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

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Running Title: Systemic delivery of EGFR antisense oligonucleotides and docetaxel

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Abbreviations: SCCHN, squamous cell carcinoma of the head and neck; EGFR, epidermal growth factor receptor; IP, intraperitoneal; IV, intravenous; SPE, solid phase extraction.

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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 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. Since 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 IP daily in athymic mice bearing 1483 human SCCHN xenografts alone or in combination with docetaxel at 2.5 mg/kg IP once a week for four 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 to 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 may be an effective treatment strategy for SCCHN.

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Introduction

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 indicating that EGFR may serve as a therapeutic target for SCCHN. Various strategies targeting EGFR have demonstrated antitumor efficacy in preclinical models. Recently the FDA 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 as well as 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

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oligonucleotides in the plasma by increasing their resistance to nuclease degradation. Preclinical studies with antisense phosphorothioate-modified oligonucleotides demonstrated successful down regulation of the anti-apoptotic proteins, X-linked inhibitor of apoptosis (XIAP) and Bcl-2, via systemic delivery in mice (Hu et al., 2003). However, emerging evidence suggests that no single treatment regimen results in sustained anti-tumor 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 taxane analogue that is active both as monotherapy as well as in combination with molecular targeting agents including bevacizumab in patients with solid tumors (Dreyfuss et al., 1996; Herbst et al., 2007; Schoffski et al., 1999). Combining EGFR inhibitors with chemotherapy has been reported to result in added tumor growth inhibition in a number of cancer cell lines including ovary, breast and colon (Ciardiello et al., 2000). The plasma, tumor, and tissue disposition of taxane analogues has been previously evaluated in preclinical models (Bissery et al., 1995; Sparreboom et al., 1998; Strychor S, 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.

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Materials and Methods

Cells and Reagents. Human squamous cell carcinoma of the head and neck (SCCHN) cells 1483 and PCI-15b used in this study are previously described well characterized cell lines (Heo et al., 1989; Sacks et al., 1988). Cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum in presence of 5% CO_2. Docetaxel (Taxotere) was obtained from Aventis Pharmaceuticals as 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 as previously described (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 (Transduction laboratories, Lexington, KY); β-Actin monoclonal mouse IgM (Oncogene Research Products, Boston, MA); phospho-AKT (Ser473; Cell Signaling Technology); phospho-Stat3 (Tyr705; Cell Signaling Technology) and Ki67 (DakoCytomation, Denmark). Apoptosis was measured by TUNEL (Roche Applied Science, 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 Sprague Dawley Inc., Indianapolis, Indiana) between the age of 5 and 6 weeks were injected with 1 x 10^6 1483 cells in 100 μl of Hanks basal salt solution subcutaneously on the left flank. Treatment was initiated 8 days post inoculation when tumors reached a average volume of 15 mm^3. Tumor volumes were calculated using the formula: length x $(width)^2/2$ where length is the largest diameter and width is the smallest diameter

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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 IP once daily for 30 days alone or in combination with docetaxel administered IP 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 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 tumor of mice administered a single dose of docetaxel (2.5 mg/kg IP) alone or in combination with EGFR antisense oligonucleotides (5 mg/kg IP). Plasma and tumor tissue were harvested at 0.25, 1, 3, 6, and 24 h post 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 d. Specifically mice (n = 5 per treatment arm) were treated for 30 d (720 h) with EGFR antisense or sense oligonucleotides administered at 5 mg/kg IP once daily for 30 days alone or in combination with docetaxel administered IP 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

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and immediately placed on ice. Plasma was harvested from the blood samples after centrifugation at 12,000 x g for 4 min at 4˚C and 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 (SPE) while tumor samples were processed using acetonitrile precipitation followed by SPE (Strychor S, 2005). Docetaxel concentrations in plasma and tumors were determined by an LC-MS assay as previously described (Parise et al., 2003).

Immunoblotting. Xenograft tumors were minced on dry ice and suspended in 500 µl of lysis buffer (10mM Tris HCl, pH 7.6, 50mM $Na_4P_2O_7$, 50mM NaF, 1mM NaV_3O_4, 1% TritonX-100 and 1X protease inhibitor cocktail tablet that included a broad spectrum potent inhibitor of protein tyrosine phosphatases [Roche, Germany]). The lysates were sonicated and the supernatant collected after centrifugation at 13,000 rpm for 5 minutes. Forty micrograms of protein was fractionated through 8% SDS polyacrylamide gels and analyzed via immunoblotting for biomarker modulation. Autoradiograms were scanned and the bands quantified using the DigiDoc 1000 digital imaging system (Alpha Innotech, San Leandro, CA). Values obtained were normalized to β-Actin levels as well as to a positive control lysates run on every gel. Cell lysates obtained from a well characterized HNSCC cell line PCI-15b was used as the positive control. Relative intensities were averaged across tumors from the same group and the cumulative results were graphed using Graphpad PRISM Version 6.

Immunohistochemistry. Four micron thick sections of paraffin-fixed tumor samples from the EGFR antisense oligonucleotide alone and EGFR sense oligonucleotide alone treatment groups were removed and adhered to slides by heating overnight at 57°C in a dry slide incubator. The

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specimens were deparaffinized, rehydrated and placed in a 1:10 diluted mixture of methanol/H_20_2 for 15 minutes at room temperature and than rinsed two times with distilled water. Slides were placed in boiling citrate buffer for 10 minutes to enable antigen retrieval and examined for Ki-67 expression. Sections from paraffin embedded tissues were also examined for apoptosis by TUNEL (Roche Applied Science, Germany) according to the manufacturer's instructions. The average number of positive cells was determined from five separate fields under 400X magnification. Cumulative findings from blinded assessments of sections by 2 independent investigators were graphed using Graphpad PRISM Version 6.

Pharmacokinetic analysis. The compartmental pharmacokinetic analysis of docetaxel in plasma and tumor was performed using maximum likelihood estimation in the ADAPT II modeling program as previously described (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 (AIC), 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 prior to 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

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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 6 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, docetaxel in combination with EGFR antisense or sense oligonucleotides was evaluated using the exact 2-tailed Kruskal-Wallis test for equality of the 3 groups and the exact 2-tailed Wilcoxon test to compare each group. The exact 2-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 to the docetaxel and EGFR sense oligonucleotide treated group using the exact 2-tailed Kruskal-Wallis test. Exact nonparametric tests were performed using StatXact with Cytel studio software (version 6.1). P values less than 0.05 was considered statistically significant.

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Results

Systemic administration of EGFR antisense oligonucleotides in combination with low dose of docetaxel has 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 may be impractical in the clinical setting. In a preliminary study we compared the antitumor efficacy of intraperitoneal (IP) versus intravenously (IV) administered EGFR antisense oligonucleotides in SCCHN tumor bearing mice and found no difference in the post treatment tumor volumes using the two routes of delivery (data not shown). The IP route was chosen for due to greater ease of administration. In order 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 IP daily for 4 weeks. Tumors in EGFR antisense oligonucleotide treated group had significantly smaller tumor volumes compared to EGFR sense oligonucleotide treated mice ($P<0.05$) (Figure 1A). Further, 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 3 paired comparisons were made among 3 treatment groups. In 2 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 Figure 1B. Mice treated with EGFR antisense oligonucleotides in combination with docetaxel had the smallest tumors among all the groups.

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Systemic administration of EGFR antisense oligonucleotides results in modulation of EGFR levels and phosphorylation of downstream signaling molecules

In order 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 to EGFR sense oligonucleotide treated mice ($P<0.05$) (Figure 2A). Mice treated with a combination of docetaxel and EGFR antisense oligonucleotides also showed reduced protein expression levels of EGFR compared to mice treated with EGFR sense oligonucleotides and docetaxel ($P<0.01$) (Figure 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 (Figures 2C and D, respectively). While 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 ($P>0.05$) (Figures 2C and D, respectively). Levels of pMAPK also remained unchanged between the two groups (Figure 2E).

Systemic administration of EGFR antisense oligonucleotides in combination with docetaxel results 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). In order to examine the effects of EGFR antisense oligonucleotides with or without docetaxel on proliferation of SCCHN tumor cells, tumors were examined by immunohistochemical analysis for Ki67 expression. The dark nuclei

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were counted and the cumulative findings from 2 independent blinded assessments of all the tumors in each group were represented in the graph. The error bars represent the ± SEM. EGFR antisense oligonucleotide treated tumors demonstrated fewer proliferating cells compared to EGFR sense oligonucleotide treated tumors (Figure 3A). Docetaxel administration further reduced the number of proliferating SCCHN cells in mice treated with EGFR antisense oligonucleotides compared to 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 2 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 ($P<0.001$) (Figure 3B).

Pharmacokinetic analysis demonstrates increased docetaxel exposure in the tumors after multiple treatments with EGFR antisense oligonucleotides

In order 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 4-compartment model with administration of docetaxel into the intraperitoneal cavity was fit to the plasma concentration versus time data as previously reported (Strychor S, 2005). The model parameters consisted of the rate constant for absorption (ka) 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}, k_{31}), and

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the elimination rate constant from the central compartment (k_{10}) (Figure 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 of docetaxel administered IV at 10 mg/kg (Strychor S, 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 S, 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 Vc values allowed us to accurately determined the rate constant describing the absorption of docetaxel from the IP 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 5th compartment describing the tumor disposition was added to the 4-compartment model described above for the plasma disposition after IP 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 IP cavity or systemic clearance.

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Administrations of antisense oligonucleotides against cAMP-dependent protein kinase (PKA) 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 (Figure 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 tumor 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 d) 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 Figures 6A and 6B, respectively. The docetaxel

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plasma concentration versus time profile is similar after administration of docetaxel alone and in combination with EGFR antisense oligonucleotides. The docetaxel tumor concentration versus time profiles after administration of docetaxel in combination with EGFR antisense is greater compared with docetaxel alone. On day 30, the mean ± SD concentration of docetaxel in tumor 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 is specific to EGFR antisense oligonucleotides administration.

Systemic administration of EGFR antisense oligonucleotides and docetaxel is well tolerated *in vivo*

Mice in this study were examined for evidence of toxicity 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 were evaluated for hemoglobin, hematocrit, albumin, red blood cells, white blood cells and for liver enzymes SGOT and SGPT (Table 2). There were no differences in hematologic and liver enzyme values among the treatment groups (Table 3). Thus, the combination of EGFR antisense and docetaxel produced no observed toxicity.

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Discussion

A number of 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 activation of EGFR which disrupts the downstream processes necessary for cell survival. Although these inhibitors demonstrate anti-tumor 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 of gene therapy vectors, we elected to target EGFR with an antisense approach.

We previously demonstrated that intratumoral injections of EGFR antisense oligonucleotides has 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 persist in the plasma up to 6 hours (Chen et al.,

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2000; Yuen et al., 1999). 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 IP 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 (Beltinger et al., 1995; Gao et al., 1993). 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. In order 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 to 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 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 effectively down modulate EGFR levels in SCCHN xenografts. Further, we observed an

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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 (Zhang et al., 2004), (Grandis et al., 1998). Combining EGFR inhibitors with chemotherapy has been reported to demonstrate enhanced antitumor effects in lung and pancreatic cells (Higgins et al., 2004; Ng et al., 2002). However, few studies examine the mechanisms behind the increased sensitivity. In order 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 were similar suggesting no acute effects of the EGFR antisense oligonucleotides on the IP absorption, systemic clearance, or tumor distribution of docetaxel. The model simulations suggests 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 anti-angiogenesis 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

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ultimately inhibit the delivery of anticancer agents to tumors (Jain, 1998). Anti-angiogenic 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 H, 2001; Viloria-Petit et al., 2001). However, the optimal sequence and timing to combine anti-angiogenic agents with chemotherapy in order 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 appears that these effects only occur after repeated dosing of the anti-angiogenic 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 may be a feasible therapeutic approach for HNSCC.

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Figure Legends

Figure 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) (∇) IP once weekly, EGFR antisense (×), sense oligonucleotides (◊) IP daily at 5 mg/kg body weight. Ten mice per group were treated with EGFR antisense (○) or sense (Δ) oligonucleotides IP 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 to 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 to the EGFR sense plus docetaxel treated tumors ($P<0.01$). (B) The 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 1 way analysis of variance of log-transformed tumor volumes.

Figure 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

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SCCHN tumors compared to control tumors treated with EGFR sense oligonucleotide with or without docetaxel ($P<0.05$ and $P<0.01$ respectively). Immunoblots demonstrate down modulation of (C) pAkt ($P = 0.023$) but not (D) pSTAT3 ($P>0.05$) and (E) pMAPK ($P>0.05$) in tumors treated with EGFR antisense oligonucleotide plus docetaxel compared to the EGFR sense oligonucleotide plus docetaxel. Numbers above the lanes indicate individual tumors from mice treated in each group. Error bars depict ± SEM.

Figure 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 (5mg/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 400X magnification in 10 separate fields. Error bars indicate ± SEM. EGFR antisense oligonucleotide treatment reduced the number of proliferating tumor cells compared to the control groups. There was a significant reduction in cell proliferation in tumors treated with docetaxel and EGFR antisense oligonucleotides compared to tumors treated with docetaxel and EGFR sense oligonucleotides as depicted in a representative photomicrograph (200 X 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 400X magnification in 4 separate fields. Error bars indicate ± SEM. EGFR antisense treatment resulted in increased apoptosis in the tumors. Combination of docetaxel and EGFR antisense oligonucleotides resulted in increased apoptosis compared to the EGFR sense oligonucleotide and docetaxel treated tumors as depicted in a representative photomicrograph (200 X magnification) of tumors from each group ($P<0.001$).

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Figure 4. 5-Compartmental model describing the plasma and tumor disposition of docetaxel after IP administration. A 5-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 4-compartment model with administration of docetaxel into the IP 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 IP cavity to the central compartment (k_a), rate constant representing clearance from the IP cavity and 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}, k_{31}), and the elimination rate constant from the central compartment (k_{10}). A 5th 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).

Figure 5: Single dose EGFR antisense oligonucleotides do not increase docetaxel levels in the tumor compared to 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 (Δ, ----) docetaxel concentration versus time profiles after administration of docetaxel in combination with EGFR antisense are presented.

Figures 6. Multiple doses of EGFR antisense oligonucleotides and docetaxel results in an increase in docetaxel levels of the tumor compared to the plasma. Concentration versus time

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profiles of docetaxel in plasma and tumor from 0 to 720 h after administration of (A) docetaxel alone and (B) docetaxel in combination with EGFR antisense oligonucleotides. In the docetaxel alone group, docetaxel was administered at 2.5 mg/kg IP once a week for four weeks. In the EGFR antisense oligonucleotide plus docetaxel group, docetaxel was administered at 2.5 mg/kg IP every week for four weeks and EGFR antisense oligonucleotide was administered at 5 mg/kg IP/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 (Δ,----) concentration versus time profiles after administration of docetaxel in combination with EGFR antisense are presented. The mean tumor concentration at 720 h (♦) is presented.

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Tables:

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

Parameter	Units	Docetaxel Alone	CV%	Docetaxel + EGFR Antisense Oligonucleotide	CV%
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 $AUC_{0\text{-}24\ h}$	ng/ml•h	179	---	156	---
Plasma $AUC_{0\text{-}720\ h}$	ng/ml•h	1,284	---	1,167	---
Tumor $AUC_{0\text{-}24\ h}$	ng/ml•h	313	---	312	---
Tumor $AUC_{0\text{-}720\ h}$	ng/ml•h	12,070	---	23,280	---
Ratio of Tumor to Plasma $AUC_{0\text{-}24\ h}$	---	1.74	---	2.00	---
Ratio of Tumor to Plasma $AUC_{0\text{-}720\ h}$	---	9.40	---	19.94	---

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Table 2. Laboratory values of hematological parameters for mice from various groups.

	Bun	Albumin	ALT (SGPT)	AST (SGOT)	Hemoglobin	Hematocrit	WBC	RBC
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

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Table 3: Kruskal-Wallis test results for equality of 6 treatment groups (including the no-treatment control). Raw and adjusted p values are shown.

Lab Test	Kruskal-Wallis asymptotic p value	Sidak-Adjusted p value	Permutation-Adjusted p value*
BUN	.1540	.7376	.6696
Albumin	.2254	.8704	.8314
SGPT	.3928	.9815	.9734
SGOT	.4708	.9938	.9886
Hemoglobin	.1016	.5757	.4900
Hematocrit	.1066	.5942	.5114
WBC	.8933	1.0	1.0
Red Cells	.0229	.1690	.0872

* Based on 5000 permutations of the original data

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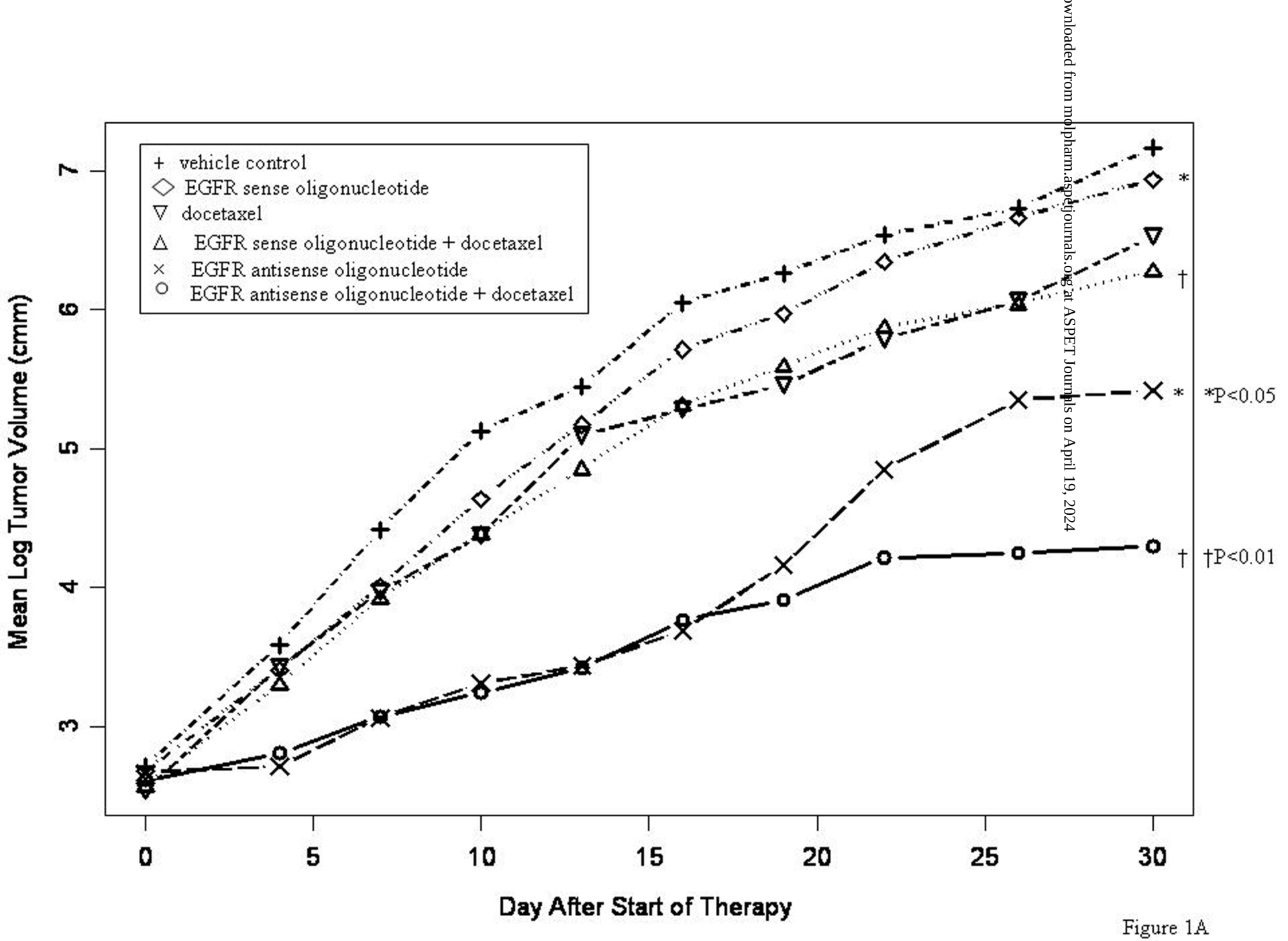

Figure 1A

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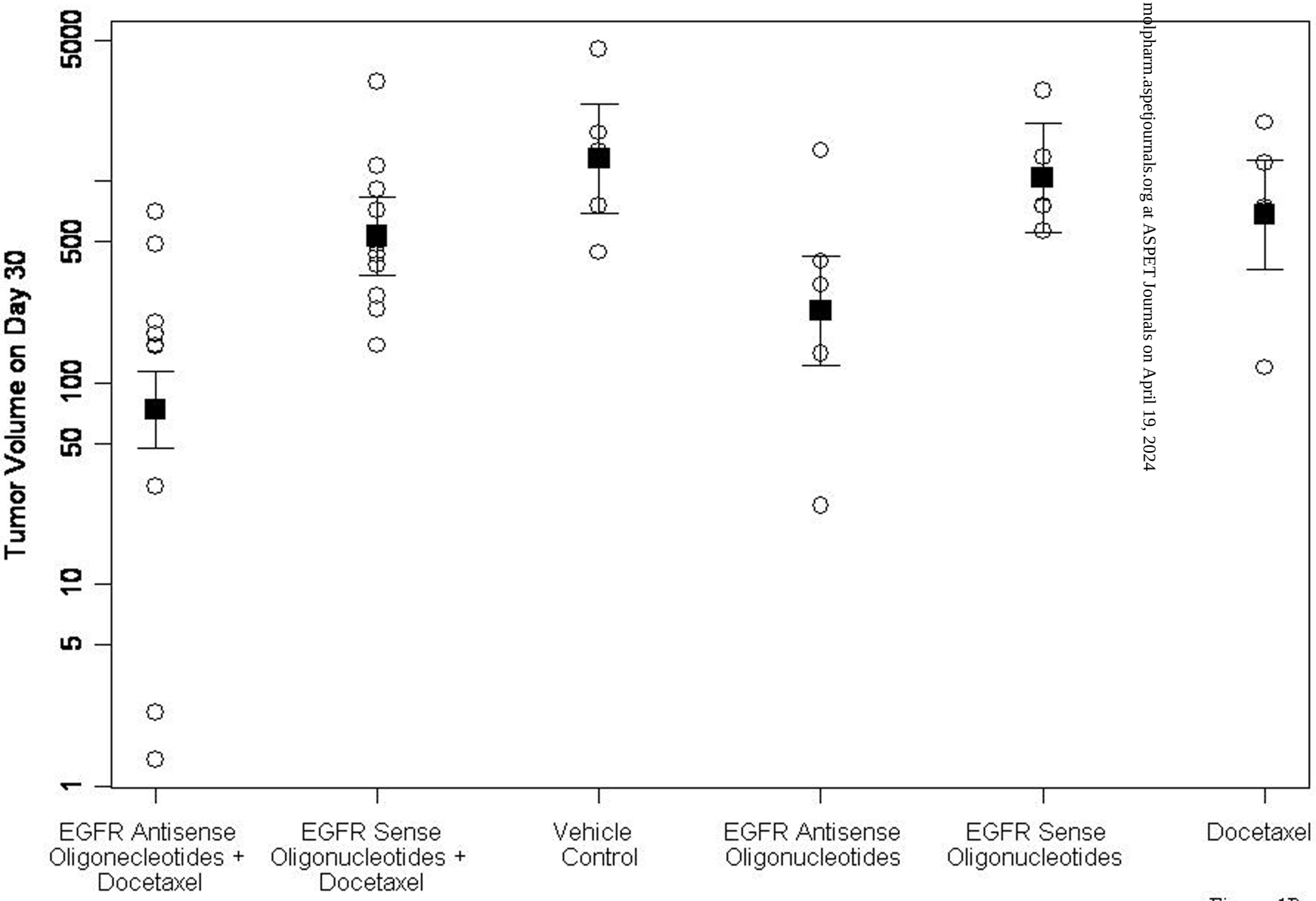

Figure 1B

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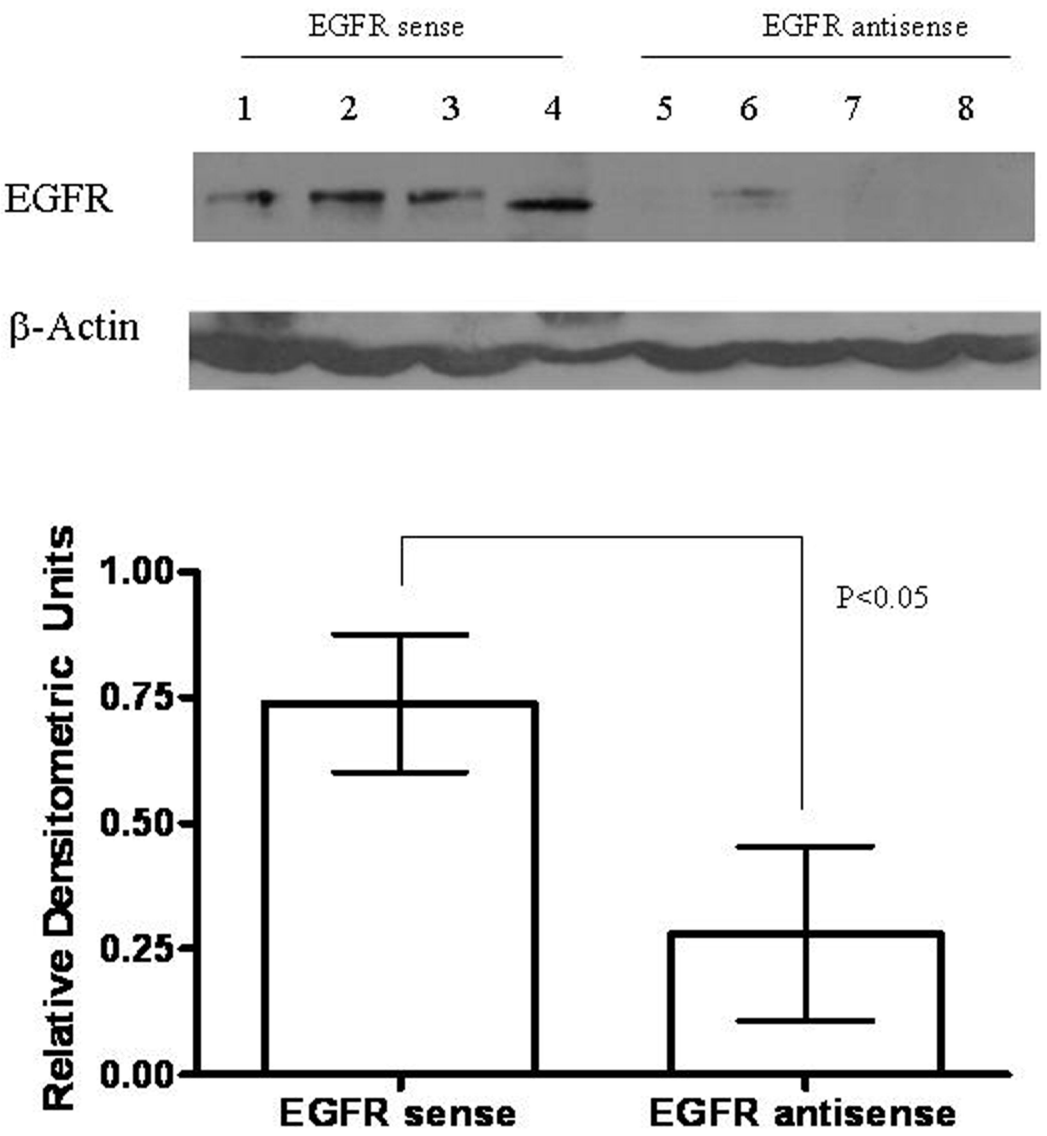

Figure 2A

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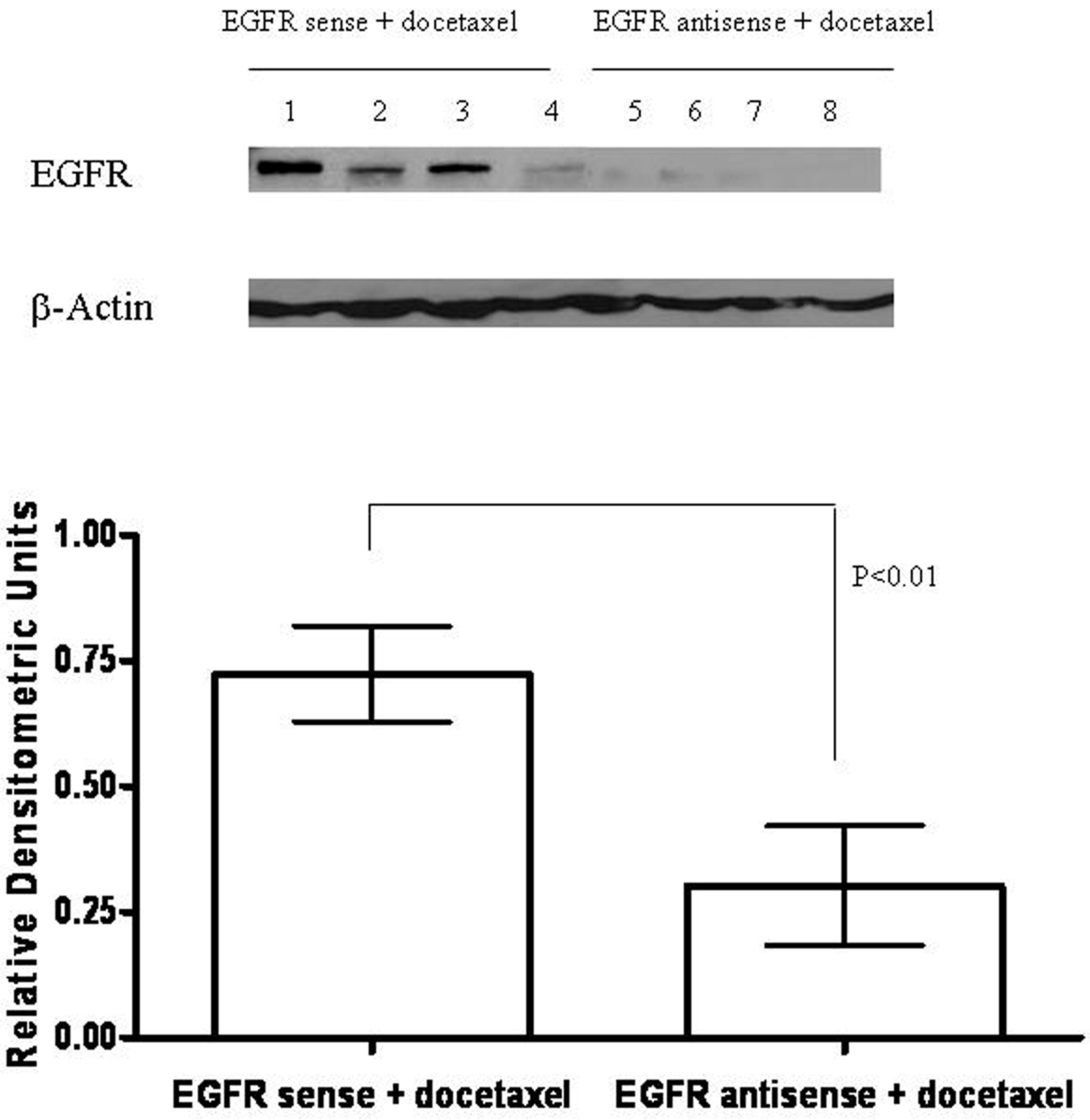

Figure 2B

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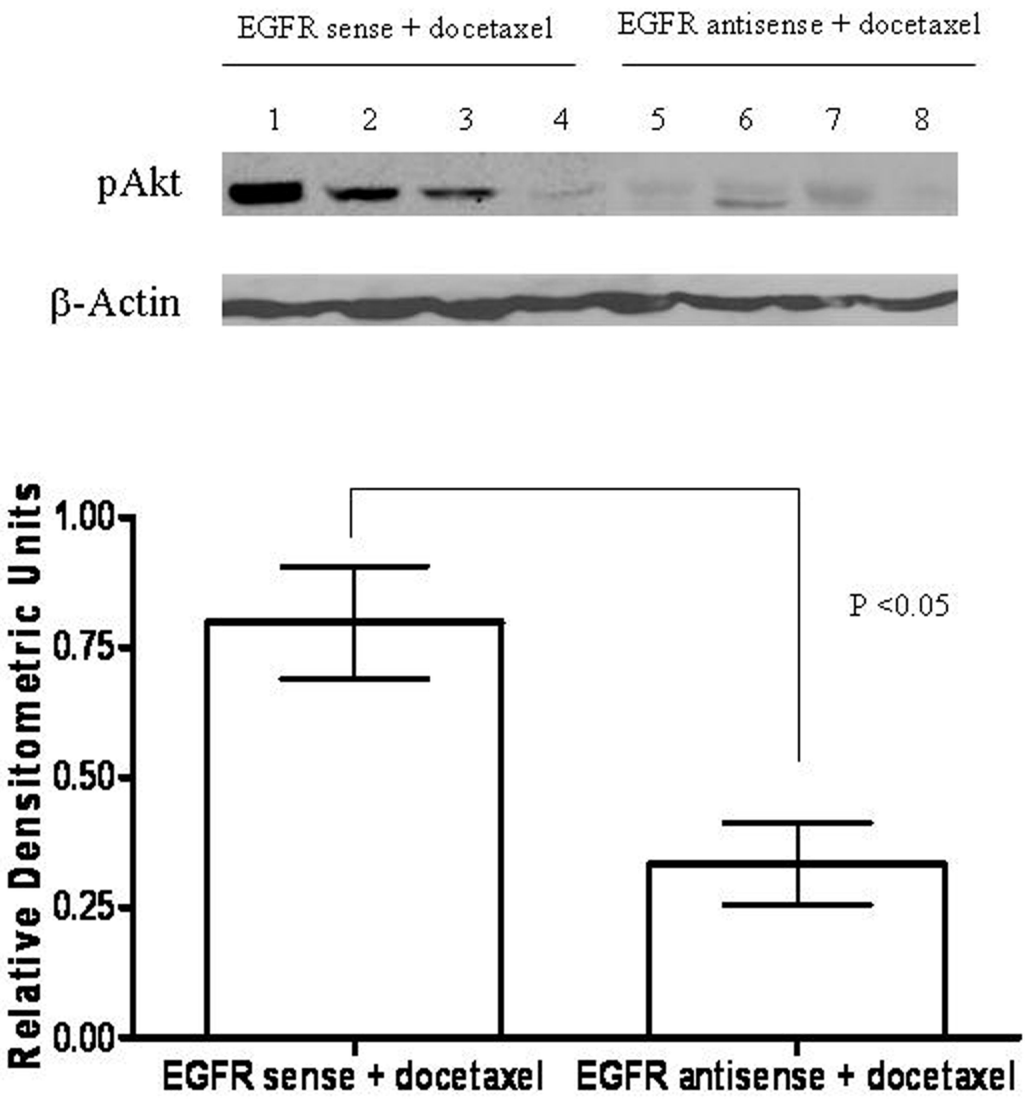

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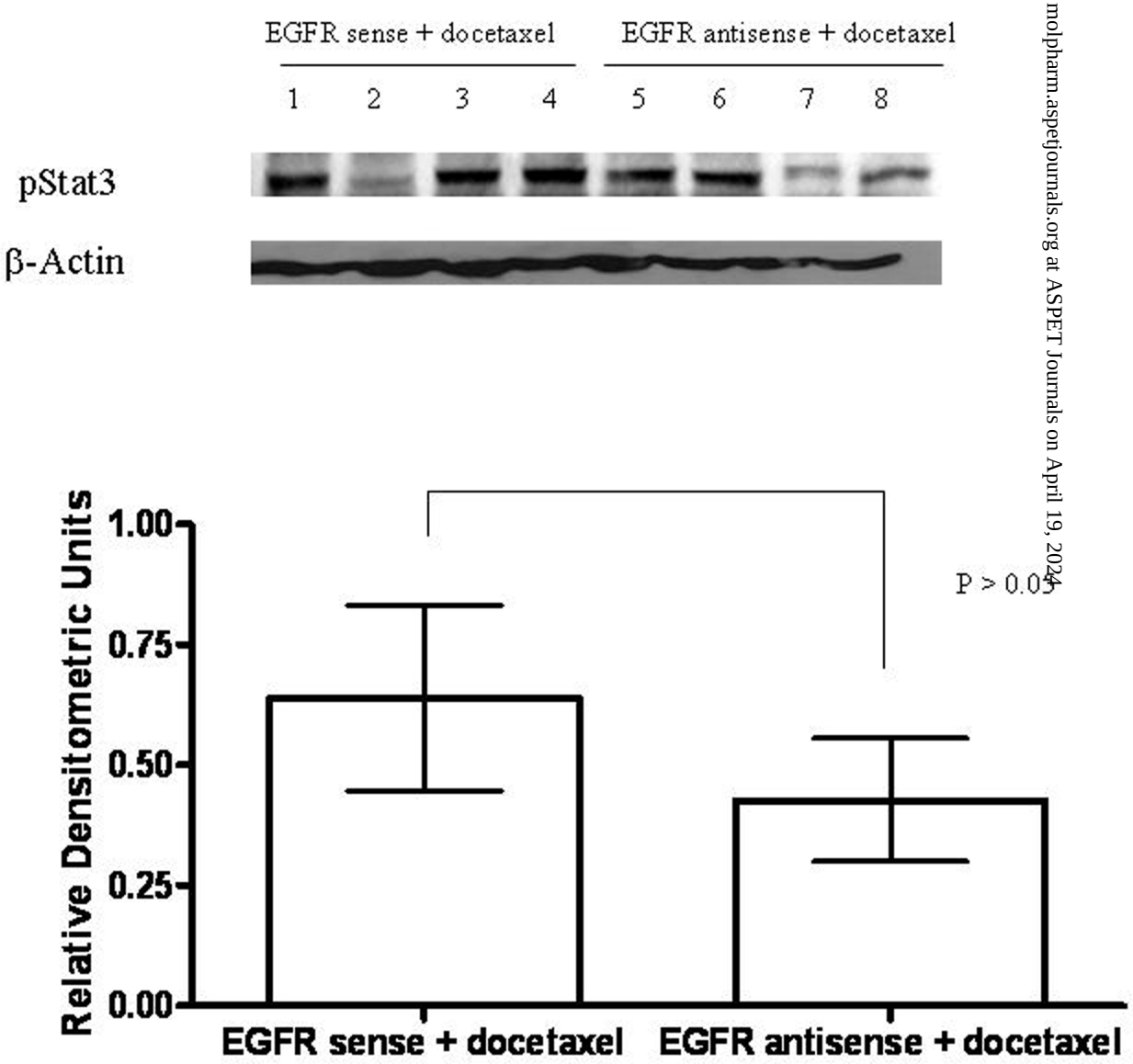

Figure 2D

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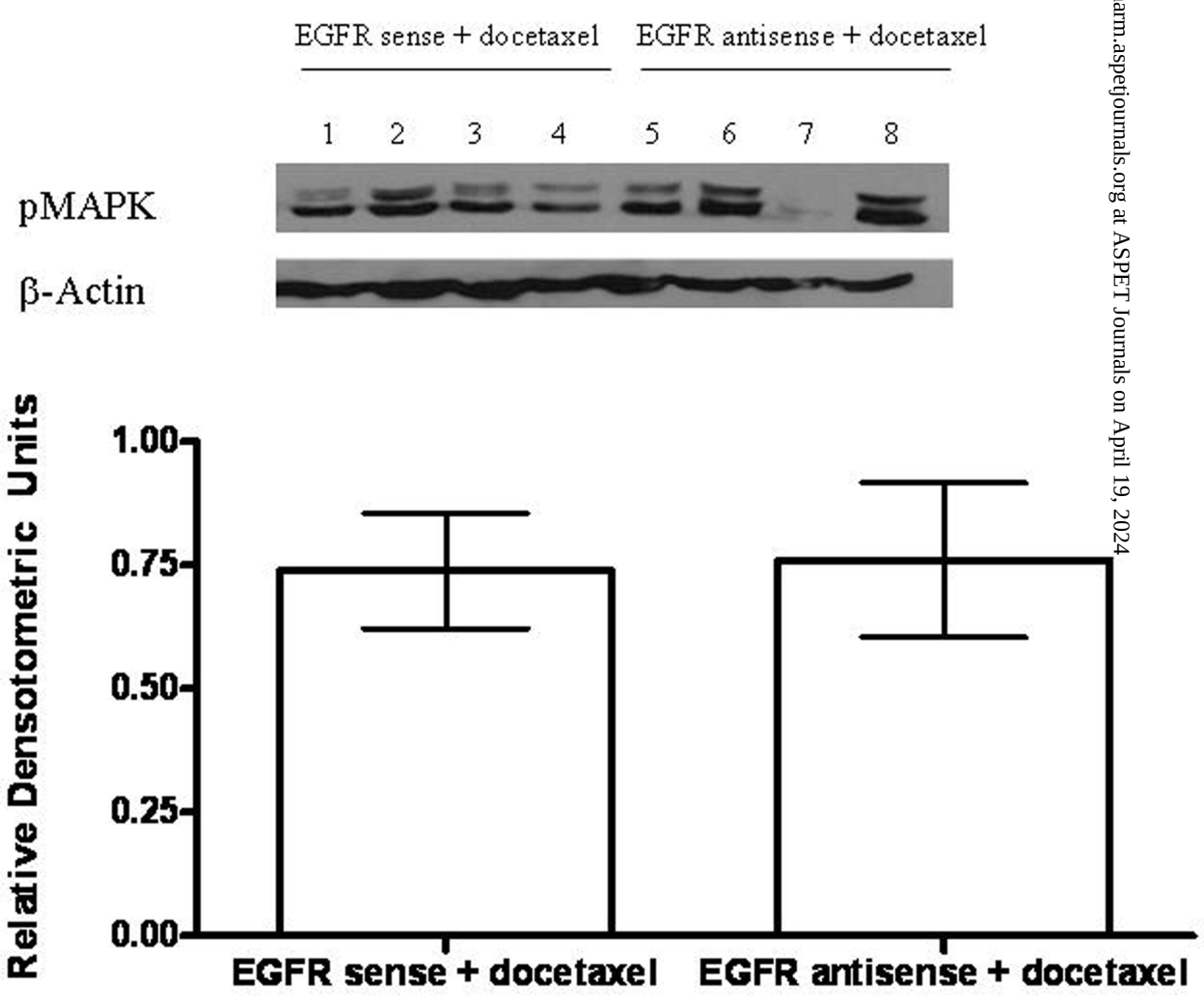
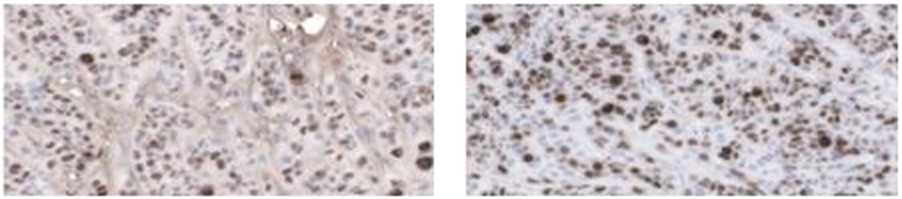


Figure 2E



EGFR anti sense + docetaxel

EGFR sense + docetaxel

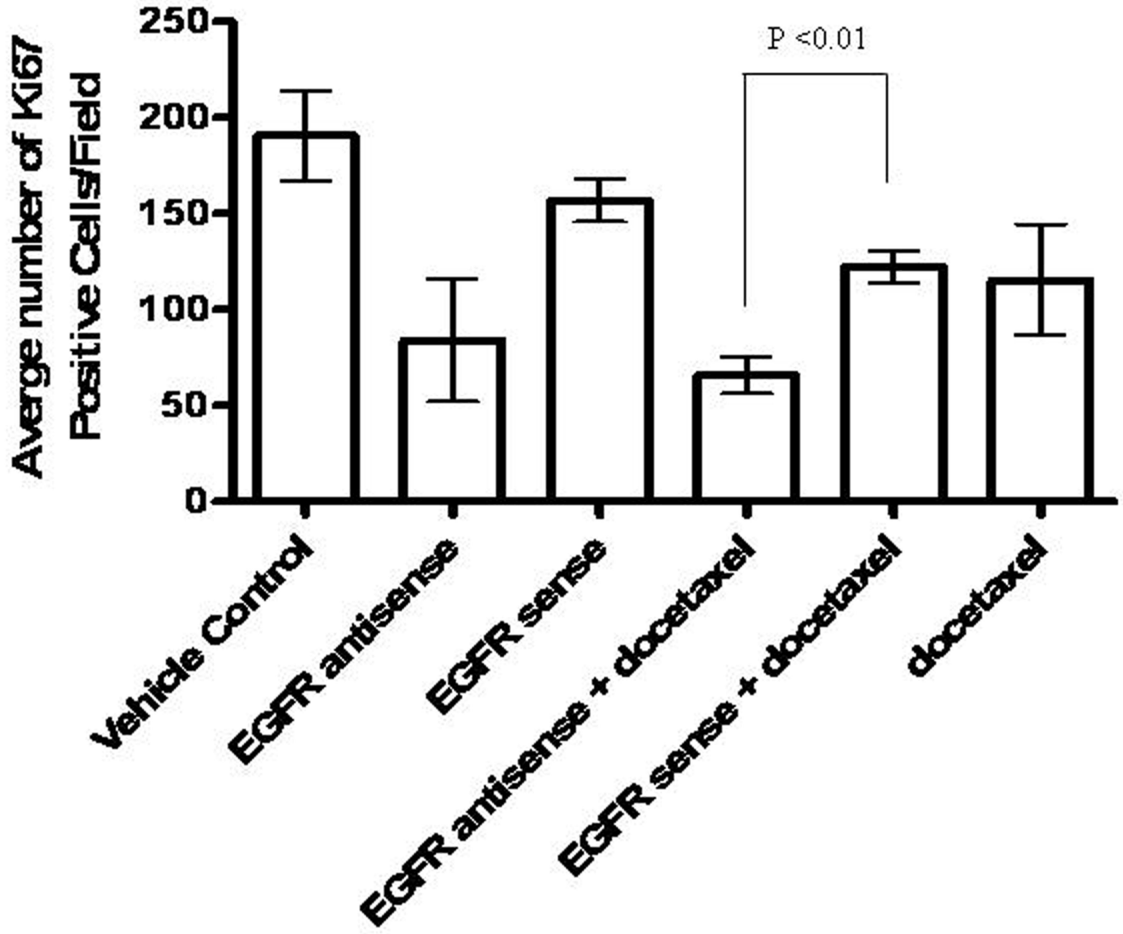


Figure 3A

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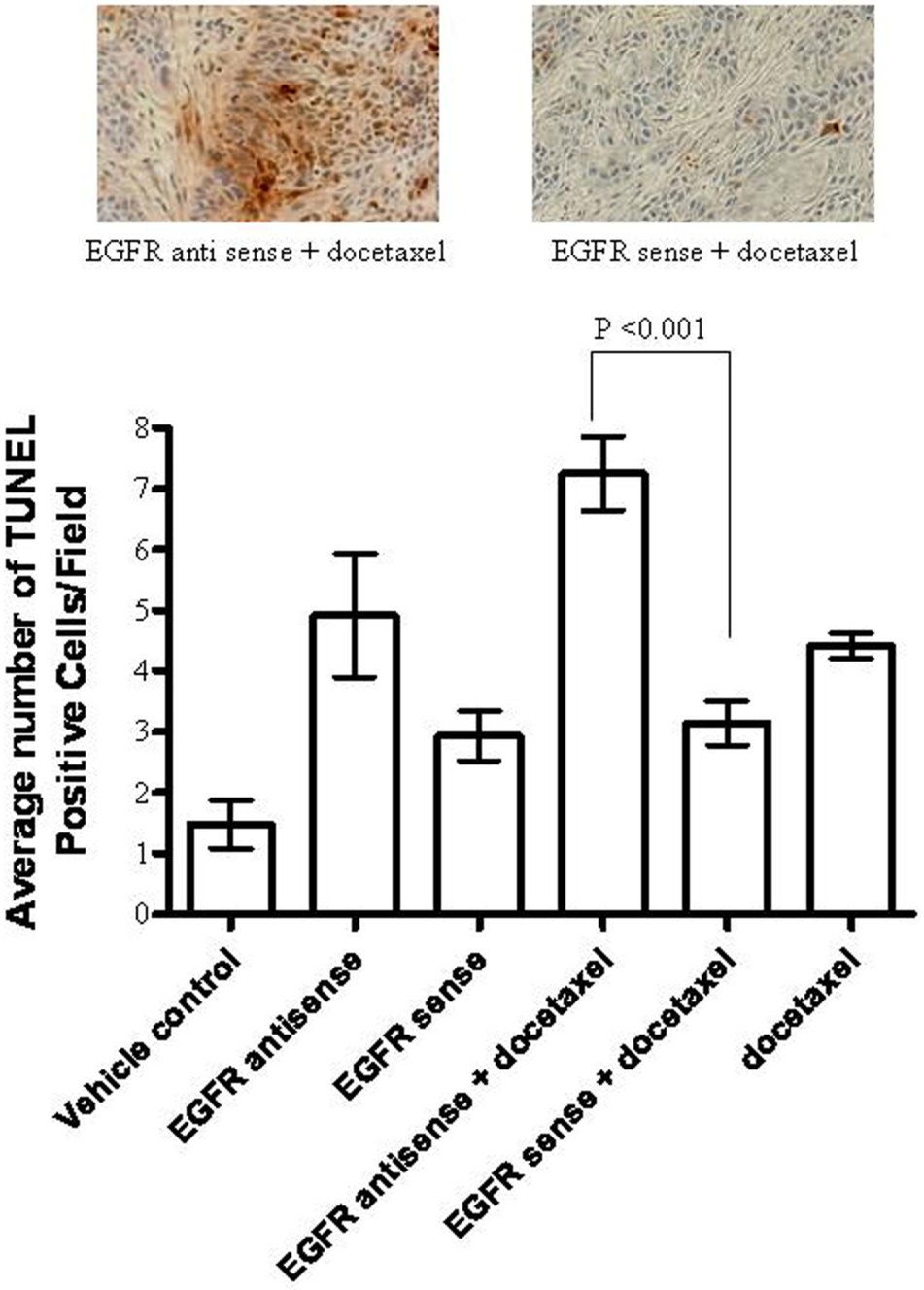

Figure 3B

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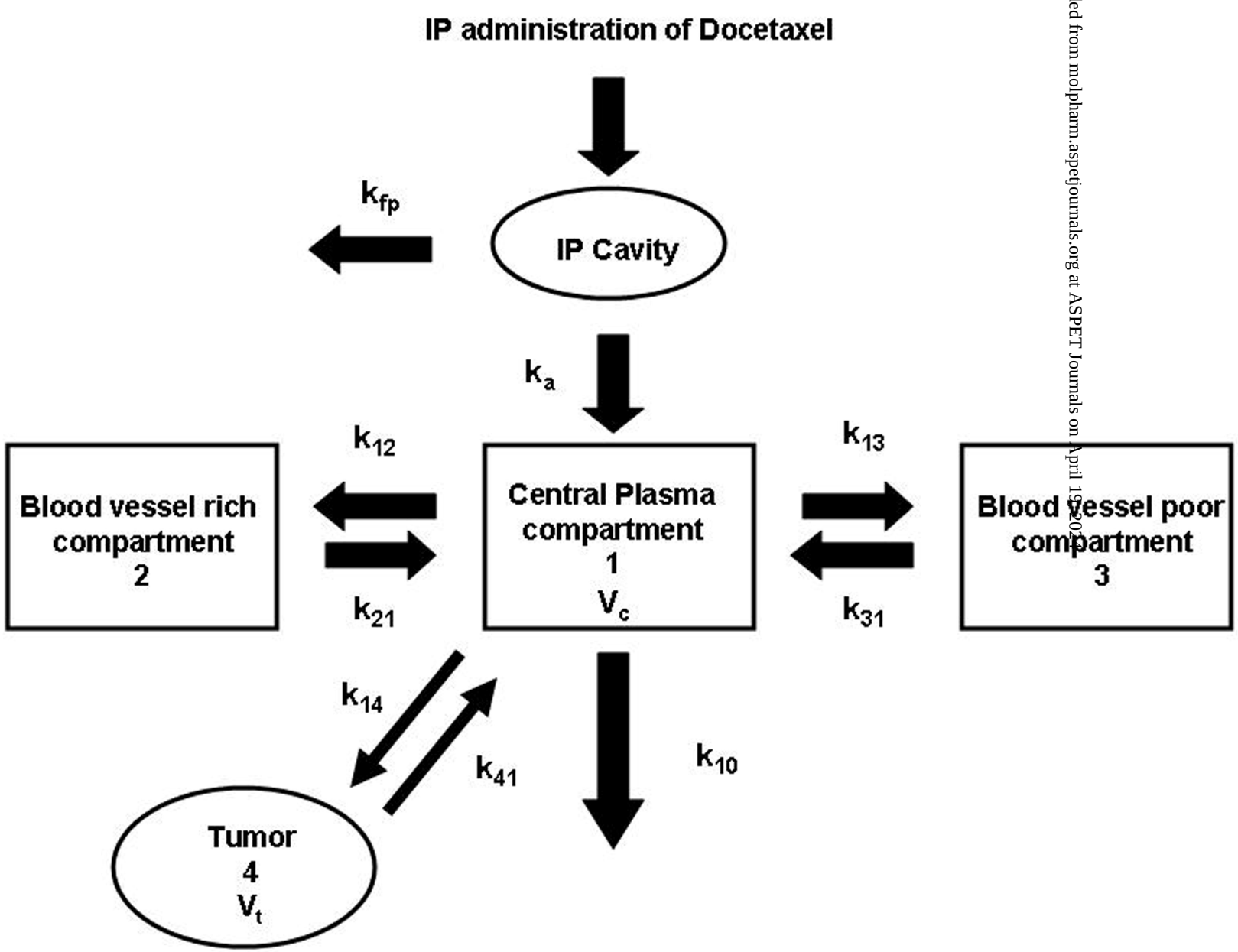

Figure 4

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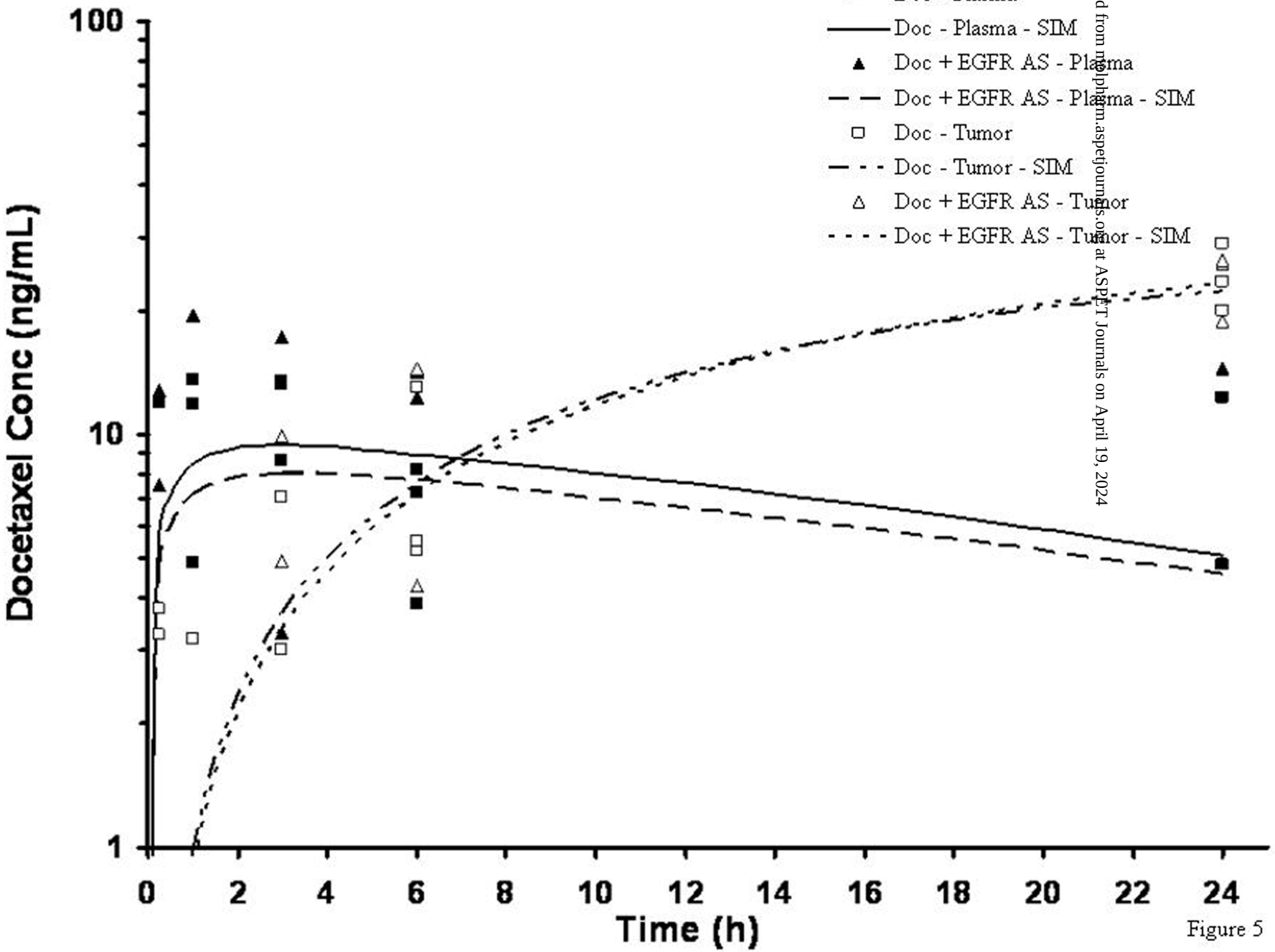

Figure 5

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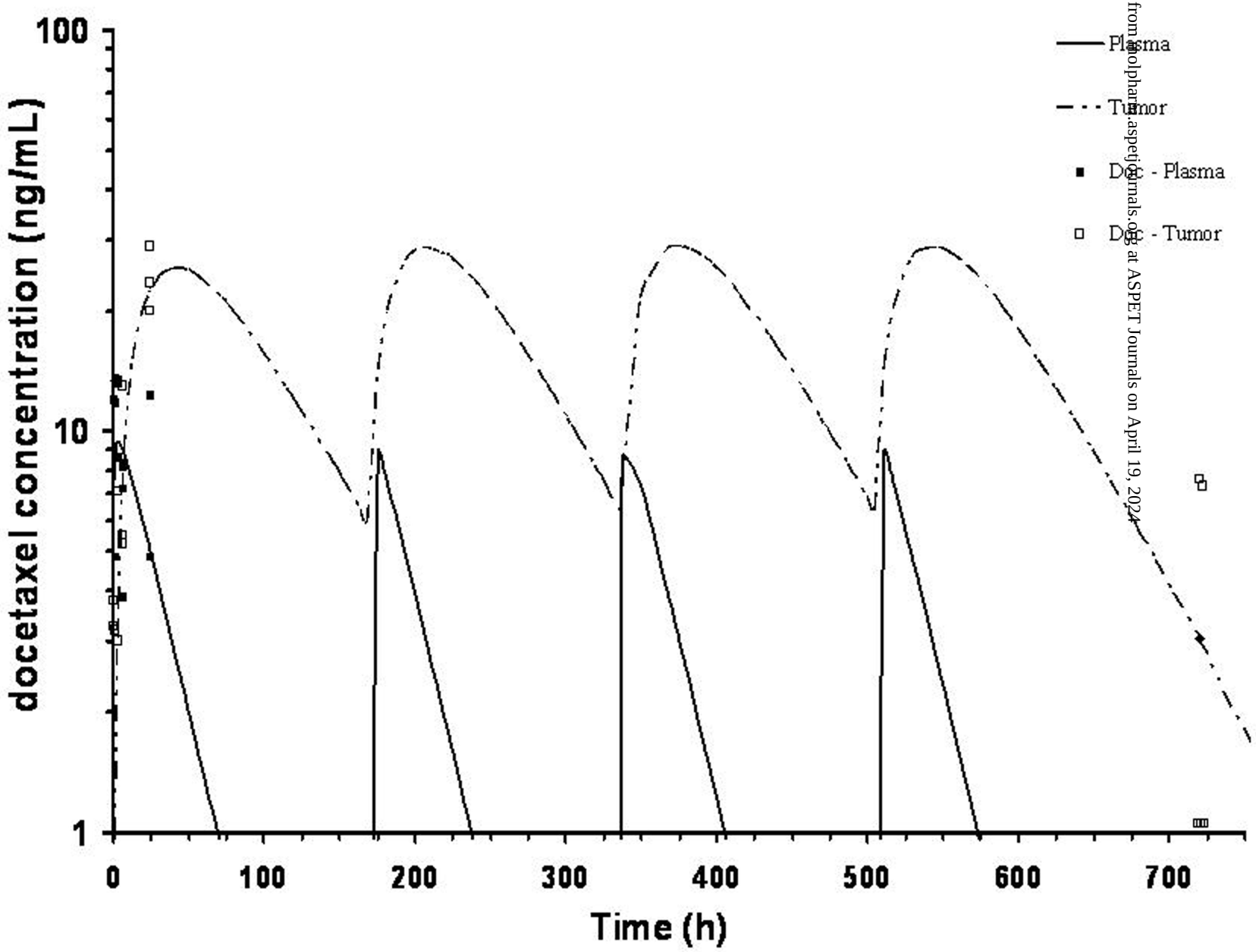

Figure 6A

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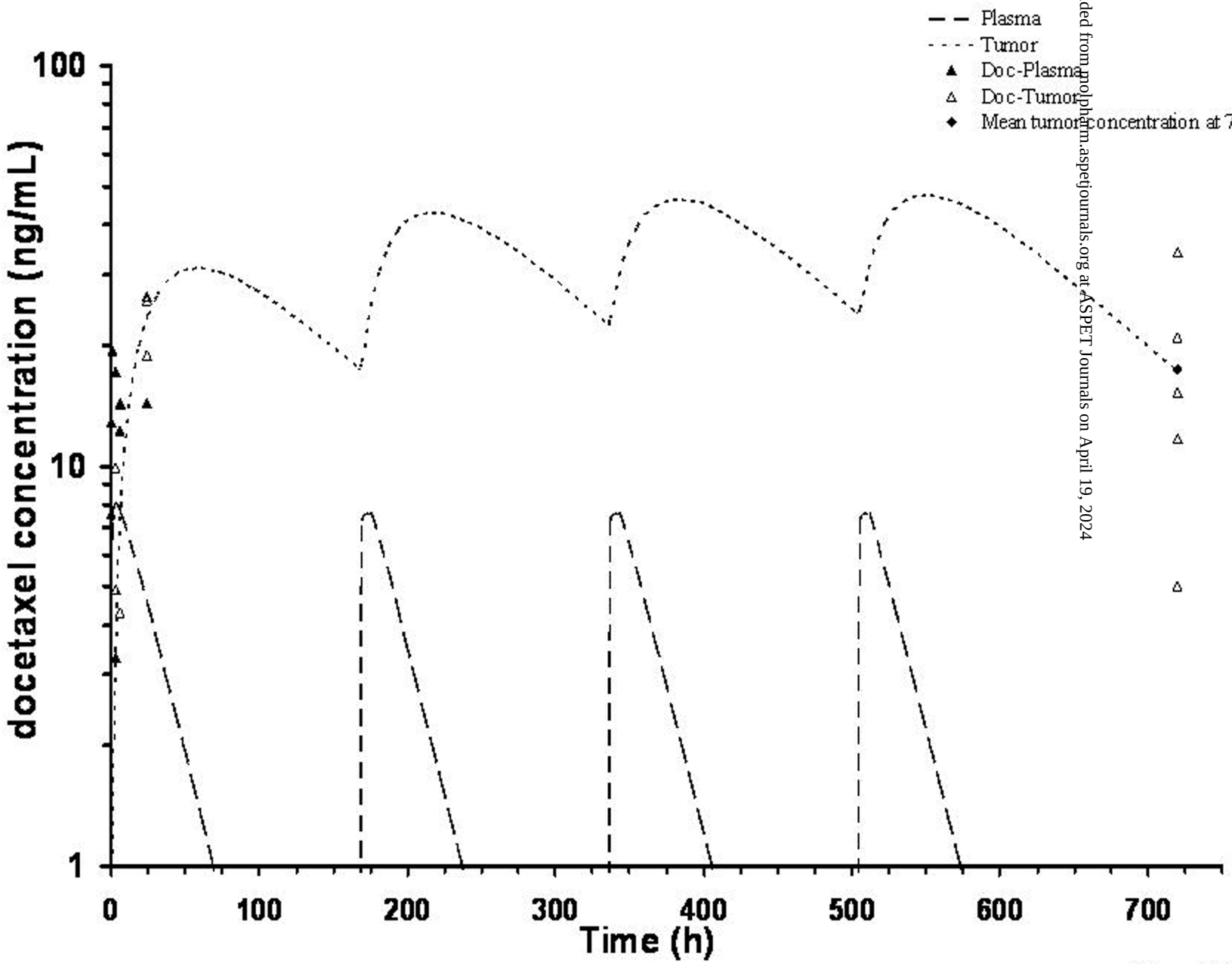

Figure 6B

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