacokinetic analysis was maximum likelihood estimation and linear models for the variance of the additive errors, respectively. Various pharmacokinetic model structures were considered to characterize the disposition of docetaxel. Akaike's Information Criteria, estimated error of the model parameters, and residual analysis were use to select the model structure that maximized the fit accuracy and simultaneously minimized the number of model parameters. The final model structure used for the pharmacokinetic analysis produced identifiable parameters.

Statistical Analysis. Tumor volumes were (natural) log transformed before analysis. Adequacy of random assignment of animals to treatment groups was checked by one-way analysis of variance. Day 30 differences were compared in an omnibus test with a one-way analysis of variance. Contrasts of specific interest were tested with a t test using the pooled estimate of standard error. P values were adjusted with the multivariate t distribution to simulate the data under the complete null hypothesis (Edwards and Berry, 1987). P values were also adjusted using the Bonferroni correction. Serum chemistries were compared among the six animal treatment groups with the Kruskal-Wallis test. P values were adjusted by the Sidak method and by permutation testing.

The tumor concentrations of docetaxel obtained after the end of the 4-week treatment regimen on day 30 after administration of docetaxel alone or docetaxel in combination with EGFR antisense or sense oligonucleotides was evaluated using the exact two-tailed Kruskal-Wallis test for equality of the three groups and the exact two-tailed Wilcoxon test to compare each group. The exact two-tailed Wilcoxon test was used to compare the tumor concentrations of docetaxel at 24 h after administration of docetaxel alone and docetaxel in combination with EGFR antisense oligonucleotides. The numbers of Ki67 or TUNEL-positive cells from the docetaxel and EGFR antisense oligonucleotide group was compared with the docetaxel and EGFR sense oligonucleotide-treated group using the exact two-tailed Kruskal-Wallis test. Exact nonparametric tests were performed using StatXact with Cytel Studio version 6.1 software (Cytel Inc., Cambridge MA). P values less than 0.05 was considered statistically significant.

Results

Systemic Administration of EGFR Antisense Oligonucleotides in Combination with Low Dose of Docetaxel Had Antitumor Efficacy against SCCHN Xenografts. We previously demonstrated that the EGFR antisense oligonucleotides inhibit SCCHN tumor growth when administered intratumorally (Niwa et al., 2003). However, intratumoral administration of EGFR antisense oligonucleotides likely reaches a limited number of tumor cells at the site of injection, and it may be impractical in the clinical setting. In a preliminary study, we compared the antitumor efficacy of i.p.- versus i.v.-administered EGFR antisense oligonucleotides in SCCHN tumor-bearing mice, and we found no difference in the post-treatment tumor volumes using the two routes of delivery (data not shown). The i.p. route was chosen due to greater ease of administration. To examine the antitumor efficacy of EGFR antisense oligonucleotides, tumor-bearing mice were treated systemically with either EGFR antisense or sense oligonucleotides at 5 mg/kg i.p. daily for 4 weeks. Tumors in EGFR antisense oligonucleotide-treated group had significantly smaller tumor volumes compared with EGFR sense oligonucleotide-treated mice ($P < 0.05$) (Fig. 1A). Furthermore, tumor volumes of mice treated with EGFR antisense oligonucleotides plus docetaxel were significantly lower than the EGFR sense oligonucleotides plus docetaxel group after the Bonferroni correction ($P < 0.01$). The Bonferroni correction was applied since three-paired comparisons were made among three treatment groups. In two of the 10 mice administered systemic EGFR antisense oligonucleotides plus docetaxel, the tumors regressed completely by day 30. Differences among the tumor volumes between the various groups on day 30 are represented in Fig. 1B. Mice treated with EGFR antisense oligonucleotides in combination with docetaxel had the smallest tumors among all the groups.

Systemic Administration of EGFR Antisense Oligonucleotides Results in Modulation of EGFR Levels and Phosphorylation of Downstream Signaling Molecules. To examine the antitumor mechanisms of EGFR targeting using antisense oligonucleotides, SCCHN tumors from mice treated with EGFR antisense oligonucleotides alone or in combination with docetaxel were examined for total EGFR levels and for activation of signaling molecules downstream of EGFR. Mice treated with EGFR antisense oligonucleotides demonstrated down-modulation of EGFR protein levels compared with EGFR sense oligonucleotide-treated mice ($P < 0.05$) (Fig. 2A). Mice treated with a combination of docetaxel and EGFR antisense oligonucleotides also showed reduced protein expression levels of EGFR compared with mice treated with EGFR sense oligonucleotides and docetaxel ($P < 0.01$) (Fig. 2B). The bar graph depicts the cumulative densitometry analyses of immunoblots from xenograft tumor cell lysates of all the mice in each group. In addition to EGFR, we examined pAkt levels and pSTAT3 levels (Fig. 2, C and D, respectively). Although pAkt was down-modulated in EGFR antisense oligonucleotides plus docetaxel-treated tumors ($P < 0.05$), there was no appreciable change in pSTAT3 levels in the tumors ($P > 0.05$) (Fig. 2, C and D, respectively). Levels of phospho-mitogen-activated protein kinase also remained unchanged between the two groups (Fig. 2E).

Systemic Administration of EGFR Antisense Oligonucleotides in Combination with Docetaxel Resulted in Reduced Proliferation and Increased SCCHN Apoptosis. We and others have previously demonstrated that inhibition of EGFR results in reduced tumor cell growth (Thomas and Grandis, 2004). To examine the effects of EGFR antisense oligonucleotides with or without docetaxel on proliferation of SCCHN tumor cells, tumors were examined by immunohistochemical

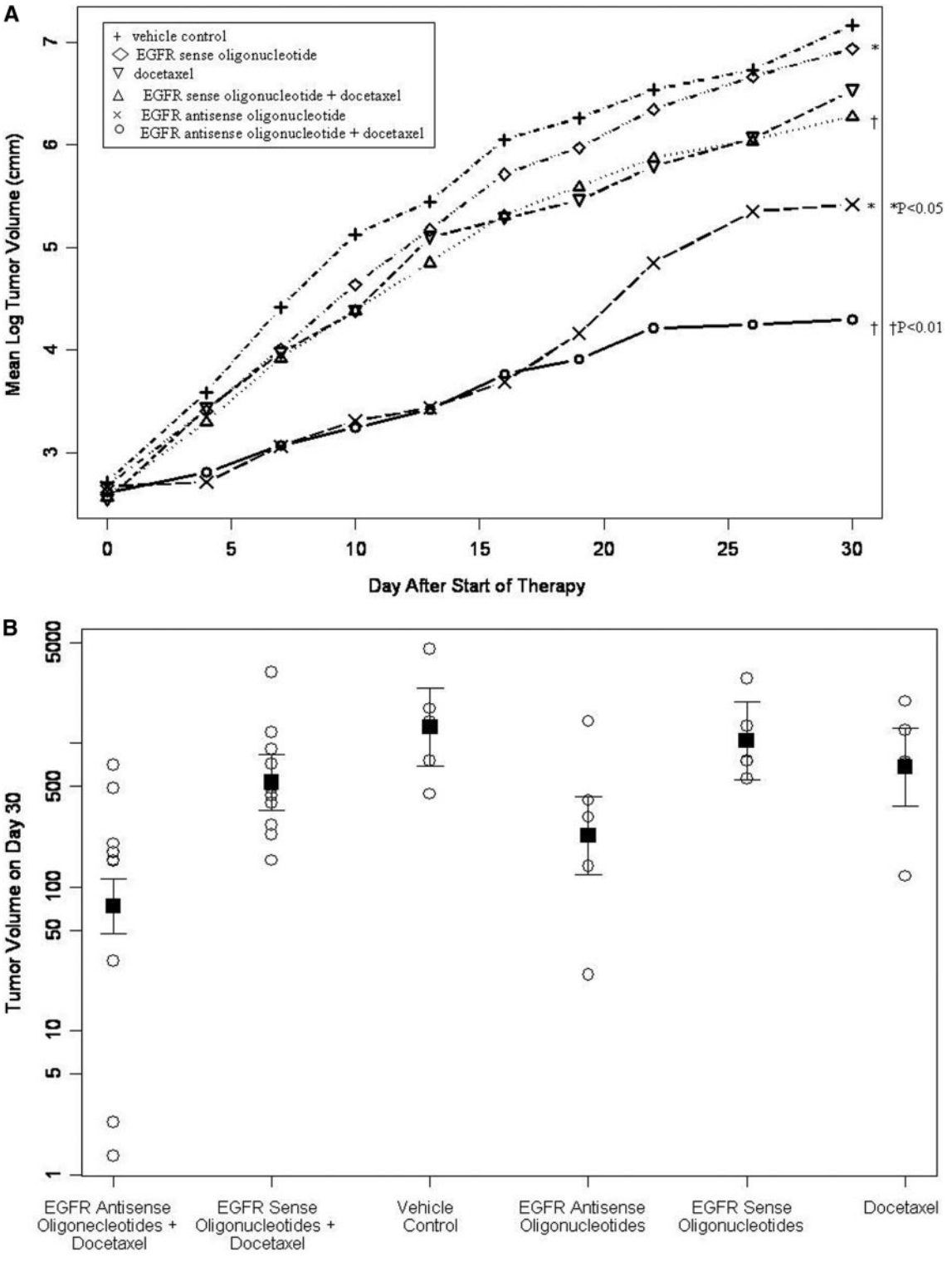

Fig. 1. Antitumor effects of systemic administration of EGFR antisense oligonucleotide monotherapy and combination therapy with docetaxel in vivo. One million (SCCHN) 1483 cells were injected subcutaneously into athymic nude mice. A, five mice per group were administered vehicle control (saline) (+), docetaxel (2.5 mg/kg) (▽) i.p. once weekly, EGFR antisense (×), and sense oligonucleotides (◇) i.p. daily at 5 mg/kg body weight. Ten mice per group were treated with EGFR antisense (○) or sense (△) oligonucleotides i.p. daily at 5 mg/kg body weight in combination with one weekly injection of docetaxel (2.5 mg/kg). The graph depicts the median tumor volume by day and treatment group. Mice treated with EGFR antisense oligonucleotides demonstrated reduced tumor growth compared with mice treated with EGFR sense oligonucleotides ($P < 0.05$). A significant reduction in tumor growth rate in the EGFR antisense oligonucleotide plus docetaxel-treated tumors was observed compared with the EGFR sense plus docetaxel-treated tumors ($P < 0.01$). B, graph depicts tumor volumes of mice in all treatment groups on day 30 of the experiment. The actual tumor volume for each mouse is shown as an open circle. The group means after log transformation are plotted as squares, and the estimated standard deviation for each group are depicted as error bars. Standard errors were derived from the residual mean squared error of the one-way analysis of variance of log-transformed tumor volumes.

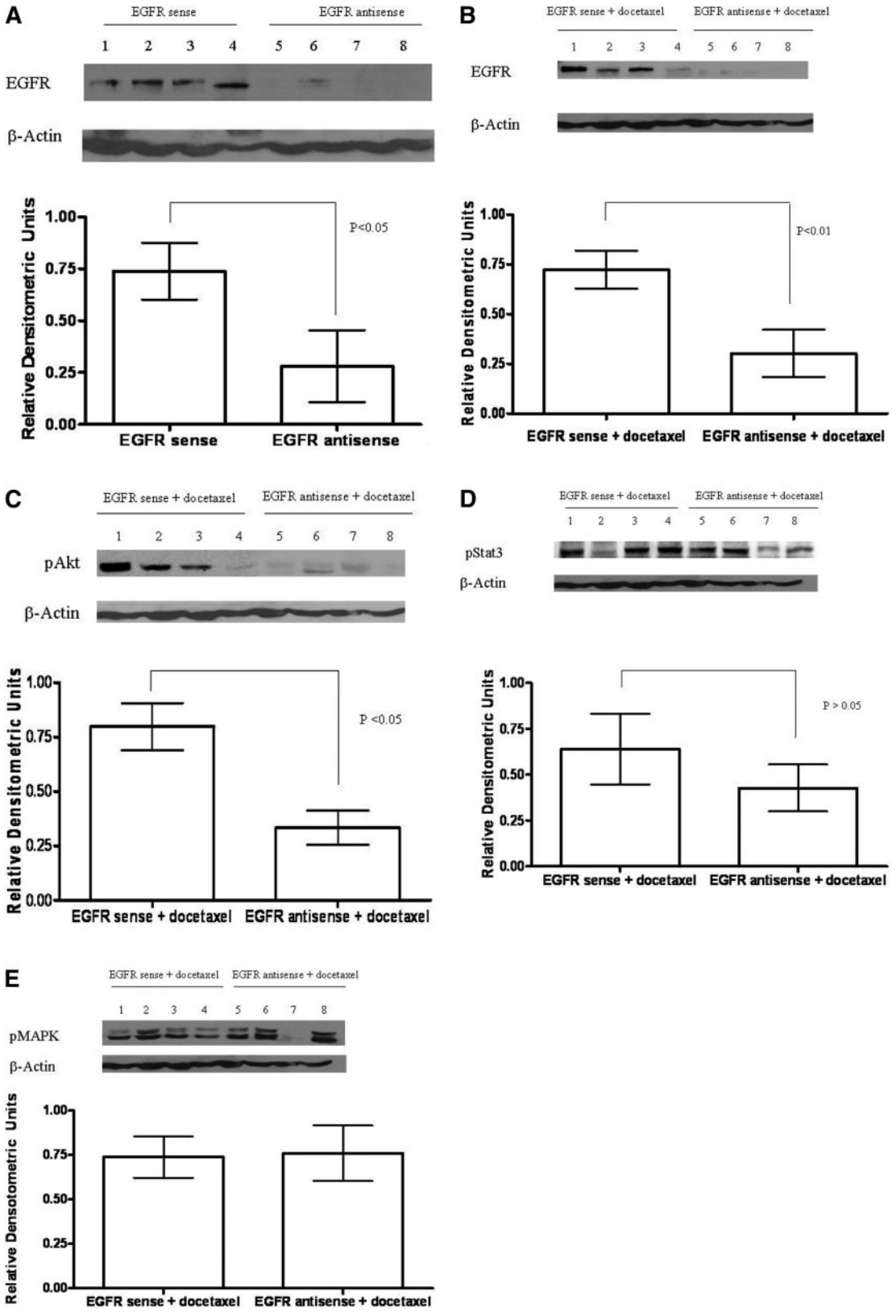

Fig. 2. EGFR antisense oligonucleotide with or without docetaxel modulates the EGFR signaling in SCCHN xenografts. EGFR antisense oligonucleotide monotherapy (A) and combination therapy with docetaxel (B) resulted in down-modulation of EGFR expression in SCCHN tumors compared with control tumors treated with EGFR sense oligonucleotide with or without docetaxel ($P < 0.05$ and $P < 0.01$, respectively). Immunoblots demonstrate down-modulation of phospho-Akt ($P = 0.023$) (C) but not pSTAT3 ($P > 0.05$) (D) and phospho-mitogen-activated protein kinase ($P > 0.05$) (E) in tumors treated with EGFR antisense oligonucleotide plus docetaxel compared with the EGFR sense oligonucleotide plus docetaxel. Numbers above the lanes indicate individual tumors from mice treated in each group. Error bars depict ± S.E.M.

analysis for Ki67 expression. The dark nuclei were counted, and the cumulative findings from two independent blinded assessments of all the tumors in each group were represented in the graph. The error bars represent the ± S.E.M. EGFR antisense oligonucleotide-treated tumors demonstrated fewer proliferating cells compared with EGFR sense oligonucleotide-treated tumors (Fig. 3A). Docetaxel administration further reduced the number of proliferating SCCHN cells in mice treated with EGFR antisense oligonucleotides compared with that in tumors treated with EGFR sense oligonucleotides and docetaxel ($P < 0.01$). We also examined the xenografts for apoptosis using TUNEL staining for DNA fragmentation. Apoptotic cell nuclei stained brown were counted, and the cumulative findings from two independent blinded assessments of all the tumors in each group were represented in the graph. The combination of EGFR antisense oligonucleotides and docetaxel induced greater apoptotic cell death in tumors as measured by the increased nuclear staining of fragmented DNA compared with the combination of EGFR sense oligonucleotides and docetaxel ($P < 0.001$) (Fig. 3B).

Pharmacokinetic Analysis Demonstrated Increased Docetaxel Exposure in the Tumors after Multiple Treatments with EGFR Antisense Oligonucleotides. To determine the mechanisms whereby the tumors demonstrated an increased sensitivity to chemotherapeutic agent docetaxel in the presence of EGFR inhibition, we examined the levels of docetaxel in the plasma and tumors of mice treated with both agents. A four-compartment model with administration of docetaxel into the intraperitoneal cavity was fit to the plasma concentration versus time data as reported previously (Strychor et al., 2005). The model parameters consisted of the rate constant for absorption (k_a) from the intraperitoneal cavity to the central plasma compartment, rate constant representing first pass clearance via the liver (k_{fp}), the volume of the central compartment (V_c), intercompartment rate constants (k_{12}, k_{21}, k_{13}, and k_{31}), and the elimination rate constant from the central compartment (k_{10}) (Fig. 4). Due to the limited concentration versus time data, especially during the elimination phase, and to get an accurate estimate of the k_a, the parameters presenting the plasma disposition were fixed to values from our prior study

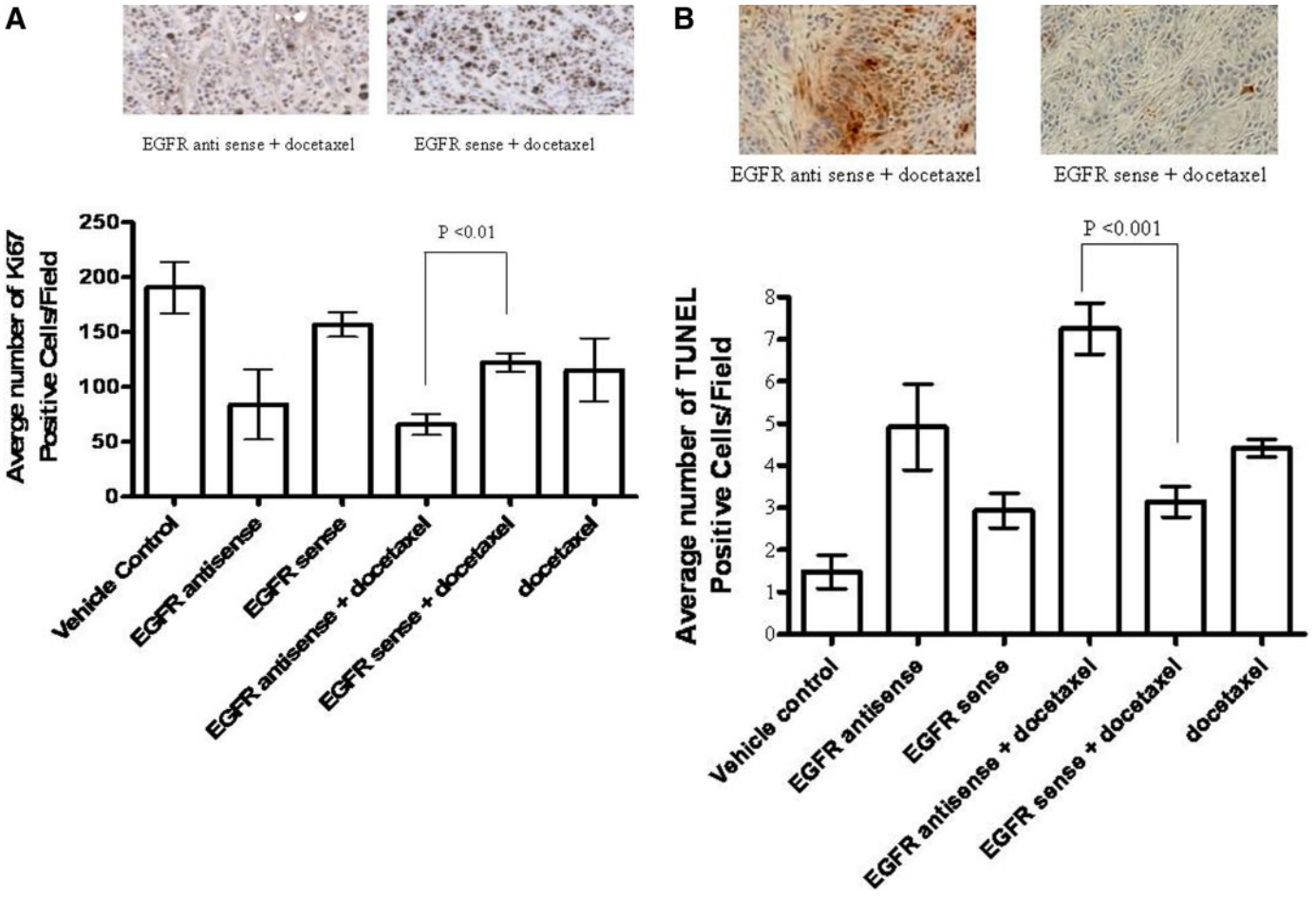

Fig. 3. Systemic EGFR antisense oligonucleotides administration reduces SCCHN tumor cell proliferation and induces apoptosis in vivo. SCCHN xenograft tumors treated with either EGFR sense or antisense oligonucleotides (5 mg/kg) were paraffin-embedded and examined for proliferation and apoptosis. A, tumor cell proliferation in various groups was assessed by the number of Ki67-positive cells at 400× magnification in 10 separate fields. Error bars indicate ± S.E.M. EGFR antisense oligonucleotide treatment reduced the number of proliferating tumor cells compared with the control groups. There was a significant reduction in cell proliferation in tumors treated with docetaxel and EGFR antisense oligonucleotides compared with tumors treated with docetaxel and EGFR sense oligonucleotides as depicted in a representative photomicrograph (200× magnification) of tumors from each group ($P < 0.01$). B, apoptotic cells were stained for fragmented DNA using TUNEL apoptosis detection kit. Positive cells were counted at 400× magnification in four separate fields. Error bars indicate ± S.E.M. EGFR antisense treatment resulted in increased apoptosis in the tumors. Combination of docetaxel and EGFR antisense oligonucleotides resulted in increased apoptosis compared with the EGFR sense oligonucleotide and docetaxel-treated tumors as depicted in a representative photomicrograph (200× magnification) of tumors from each group ($P < 0.001$).

of docetaxel administered i.v. at 10 mg/kg (Strychor et al., 2005). The V_c, k_{12}, k_{21}, k_{13}, k_{31}, and k_{10} were fixed at 1.22 l/m^2, 2.06 h^{-1}, 2.02 h^{-1}, 0.66 h^{-1}, 0.17 h^{-1}, and 3.78 h^{-1}, respectively (Strychor et al., 2005). The corresponding half-life and clearance in all studies were fixed at 0.2 h and 4.6 l/h/m^2 (Zamboni et al., 1999). The systemic disposition of docetaxel was similar in both studies, and fixing the k_{10} and V_c values allowed us to accurately determined the rate constant describing the absorption of docetaxel from the i.p. cavity. The area under the concentration versus time curves (AUC) of docetaxel in plasma (AUC_{Plasma}) and tumor (AUC_{Tumor}) from 0 to 24 h were estimated using the log trapezoidal method by simulating the concentration versus time data based upon model-specific parameters.

A fifth compartment describing the tumor disposition was added to the four-compartment model described above for the plasma disposition after i.p. administration. The model structure was based on our prior model of tumor disposition (Zamboni et al., 1999). The model parameters describing the tumor disposition were the rate constants from the plasma to the tumor (k_{14}) and from the tumor to the plasma (k_{41}) and the volume of the tumor compartment (V_t). The V_t was fixed to 0.17 l/m^2 based on our previous study (Zamboni et al., 1999). The docetaxel tumor AUC from 0 to 720 h after administration of docetaxel in combination with EGFR antisense oligonucleotides and docetaxel alone was 23,280 and 12,070 ng/ml · h, respectively (Table 1). The pharmacokinetic model parameters suggest that the increase in docetaxel tumor exposure in combination with EGFR antisense oligonucleotides is due to tumor-related factors (i.e., k_{14}) induced by EGFR antisense oligonucleotides and not associated with altered absorption of docetaxel from the i.p. cavity or systemic clearance.

Administrations of antisense oligonucleotides against cAMP-dependent protein kinase has been reported to increase the tissue and tumor uptake of chemotherapeutic agent irinotecan in athymic nude mice (Wang et al., 2002). To evaluate the effect of a single dose of EGFR antisense oligonucleotides on the plasma and tumor uptake of docetaxel, pharmacokinetic studies in plasma and tumor were performed from 0 to 24 h after administration of docetaxel alone or in combination with EGFR antisense oligonucleotides. The concentration versus time profiles of docetaxel in plasma and tumor from 0 to 24 h after a single dose of docetaxel alone or 30 min after administration of EGFR antisense oligonucleotides were similar (Fig. 5). Overall, the plasma and tumor concentrations of docetaxel were near the lower limit of quantitation (1 nM). The tumor concentrations of docetaxel at 24 h after administration of docetaxel alone and in combination with EGFR antisense oligonucleotides were 24.2 ± 4.6 and 23.8 ± 4.3 ng/ml, respectively ($P > 0.05$). The pharmacokinetic parameters of docetaxel after administration of docetaxel alone and in combination with EGFR antisense oligonucleotides are summarized in Table 1. The parameters describing the intratumoral and systemic disposition of docetaxel were found to be similar after administration of docetaxel alone and in combination with the EGFR antisense oligonucleotides. These results suggest that although there is an accumulation of docetaxel in tumors relative to plasma over 24 h, a single dose of the EGFR antisense oligonucleotide does not alter the plasma or tumor levels of docetaxel.

To evaluate the effect of repeated administration of EGFR antisense oligonucleotides on the tumor disposition of docetaxel, pharmacokinetic studies in tumor were performed at 720 h (30 days) after administration of docetaxel alone and in combination with EGFR antisense or sense oligonucleotides. The concentration versus time profiles of docetaxel in plasma and tumor from 0 to 720 h after repeated administration of docetaxel alone and in combination with EGFR antisense oligonucleotides are represented in Fig. 6, A and B, respectively. The docetaxel plasma concentration versus time profile is similar after administration of docetaxel alone and in combination

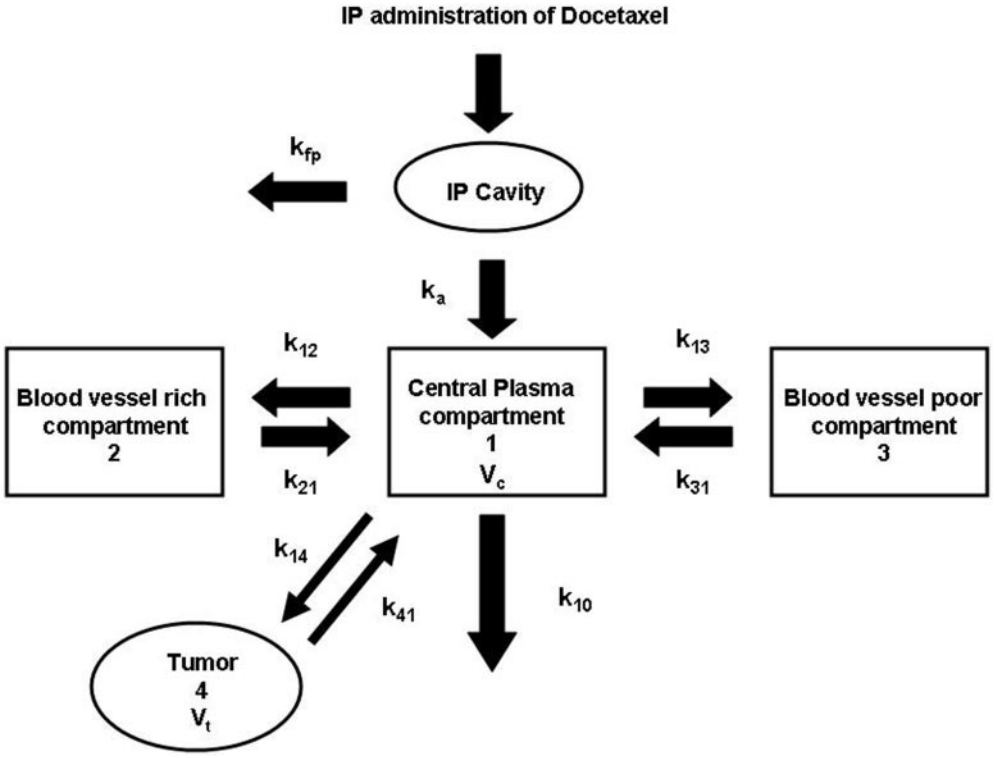

Fig. 4. Five-compartmental model describing the plasma and tumor disposition of docetaxel after i.p. administration. A five-compartment model was fit to the plasma and tumor concentration versus time data after administration of docetaxel alone and in combination with the EGFR antisense. A four-compartment model with administration of docetaxel into the i.p. cavity was fit to the plasma concentration versus time data. The model parameters representing the plasma disposition consisted of the rate constant for absorption from the i.p. cavity to the central compartment (k_a), the rate constant representing clearance from the i.p. cavity and first pass clearance via the liver (k_{fp}), the volume of the central compartment (V_c), the intercompartment rate constants (k_{12}, k_{21}, k_{13}, and k_{31}), and the elimination rate constant from the central compartment (k_{10}). A fifth compartment was used to represent the tumor disposition of docetaxel. The model parameters describing the tumor disposition were the rate constants from the plasma to the tumor (k_{pt}) and from the tumor to the plasma (k_{tp}) and the volume of the tumor compartment (V_t).

with EGFR antisense oligonucleotides. The docetaxel tumor concentration versus time profiles after administration of docetaxel in combination with EGFR antisense are greater compared with docetaxel alone. On day 30, the mean ± S.D. concentration of docetaxel in tumors after administration of docetaxel in combination with the EGFR antisense oligonucleotides (17.4 ± 11.0 ng/ml) was significantly greater than after administration of docetaxel alone (3.1 ± 4.2 ng/ml) or docetaxel in combination with EGFR sense oligonucleotides (5.4 ± 8.1 ng/ml) ($P < 0.05$). The docetaxel tumor concentrations after administration of docetaxel alone or docetaxel in combination with EGFR sense oligonucleotides were similar ($P > 0.05$), indicating that the increased tumor concentrations of docetaxel are specific to EGFR antisense oligonucleotides administration.

Systemic Administration of EGFR Antisense Oligonucleotides and Docetaxel Was Well Tolerated in Vivo. Mice in this study were examined for evidence of toxicity resulting from due to systemic administration of EGFR antisense oligonucleotides with or without docetaxel. Blood from mice treated with EGFR antisense or sense oligonucleotides alone and in combination with docetaxel was evaluated for hemoglobin, hematocrit, albumin, red blood cells, and white blood cells and for liver enzymes serum glutamic-oxalocetic transaminase and serum glutamic-pyruvic transaminase (Table 2). There were no differences in hematological and liver enzyme values among the treatment groups (Table 3). Thus, the combination of EGFR antisense and docetaxel produced no observed toxicity.

Discussion

Several small molecule inhibitors and antibodies targeting EGFR have been tested in SCCHN patients. Most EGFR inhibitors work by blocking the ligand binding domain or the

TABLE 1
Pharmacokinetic parameters of docetaxel after administration of docetaxel alone and in combination with EGFR antisense oligonucleotides

Parameter	Units	Docetaxel Alone	Coefficient of Variation	Docetaxel + EGFR Antisense Oligonucleotide	Coefficient of Variation
			%		%
k_a	h^{-1}	0.0036	16.2	0.0031	18.1
k_{fp}	h^{-1}	0.0332	19.4	0.0313	18.8
k_{14}	h^{-1}	0.0208	15.0	0.0224	14.5
K_{41}	h^{-1}	0.0159	22.0	0.0075	13.5
Plasma $AUC_{0-24\ h}$	ng/ml · h	179		156	
Plasma $AUC_{0-720\ h}$	ng/ml · h	1284		1167	
Tumor $AUC_{0-24\ h}$	ng/ml · h	313		312	
Tumor $AUC_{0-720\ h}$	ng/ml · h	12,070		23,280	
Ratio of tumor-to-plasma $AUC_{0-24\ h}$		1.74		2.00	
Ratio of tumor-to-plasma $AUC_{0-720\ h}$		9.40		19.94	

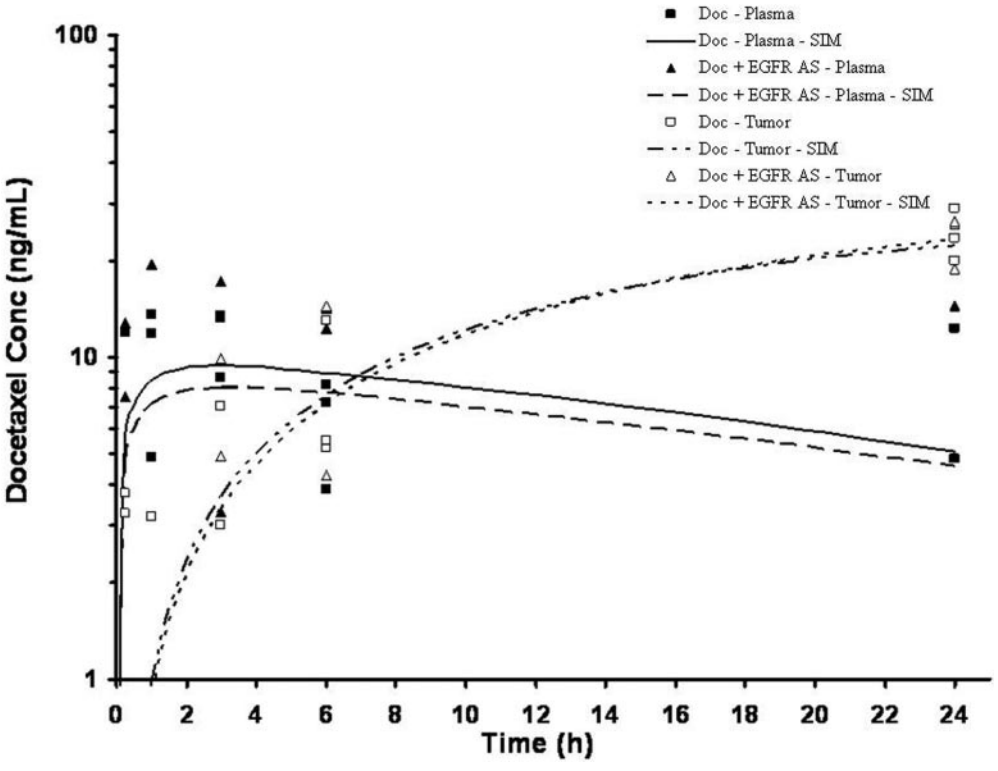

Fig. 5. Single-dose EGFR antisense oligonucleotides do not increase docetaxel levels in the tumor compared with the plasma in 24 h. The concentration versus time profiles of docetaxel in plasma and tumor from 0 to 24 h after administration of docetaxel (2.5 mg/kg) alone or in combination with EGFR antisense oligonucleotides (5 mg/kg) are depicted in the graph. Individual data and best-fit line for plasma (■, —) and tumor (□, — - -) docetaxel concentration versus time profiles after administration of docetaxel alone are presented. Individual data and best fit line for plasma (▲, – –) and tumor (△, - - - -) docetaxel concentration versus time profiles after administration of docetaxel in combination with EGFR antisense are presented.

activation of EGFR, which disrupts the downstream processes necessary for cell survival. Although these inhibitors demonstrate antitumor efficacy in approximately 10% of the patients, the lack of more robust responses may be due to incomplete blockade of the receptor and high receptor turnover (Thomas and Grandis, 2004). A direct comparison between EGFR tyrosine kinase inhibitors and EGFR antisense oligonucleotides demonstrated that down-modulating protein expression may have more antitumor efficacy than inhibiting receptor activation (Rubin Grandis et al., 1997). We have previously demonstrated that down-modulation of EGFR protein expression with liposome-mediated antisense gene therapy is an effective strategy for the treatment of SCCHN tumors (He et al., 1998). However, given the potential toxicity of liposomes, difficulty with formulating liposomal products, and the concerns regarding systemic delivery

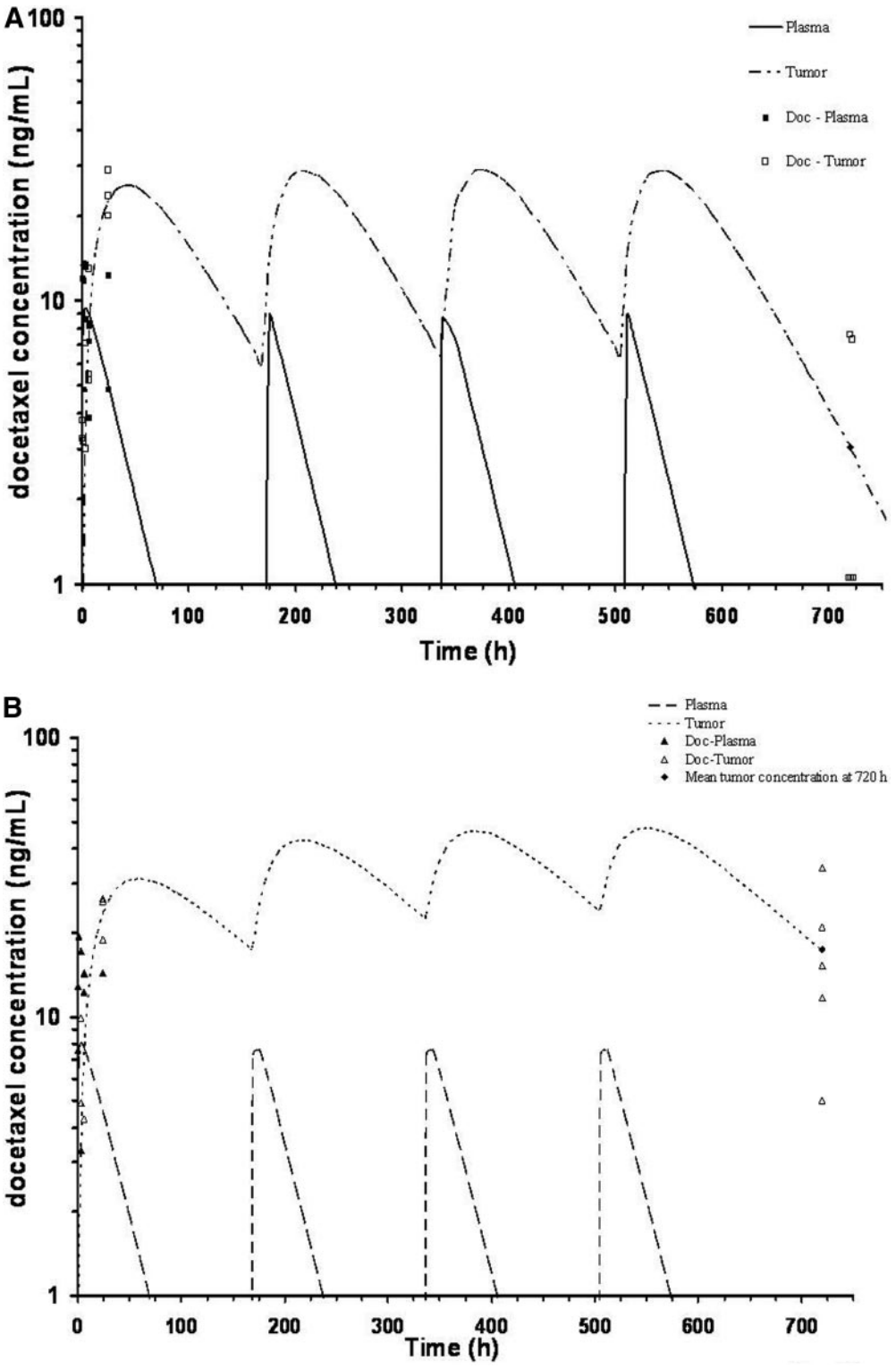

Fig. 6. Multiple doses of EGFR antisense oligonucleotides and docetaxel results in an increase in docetaxel levels of the tumor compared with the plasma. Concentration versus time profiles of docetaxel in plasma and tumor from 0 to 720 h after administration of docetaxel alone (A) and docetaxel in combination with EGFR antisense oligonucleotides (B). In the docetaxel alone group, docetaxel was administered at 2.5 mg/kg i.p. once a week for 4 weeks. In the EGFR antisense oligonucleotide plus docetaxel group, docetaxel was administered at 2.5 mg/kg i.p. every week for 4 weeks, and EGFR antisense oligonucleotide was administered at 5 mg/kg i.p. daily for 4 weeks. Individual data and best-fit line for docetaxel plasma (■, —) and tumor (□, — - -) concentration versus time profiles after administration of docetaxel alone are presented. Individual data and best-fit line for docetaxel plasma (▲, – –) and tumor (△, - - - -) concentration versus time profiles after administration of docetaxel in combination with EGFR antisense are presented. The mean tumor concentration at 720 h (♦) is presented.

of gene therapy vectors, we elected to target EGFR with an antisense approach.

We previously demonstrated that intratumoral injections of EGFR antisense oligonucleotides have antitumor efficacy against SCCHN xenografts (Niwa et al., 2003). Although oral cavity tumors are relatively accessible for intratumoral injections, therapies that rely on intratumoral delivery are unlikely to reach all tumor cells in all locations. Hence, we have elected to pursue therapeutic targeting of EGFR using systemic administration of antisense EGFR oligonucleotides. DNA with a phosphodiester backbone is susceptible to enzymatic degradation with half-lives of approximately 5 min in plasma (Eder et al., 1991). The phosphorothioate modification of the backbone serves to protect the oligonucleotides from endogenous nuclease degradation, increasing the plasma half-life, and they persist in the plasma up to 6 h (Yuen et al., 1999; Chen et al., 2000). Phosphorothioated antisense oligonucleotides have been administered systemically in cancer patients with minimal toxicity and efficient modulation of target genes (Waters et al., 2000). Previous reports demonstrate that phosphorothioate oligonucleotides have similar pharmacokinetic profiles when administered via intravenous, intraperitoneal, or subcutaneous routes (Agrawal, 1996).

Daily i.p. injections of EGFR antisense oligonucleotides reduced SCCHN tumor volume. Administration of EGFR antisense oligonucleotides alone and in combination with docetaxel was well tolerated. Oligonucleotides are negatively charged polyanions that could potentially result in poor cellular uptake. However, phosphorothioate oligonucleotides can enter the cytoplasm via several mechanisms, including pinocytosis (Gao et al., 1993; Beltinger et al., 1995). Our studies demonstrate that systemic administration of phosphorothioate-modified oligonucleotides is well tolerated in mice. Systemic administration of EGFR antisense oligonucleotides also reduced target gene expression. To maximize the antitumor efficacy of EGFR down-modulation we examined the effect of combining systemic delivery of EGFR antisense oligonucleotides with a chemotherapeutic agent. Combining EGFR targeting strategies with chemotherapy or radiation has been reported to enhance the antitumor efficacy compared with administration of the agents alone (Mendelsohn, 2001). In a recent report, combining radiation with the EGFR inhibitor cetuximab increased patient survival rates compared with patients administered radiation alone (Bonner et al., 2006).

We have previously demonstrated that targeting EGFR with intratumoral administration of EGFR antisense oligonucleotides results in down-modulation of EGFR and it attenuates the activation of downstream signaling molecules, including Akt (Niwa et al., 2003). Here, we demonstrate for the first time that systemically administered EGFR antisense oligonucleotides ef-

TABLE 2
Laboratory values of hematological parameters for mice from various groups

	BUN	Albumin	ALT	AST	Hb	Hct	WBC	RBC
	mg/dl	*g/dl*	*U/l*	*U/l*	*g/dl*	*%*	$\times 10^3/\mu l$	$\times 10^6/\mu l$
EGFR antisense + docetaxel	22	2.4	26	154	12.1	34.8	3.5	4.93
	29	2.7	21	150	13.2	35.9	2.3	6.83
	27	2.1	26	172	11.6	36.9	1.1	6.28
	29	2.5	19	130	12.4	34.2	3.4	6.19
	25	1.8	20	128	11.4	32.5	0.5	5.98
	24	2.2	22	173	11.3	35.6	5.6	6.92
	26	2.6	20	109	11.8	32.2	2.5	5.87
	24	2.4	26	182	10.9	30.8	1.2	4.16
	28	2.7	17	129	13.4	38.1	2.8	7.39
	32	2.4	24	160	11.6	31.8	1.2	6.27
EGFR sense + docetaxel	25	2.5	19	106	12.1	33.8	2.3	5.58
	27	2.2	21	181	12.9	35	3.4	6.85
	29	2.7	21	147	12.8	35.5	1	7.29
	28	2	23	117	11.4	31.8	2.8	6.35
	29	2.7	115	222	12.6	35.5	0.8	6.56
	27	2.1	61	146	12.4	34.5	0.5	6.84
	29	2.7	23	138	12.4	34.5	2.9	6.65
	27	2.8	24	128	13.5	39.9	4.9	7.77
	25	2.5	19	108	13	35.4	3.5	6.53
	27	2.5	23	123	12.7	35.9	2.1	6.75
Vehicle control	22	2.6	22	130	12.6	31.5	2.2	6.95
	25	2.1	21	128	11.7	31.7	1.6	6.1
	27	2.5	23	116	13.7	45.1	1	7.23
	18	1.8	15	213	7.6	20.5	1.1	3.27
	26	2.6	23	125	11.3	33.1	3.2	5.63
EGFR antisense	26	2.8	25	103	12.3	34.5	1.4	6.03
	24	2.7	25	137	11.2	32.1	4.4	5.02
	24	2.7	32	119	11.9	33.9	2.9	6.65
EGFR sense	23	2.2	23	143	11.8	32.8	1.9	6.29
	23	2.5	24	130	12.2	35.1	2	6.98
	25	2.7	19	135	13.5	40.2	1.6	7.34
	25	2.7	27	211	12.8	39	6	7.25
	27	2.7	23	146	13	35.8	1.4	7.47
Docetaxel	18	2.3	21	198	13.5	44.2	3	7.4
	23	2.3	21	116	13.1	35.9	1.2	7.32
	31	3.4	38	196	12.3	34.9	5	6.81
	29	2.4	26	127	13.2	38.7	1.9	7.7

BUN, blood urea nitrogen; ALT, alanine aminotransferase (serum glutamic-pyruvic transaminase); AST, aspartate aminotransferase (serum glutamic-oxalacetic transaminase); Hb, hemoglobin; Hct, hematocrit; WBC, white blood cells; RBC, red blood cells.

fectively down-modulate EGFR levels in SCCHN xenografts. Furthermore, we observed an attenuation of Akt phosphorylation on EGFR antisense oligonucleotide treatment. In addition to examining effects on biomarkers, we examined the effects of EGFR down-modulation on tumor cell proliferation and apoptosis. We have previously demonstrated that EGFR activation triggers downstream signaling pathways that result in increased HNSCC proliferation and survival (Grandis et al., 1998; Zhang et al., 2004). Combining EGFR inhibitors with chemotherapy has been reported to demonstrate enhanced antitumor effects in lung and pancreatic cells (Ng et al., 2002; Higgins et al., 2004). However, few studies have examined the mechanisms behind the increased sensitivity. To examine the effects of EGFR antisense oligonucleotide administration on the pharmacokinetics of docetaxel in vivo, we evaluated the plasma and tumor disposition of an anticancer agent after single and repeated administration of an EGFR antisense oligonucleotide.

The plasma and tumor disposition of docetaxel from 0 to 24 h after administration of a single dose of docetaxel alone or in combination with EGFR antisense oligonucleotides was similar, suggesting no acute effects of the EGFR antisense oligonucleotides on the i.p. absorption, systemic clearance, or tumor distribution of docetaxel. The model simulations suggest there is a higher and prolonged docetaxel tumor exposure from 24 h to 30 days (720 h) after administration of docetaxel in combination with EGFR antisense oligonucleotides compared with docetaxel alone. However, additional plasma and tumor samples of docetaxel during this interval are required to confirm these results. The levels of docetaxel in the tumor on day 30 after administration of docetaxel in combination with EGFR antisense oligonucleotide was significantly greater compared with docetaxel alone or in combination with EGFR sense oligonucleotides. A potential mechanism associated with the increased tumor delivery of chemotherapeutic agents in combination with antiangiogenesis agents is known as pruning (Jain, 2001). Unlike normal blood vessels, tumor vessels are structurally and functionally abnormal, which can increase the resistance to blood flow, increase interstitial hypertension, and ultimately inhibit the delivery of anticancer agents to tumors (Jain, 1998). Antiangiogenic agents prune the immature and inefficient blood vessels by eliminating the excess endothelial cells, which results in normalization of vasculature (McDonald and Baluk, 2002). Normalization of the tumor vasculature improves the penetration of molecules in tumors (Tong et al., 2004). The normalization of tumor vasculature during tumor regression has been reported for imatinib, cetuximab, and trastuzumab (Jain, 2001; Uehara et al., 2001; Viloria-Petit et al., 2001). However, the optimal sequence and timing to combine antiangiogenic agents with chemotherapy to improve the delivery of anticancer agents to tumors and deprive the tumor of its blood supply are currently unknown (Jain, 2001). Based on the results of our study, it seems that these effects only occur after repeated dosing of the antiangiogenic therapy and that the effects are tumor-specific.

Thus, systemic administration of EGFR antisense oligonucleotides is feasible for down-modulating EGFR levels in SCCHN tumors without any apparent systemic toxicity. Combination therapy with EGFR antisense oligonucleotides and low doses of docetaxel results in an increase in intratumoral concentrations of docetaxel, thereby enhancing the antitumor effects of the single agents, and it may be a feasible therapeutic approach for HNSCC.

TABLE 3

Kruskal-Wallis test results for equality of six treatment groups (including the no-treatment control)

Raw and adjusted *P* values are shown.

Lab Test	*P* Values		
	Kruskal-Wallis Asymptotic	Sidak-Adjusted	Permutation-Adjusted[a]
Blood urea nitrogen	0.1540	0.7376	0.6696
Albumin	0.2254	0.8704	0.8314
Alanine aminotransferase	0.3928	0.9815	0.9734
Aspartate aminotransferase	0.4708	0.9938	0.9886
Hemoglobin	0.1016	0.5757	0.4900
Hematocrit	0.1066	0.5942	0.5114
White blood cells	0.8933	1.0	1.0
Red blood cells	0.0229	0.1690	0.0872

[a] Based on 5000 permutations of the original data.

References

Agrawal S (1996) Antisense oligonucleotides: towards clinical trials. *Trends Biotechnol* **14:**376–387. 8987636

Beltinger C, Saragovi HU, Smith RM, LeSauteur L, Shah N, DeDionisio L, Christensen L, Raible A, Jarett L, and Gewirtz AM (1995) Binding, uptake, and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. *J Clin Invest* **95:**1814–1823. 7706488

Bissery MC, Nohynek G, Sanderink GJ, and Lavelle F (1995) Docetaxel (Taxotere): a review of preclinical and clinical experience. Part I: preclinical experience. *Anticancer Drugs* **6:**339–355, 363–338.

Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, et al. (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med* **354:**567–578. 16467544

Chen HX, Marshall JL, Ness E, Martin RR, Dvorchik B, Rizvi N, Marquis J, McKinlay M, Dahut W, and Hawkins MJ (2000) A safety and pharmacokinetic study of a mixed-backbone oligonucleotide (GEM231) targeting the type I protein kinase A by two-hour infusions in patients with refractory solid tumors. *Clin Cancer Res* **6:**1259–1266. 10778949

Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, Bianco AR, and Tortora G (2000) Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* **6:**2053–2063. 10815932

Clarke SJ and Rivory LP (1999) Clinical pharmacokinetics of docetaxel. *Clin Pharmacokinet* **36:**99–114. 10092957

Dreyfuss AI, Clark JR, Norris CM, Rossi RM, Lucarini JW, Busse PM, Poulin MD, Thornhill L, Costello R, and Posner MR (1996) Docetaxel: an active drug for squamous cell carcinoma of the head and neck. *J Clin Oncol* **14:**1672–1678. 8622087

Eder PS, DeVine RJ, Dagle JM, and Walder JA (1991) Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3′ exonuclease in plasma. *Antisense Res Dev* **1:**141–151. 1841656

Edwards D and Berry JJ (1987) The efficiency of simulation-based multiple comparisons. *Biometrics* **43:**913–928. 3427176

Gao WY, Storm C, Egan W, and Cheng YC (1993) Cellular pharmacology of phosphorothioate homooligodeoxynucleotides in human cells. *Mol Pharmacol* **43:**45–50. 8423768

Grandis JR, Chakraborty A, Zeng Q, Melhem MF, and Tweardy DJ (1998) Down-modulation of TGF-alpha protein expression with antisense oligonucleotides inhibits proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells. *J Cell Biochem* **69:**55–62. 9513046

He Y, Zeng Q, Drenning SD, Melhem MF, Tweardy DJ, Huang L, and Grandis JR (1998) EGFR antisense RNA transcribed from the U6 promotor inhibits human squamous cell carcinoma growth in vivo. *J Natl Cancer Inst* **90:**1080–1087. 9672256

Heo DS, Snyderman C, Gollin SM, Pan S, Walker E, Deka R, Barnes EL, Johnson JT, Herberman RB, and Whiteside TL (1989) Biology, cytogenetics, and sensitivity to immunological effector cells of new head and neck squamous cell carcinoma lines. *Cancer Res* **49:**5167–5175. 2766286

Herbst RS, O'Neill VJ, Fehrenbacher L, Belani CP, Bonomi PD, Hart L, Melnyk O, Ramies D, Lin M, and Sandler A (2007) Phase II study of efficacy and safety of bevacizumab in combination with chemotherapy or erlotinib compared with chemotherapy alone for treatment of recurrent or refractory non-small-cell lung cancer. *J Clin Oncol* **25:**4743–4750. 17909199

Higgins B, Kolinsky K, Smith M, Beck G, Rashed M, Adames V, Linn M, Wheeldon E, Gand L, Birnboeck H, et al. (2004) Antitumor activity of erlotinib (OSI-774, Tarceva) alone or in combination in human non-small cell lung cancer tumor xenograft models. *Anticancer Drugs* **15:**503–512. 15166626

Hu Y, Cherton-Horvat G, Dragowska V, Baird S, Korneluk RG, Durkin JP, Mayer LD, and LaCasse EC (2003) Antisense oligonucleotides targeting XIAP induce apoptosis and enhance chemotherapeutic activity against human lung cancer cells in vitro and in vivo. *Clin Cancer Res* **9:**2826–2836. 12855663

Jain RK (1998) The next frontier of molecular medicine: delivery of therapeutics. *Nat Med* **4:**655–657. 9623964

Jain RK (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* **7:**987–989. 11533692

McDonald DM and Baluk P (2002) Significance of blood vessel leakiness in cancer. *Cancer Res* **62:**5381–5385. 12235011
Mendelsohn J (2001) The epidermal growth factor receptor as a target for cancer therapy. *Endocr Relat Cancer* **8:**3–9. 11350723
Ng SS, Tsao MS, Nicklee T, and Hedley DW (2002) Effects of the epidermal growth factor receptor inhibitor OSI-774, Tarceva, on downstream signaling pathways and apoptosis in human pancreatic adenocarcinoma. *Mol Cancer Ther* **1:**777–783. 12492110
Niwa H, Wentzel AL, Li M, Gooding WE, Lui VW, and Grandis JR (2003) Antitumor effects of epidermal growth factor receptor antisense oligonucleotides in combination with docetaxel in squamous cell carcinoma of the head and neck. *Clin Cancer Res* **9:**5028–5035. 14581378
Parise RA, Ramanathan RK, Zamboni WC, and Egorin MJ (2003) Sensitive liquid chromatography-mass spectrometry assay for quantitation of docetaxel and paclitaxel in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* **783:** 231–236. 12450543
Rubin Grandis J, Chakraborty A, Melhem MF, Zeng Q, and Tweardy DJ (1997) Inhibition of epidermal growth factor receptor gene expression and function decreases proliferation of head and neck squamous carcinoma but not normal mucosal epithelial cells. *Oncogene* **15:**409–416. 9242377
Sacks PG, Parnes SM, Gallick GE, Mansouri Z, Lichtner R, Satya-Prakash KL, Pathak S, and Parsons DF (1988) Establishment and characterization of two new squamous cell carcinoma cell lines derived from tumors of the head and neck. *Cancer Res* **48:**2858–2866. 2452013
Schöffski P, Catimel G, Planting AS, Droz JP, Verweij J, Schrijvers D, Gras L, Schrijvers A, Wanders J, and Hanauske AR (1999) Docetaxel and cisplatin: an active regimen in patients with locally advanced, recurrent or metastatic squamous cell carcinoma of the head and neck. Results of a phase II study of the EORTC Early Clinical Studies Group. *Ann Oncol* **10:**119–122. 10076732
Sparreboom A, van Tellingen O, Nooijen WJ, and Beijnen JH (1998) Preclinical pharmacokinetics of paclitaxel and docetaxel. *Anticancer Drugs* **9:**1–17. 9491787
Strychor SEJ, Parise RA, Egorin MJ, Joseph E, and Zamboni WC (2005) Plasma, tumor, and tissue disposition of docetaxel in SCID mice bearing SKOV-3 human ovarian xenografts, in Proceedings of the American Association for Cancer Research; 2005 April 16–20; Anaheim, CA. Vol. 46, pp 938, American Association for Cancer Research, Philadelphia, PA.
Thomas SM and Grandis JR (2004) Pharmacokinetic and pharmacodynamic properties of EGFR inhibitors under clinical investigation. *Cancer Treat Rev* **30:**255–268. 15059649
Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, and Jain RK (2004) Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res* **64:**3731–3736. 15172975
Uehara HKS, Karashima T, Zheng L, and Fidler IJ (2001) Blockade of PDGF-R signaling by STI-571 inhibits angiogenesis and growth of human prostate cancer cells in the bone of nude mice, in Proceedings of the American Association for Cancer Research; 2001 March 24–28; New Orleans, LA. Vol. 46, pp 407, American Association for Cancer Research, Philadephia, PA.
Viloria-Petit A, Crombet T, Jothy S, Hicklin D, Bohlen P, Schlaeppi JM, Rak J, and Kerbel RS (2001) Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. *Cancer Res* **61:**5090–5101. 11431346
Wang H, Hang J, Shi Z, Li M, Yu D, Kandimalla ER, Agrawal S, and Zhang R (2002) Antisense oligonucleotide targeted to RIalpha subunit of cAMP-dependent protein kinase (GEM231) enhances therapeutic effectiveness of cancer chemotherapeutic agent irinotecan in nude mice bearing human cancer xenografts: in vivo synergistic activity, pharmacokinetics and host toxicity. *Int J Oncol* **21:**73–80. 12063552
Wang H, Nan L, Yu D, Agrawal S, and Zhang R (2001) Antisense anti-MDM2 oligonucleotides as a novel therapeutic approach to human breast cancer: in vitro and in vivo activities and mechanisms. *Clin Cancer Res* **7:**3613–3624. 11705884
Waters JS, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F, and Cotter FE (2000) Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* **18:**1812–1823. 10784621
Yuen AR, Halsey J, Fisher GA, Holmlund JT, Geary RS, Kwoh TJ, Dorr A, and Sikic BI (1999) Phase I study of an antisense oligonucleotide to protein kinase C-alpha (ISIS 3521/CGP 64128A) in patients with cancer. *Clin Cancer Res* **5:**3357–3363. 10589745
Zamboni WC, Houghton PJ, Hulstein JL, Kirstein M, Walsh J, Cheshire PJ, Hanna SK, Danks MK, and Stewart CF (1999) Relationship between tumor extracellular fluid exposure to topotecan and tumor response in human neuroblastoma xenograft and cell lines. *Cancer Chemother Pharmacol* **43:**269–276. 10071976
Zhang Q, Thomas SM, Xi S, Smithgall TE, Siegfried JM, Kamens J, Gooding WE, and Grandis JR (2004) SRC family kinases mediate epidermal growth factor receptor ligand cleavage, proliferation, and invasion of head and neck cancer cells. *Cancer Res* **64:**6166–6173. 15342401

Address correspondence to: Dr. Sufi Mary Thomas, University of Pittsburgh, 200 Lothrop St., W915 BST, Pittsburgh, PA 15213. E-mail: smt30@pitt.edu